LINKING TREATMENT TARGET IDENTIFICATION TO BIOLOGICAL MECHANISMS UNDERLYING MOOD DISORDERS

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LINKING TREATMENT TARGET IDENTIFICATION TO BIOLOGICAL MECHANISMS UNDERLYING MOOD DISORDERS

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GRP Receptor Regulates Depression Behavior *via* Interaction With 5-HT2a Receptor

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Objective: Accumulating evidences indicate that gastrin-releasing peptide receptor (GRPR) may contribute to the pathophysiology of depression. However, the mechanism of the involvement of GRPR in the progression of depression remains unclear. Here, we showed the extent to which stress and antidepressant treatment impact GRPR expression, and explored the interactions between 5-HT2a receptor (5-HT2aR) and GRPR at the cellular level.

Methods: The rat depression models were created with chronic unpredictable mild stress (CUMS). Then, these rats were treated with fluoxetine for 4 weeks after CUMS. We measured body weight and performed behavioral tests to determine the effects of stress and fluoxetine on depressive-like behaviors. Real-time PCR and western blotting were used to measure the mRNA and protein expression levels of GRPR in the hypothalamus. Then, Flag-tagged protein (pcmv-Flag-5HT2aR) and Myc-tagged protein (pcmv-Myc-GRPR) expression vectors were constructed, identified, and transfected into human embryo kidney 293 (HEK293) cells. The interaction between 5-HT2aR and GRPR was detected by coimmunoprecipitation and double-label immunofluorescence.

Results: The rats subjected to 4 weeks of CUMS showed depressive-like behaviors, including decreased body weight, sucrose preference, and distance traveled, rearing frequency and velocity in the open field test and increased immobility time in the forced swimming test. Fluoxetine treatment reversed CUMS-induced depressive-like behavior. The mRNA and protein expression of GRPR in the hypothalamus was significantly increased after 4 weeks CUMS exposure, and treatment with fluoxetine reversed these changes. Coimmunoprecipitation showed that 5-HT2aR and GRPR combine with each other *in vitro*. Immunofluorescence revealed that the 5-HT2aR and GRPR were colocalization in both the cell membrane and cytoplasm.

Conclusion: Our study enhances the understanding of the involvement of GRPR in depression. This study also provides *in vitro* experimental evidence of the interaction

between 5-HT2aR and GRPR, which may play an important role in the pathogenesis of depression.

Keywords: 5-HT2a receptor, gastrin-releasing peptide receptor, chronic unpredictable mild stress, interaction, depression

INTRODUCTION

Depression is a common and complex mental disorder. It is associated with enormous adverse effects in humans and high costs to society and healthcare systems (1, 2), and the pathogenesis of depression is unclear. Over the past 30 years, 5-HT has been a major target of antidepressant drugs, such as 5-HT reuptake inhibitors (SSRIs), the antidepressant effects of which may involve many types of 5-HT receptors (3). Dysfunction of the serotonergic system is closely related to the pathogenesis of depression, and several genetic studies have focused on genes encoding 5-HT2a receptor (5-HT2aR) (4, 5). The 5-HT2aR is a subtype of the 5-HT2 receptor and belongs to the seven transmembrane-spanning receptor family, which is coupled *via* G q/11 to the inositol triphosphate (IP3)/protein kinase C (PKC)/calcium pathway. 5-HT2aR is highly expressed in several brain regions that are mainly involved in the regulation of emotions, such as the hippocampus, the amygdala, the thalamus, and several cortical areas (6). In preclinical studies, 5-HT2aR mRNA and protein expression were shown to be significantly upregulated in the frontal cortex of stressed rats (7). An increasing number of studies have found the antidepressant-like effects of 5-HT2aR selective antagonists in rodents (8–10). Moreover, increased 5-HT2aR density has been confirmed in depressed patients (11). Postmortem studies have also shown increased 5-HT2aR in unmedicated depressed patients (12). Together, these studies highlight the important roles of 5-HT2aR in the pathology of depression.

Gastrin-releasing peptide receptor (GRPR) belongs to the G-protein coupled receptor (GPCR) superfamily and plays a role in several aspects of emotional responses (13). GRPR is a type of bombesin receptor in humans, mice, and rats that consists of 384 amino acids and was cloned from 3T3 cells. GRPR is directly coupled to the Gq type of G protein and is primarily associated with an increased cellular (Ca2+) and activation of the phospholipase C (PLC)/PKC and extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways (14). Gastrin-releasing peptide (GRP) acts by binding to the GRP receptor, and consistent evidence has proposed that GRP might act as a stress mediator. Merali et al. found that chronic restraint exposure is associated with increased levels of GRP in the anterior pituitary (15). Rats given a systemic injection of corticosterone show enhanced release of GRP in the amygdala and medial prefrontal cortex in response to an acute stressor (16). Furthermore, several studies have shown that the dysfunction of the hypothalamic pituitary adrenal (HPA) axis is mainly involved in the course and progression of depression (17). Considerable evidence suggests that the expression of GRPR in stress-related brain areas including the hypothalamus, hippocampus, and amygdala is involved in the regulation of the HPA axis (18, 19). These data demonstrate the critical role of the GRP/GRPR system in the modulation of depressive-like behavior.

Previous studies have shown that GRP binds preferentially to GRPR, which increases 5-HT neuronal activity in the paraventricular nucleus (PVN) (20). In our previous study, we observed that GRPR mRNA and protein levels are markedly increased in the hypothalamus of CUMS-exposed mice and that treatment with fluoxetine reverses these changes (21). SSRIs are effective in the treatment of depression. There are different families and subtypes of 5-HT receptors, and 5-HT2aR may be involved in the antidepressant effects of SSRIs (22). The administration of fluoxetine and a reduction in either 5-HT2AR or GRPR is associated with a reduction in depression behavior. However, little is known about the molecular mechanisms of the interaction between these two important neurotransmitter systems. In this study, we used the chronic unpredictable mild stress (CUMS) to establish a depressive-like phenotype, and treatment with the antidepressant fluoxetine. We performed the behavioral tests to detect the effects of stress and fluoxetine on anhedonia and activity. We measured the mRNA and protein expression levels of GRPR in the hypothalamus. Human embryo kidney 293 (HEK 293) cells have become the mammalian cell line of choice for the production of recombinant proteins because they are easy to culture and exhibit high transfection efficiency. Transient expression in HEK293 cells provides a way of rapidly assessing the protein function. Therefore, in this study, Flag-tagged protein (pcmv-Flag-5HT2aR) and Myc-tagged protein (pcmv-Myc-GRPR) protein expression vectors were constructed, identified, and transfected into HEK293 cells. Coimmunoprecipitation and double immunofluorescence were used to explore the interaction between 5-HT2aR and GRPR at the cellular level.

MATERIAL AND METHODS

Animals

Male Sprague Dawley (SD) rats, weighing 180 g to 200 g, were obtained from the Company of Experimental Animals of Hunan slilaike jingda. The rats were housed in cages and maintained in a standard animal room (12 h/12 h light/dark cycle; 22 ± 2°C; food and water *ad libitum*). Before the CUMS procedure, the rats were acclimated to the environment for 1 week. All procedures were carried out in accordance with the guidelines of the P.R. China legislation on the ethical care and use of laboratory animals, and the Institutional Animals Care Committee of Renmin Hospital of Wuhan University approved the experimental protocols.

Experimental Groups and Drug Treatment

The rats were randomly divided into three groups (n = 10/group): the chronic unpredictable mild stress (CUMS) + normal saline group (CUMS group); the CUMS + fluoxetine group (fluoxetine group); and the control group. At the end of the CUMS procedure, normal saline was administered daily to the CUMS group rats for 4 weeks by intraperitoneal injection. Fluoxetine (Aladdin; F131623) was diluted in normal saline and intraperitoneally administered to the fluoxetine group rats. A previous work showed that a 4-week course of fluoxetine at a dose of 10 mg/kg produces antidepressant effects. The experimental design is shown in **Figure 1**.

CUMS Procedure

After 1 week of the acclimatization period, the depression model was established by CUMS as described previously with slight modification (23). The rats in the CUMS group and fluoxetine group were subjected to seven different stressors for 4 weeks. They were exposed to food deprivation for 24 h; water deprivation for 24 h; 45° cage tilt for 24 h; swimming in 4°C ice water for 5 min; tail clamping for 3 min; damp sawdust for 24 h; lights on overnight. Each animal received one of these stressors per day and the same stressor was not presented consecutively over 2 days.

Body Weight and Behavioral Tests

We measured the weights of the rats before and after the CUMS procedure, and the rats were again weighed after the fluoxetine treatment. The sucrose preference test, forced swimming test, and open field test were conducted before the CUMS procedure began, at the end of the 4-week CUMS period and after fluoxetine was administered. The sucrose preference test was performed as described previously with minor modifications to quantify anhedonia (24). Before the test, the rats were trained to consume sucrose solution (1%). After adaptation, the rats were deprived of water for 24 h. Subsequently, each rat was given two bottles, one containing 1% sucrose solution and the other containing tap water, for 24 h (the positions of the bottles were changed after 12 h). Sucrose preference was calculated by the following formula: sucrose preference (%) = (sucrose intake/total fluid intake) × 100%. In the forced swimming test, the rats were placed individually in a cylindrical tank that they were unable to exit. The immobility time, the length of time during which the rats remained still without struggling or used only minor movements to keep themselves afloat, was recorded. Each rat was forced to swim for 6 min, and the total time spent immobile during the final 4 min was recorded. The open field test was used to detect the spontaneous activity of the rats. The apparatus consisted of a square 100 × 100 cm area with 35-cm high walls. A rat was placed in the center of the rectangular cage and observed using a video tracking system for 5 min (Ethovision XT 11.5). The parameters that were assessed were the distance traveled, the speed and the frequency of rearing.

Sample Collection

The samples were collected after the final behavioral tests. The hypothalamus was immediately isolated after the rats were sacrificed under deep anesthesia. The samples were stored at −80°C until use.

Plasmid Construction

5-HT2aR and GRPR cDNA sequences were synthesized by the Wuhan Tianyi Huiyuan Company. The 5-HT2aR and GRPR genes were amplified by polymerase chain reaction (PCR), and the following primer sequences were used: 5-HT2aR (forward: 5'-CCCAAGCTTATGGAAATTCTCTGTG-3'; reverse: 5'-CCGGAATTCTCACACACAGCTAACC-3') and GRPR (forward: 5'-CAGCTCGAGATGGCTCCAAATAAT-3'; reverse: 5'-CCGGAATTCCTAGACATACCCCT-3'). The PCR conditions were as follows: 1 cycle of 95°C for 5 min and then 38 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 15 s. The PCR products were analyzed by 1% agarose gel electrophoresis and purified with a DNA purification kit (OMEGA; D2500-01). Then, 5-HT2aR cDNA was subcloned into the pcmv-flag vector at the EcoR I and Hind III sites, and the GRPR cDNA was subcloned into the pcmv-myc vector at the EcoR I and Xho I sites. The plasmids were transformed into DH5α by the heat shock method and extracted with a plasmid mini kit (OMEGA; D6943-01). Finally, the pcmv-Flag-5HT2aR and pcmv-Myc-GRPR plasmids were identified by restriction enzyme digestion.

Cell Culture and Transfection

HEK293 cells were obtained from the microbiology laboratory of the School of Basic Medicine, Wuhan University. The cells were cultured in DMEM (Gibco, 21885108) supplemented with 10% FBS and gentamicin and incubated at 37°C in 5% CO_2. The cells were seeded in 10-cm culture dishes 24 h before transfection. The pcmv-Flag-5HT2aR and pcmv-Myc-GRPR plasmids were

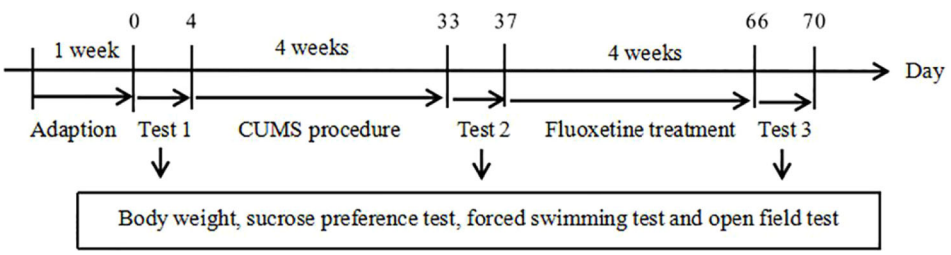

FIGURE 1 | Experimental procedures.

cotransfected with Lipofectamine 2000 reagent (Thermo,11668019) according to the manufacturer's instructions. Single transfection of the pcmv-Flag-5HT2aR or pcmv-Myc-GRPR plasmid was performed in the control groups.

RNA Extraction and RT-PCR

Total RNA was extracted from the hypothalamus using Trizol reagent (Ambion, 15596-026) and purified according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometer at A260/A280 nm. Reverse transcription was performed with mRNA (3 μg) using a first-strand synthesis kit (Thermo, K1691) for cDNA synthesis. cDNAs were subsequently amplified by PCR with the following specific primers: GAPDH (forward: 5'-TGTGAAGC TCATTTCCTGGTATG-3', reverse: 5'-AGGGCCTCTCTCTTG CTCTC-3') and GRPR (forward: 5'-GCAGGATTGGCTGCA AACTG-3', reverse: 5'-ATTGGCCTGACGATGGCTTT-3'). PCR was carried out as follows: 1 cycle of 95°C for 5 min followed by 39 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 10 min. The expression of GRPR mRNA was analyzed by $2^{-\Delta\Delta CT}$ method, and was normalized to GAPDH as a reference gene. Each experiment had more than three independent replicates.

Protein Extraction and Western Blotting

Western blotting was performed to test the expression of GRPR in the hypothalamus of rats and the cultured HEK 293 cells. Total protein from each group was extracted in 1 ml of RIPA buffer (Beyotime, P0013B) with 1% PMSF and protease inhibitor cocktail. The hypothalamus tissues were homogenized in ice cold RIPA lysis buffer. The HEK293 cells were washed with PBS 48 h after transfection and lysed with ice cold RIPA lysis buffer. Tissues and cells were centrifuged for 15 min at 12,000 rpm at 4°C, and the supernatants were collected and stored at −80°C. The concentration of proteins was determined by the BCA assay (BCA Protein Assay Kit, Thermo,23228). Then, 12% polyacrylamide gel electrophoresis was used to separate the protein samples, and then the proteins were transferred onto PVDF membranes (Merck millipore, ISEQ00010). The PVDF membranes were blocked in 5% non-fat dry milk at room temperature for 1 h. Then, the membranes were incubated with anti-GRPR antibody (1:200) (Santa Cruz, sc-32904), anti-Flag antibody (1:2,000) (Abbkine; A02010), and/or anti-Myc antibody (1:2,000) (Abbkine; A02061) at 4°C overnight. The following day the membranes were washed with TBST for three times. Then the membranes were incubated with secondary antibodies (1:5,000) at room temperature for 2 h. The immunoreactions were visualized with a chemiluminescence kit (Beyotime, P0018AM). GAPDH was used as a loading control to analyze the relative protein expression of GRPR. The intensity of the protein bands was calculated by ImageJ software. Each experiment was performed more than three times.

Coimmunoprecipitation Analysis

The protein samples were preincubated with anti-Flag antibody (1:200) at 4°C overnight. Then, 50% agarose beads were washed with PBS, incubated at 4°C for 2 h, centrifuged to remove the nonspecific binding protein, and added to the samples. Rabbit IgG was used as a negative control. The antigen-antibody complexes proteins were separated using 10% polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk for 1 h, and were incubated with anti-Myc antibody (1:2,000) at 4°C overnight. Similarly, the protein samples were preincubated with anti-Myc antibody (1:200), and the antigen-antibody complex proteins were detected by anti-Flag antibody (1:2,000). Finally, the immunoreactions were performed with a chemiluminescence kit after incubation with secondary antibodies (1:5,000).

Double-Label Immunofluorescence and Confocal Microscopy

Double-label immunofluorescence was used to analyze 5-HT2aR and GRPR distribution in HEK293 cells. After 48 h of the transfection, HEK293 cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100. The cells were blocked with 5% non-fat dry milk for 1 h. Then, the cells were incubated in a mixture of mouse anti-Flag monoclonal antibody (1:200) and rabbit anti-Myc polyclonal antibody (1:200) overnight at 4°C. The second day, the cells were washed with PBS several times and incubated with DyLight488-conjugated goat anti-mouse IgG (Abbkine; A23210) and DyLight594-conjugated goat anti-rabbit IgG (Abbkine; A23420) in the dark at room temperature for 1 h. Nuclei were stained with DAPI for 5 min. After staining, the fluorescence signals were examined with a laser-scanning confocal microscope.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software, and all data were reported as the means ± standard error of mean (SEM). The data were analyzed by one-way ANOVA test followed by LSD *post hoc* test. Statistical significance was set at $P < 0.05$.

RESULTS

Body Weight and Behavioral Tests

As shown in **Figure 2**, before the CUMS procedure, no significant difference was observed among the three groups, but the CUMS group and the fluoxetine group were significantly different from the control group following 4 weeks of CUMS. The CUMS group and the fluoxetine group rats showed a lower index of body weight ($F = 43.48$, $P < 0.05$) and sucrose preference ($F = 102.94$, $P < 0.05$), decreased distance traveled ($F = 12.93$, $P < 0.05$), rearing frequency ($F = 12.14$, $P < 0.05$) and velocity ($F = 17.87$, $P < 0.05$) in the open field test and an increased immobility time in the forced swimming test ($F = 35.75$, $P < 0.05$). Compared with the CUMS group, the fluoxetine groups exhibited significantly increased the body weight ($F = 120.14$, $P < 0.05$), sucrose preference ($F = 68.11$, $P < 0.05$), and the distance traveled ($F = 20.34$, $P < 0.05$), rearing frequency ($F = 10.48$, $P < 0.05$), and velocity ($F = 16.13$, $P < 0.05$) in the open

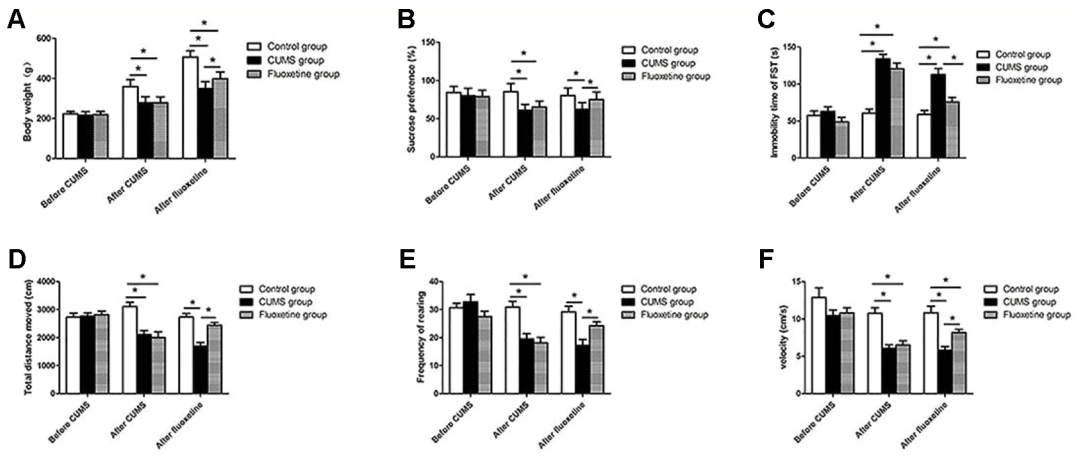

FIGURE 2 | Body weight **(A)**, sucrose preference **(B)**, forced swimming test **(C)**, total distance moved **(D)**, rearing frequency **(E)**, and velocity **(F)**. **P < 0.05.*

field test and decreased immobility time in the forced swimming test (F = 16.70, $P < 0.05$).

GRPR mRNA Expression

As shown in **Figure 3**, significant differences in the mRNA expression of GRPR were observed in the hypothalamus of rats between groups. Compared with that in rats from the control group, the mRNA expression of GRPR was significantly increased in rats from the CUMS group, and fluoxetine treatment significantly downregulated the mRNA expression of GRPR in rats from the fluoxetine group (F = 17.32, $P < 0.05$).

GRPR Protein Expression

Figure 4 shows the analysis of GRPR protein using western blotting in hypothalamic tissue. The GRPR protein level in the CUMS group was significantly increased compared with that in the control group and the fluoxetine group (F = 19.38, $P < 0.05$).

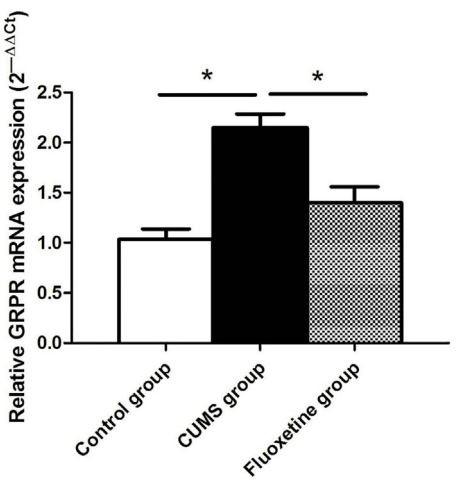

FIGURE 3 | GRPR mRNA expression in the hypothalamus between groups. **P < 0.05.*

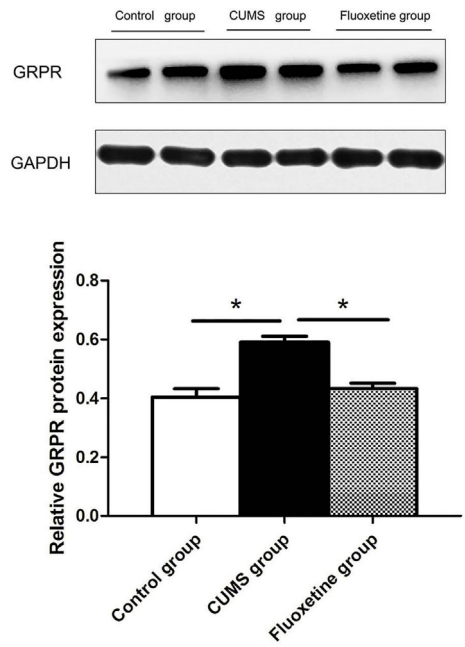

FIGURE 4 | GRPR protein expression in the hypothalamus between groups. **P < 0.05.*

5-HT2aR Is a GRPR-Interacting Protein In *Vitro*

To investigate whether 5-HT2aR is functionally associated with GRPR, coimmunoprecipitation was performed *in vitro*. The pcmv-Flag-5HT2aR and pcmv-Myc-GRPR plasmids were constructed, identified, and cotransfected or singly transfected

into HEK293 cells. First, protein from the cotransfected cells was immunoprecipitated with anti-Flag antibody and analyzed by western blotting with anti-Myc antibody. The results showed that a GRPR band was detected in the immunoprecipitate of the anti-Flag antibody. Then, anti-Myc antibody was used for immunoprecipitation, and anti-Flag antibody was used for western blotting. A 5-HT2aR band was detected in the immunoprecipitate of the anti-Myc antibody. However, neither a 5-HT2aR band nor a GRPR band were detected in singly transfected cells immunoprecipitated with antibodies for both proteins. The protein bands were also not present in the immunoprecipitate of rabbit IgG (**Figures 5A, B**).

Colocalization of 5-HT2aR and GRPR

To further determine the expression pattern of 5-HT2aR and GRPR, we used the anti-Flag antibody and anti-Myc antibody specifically bound to HEK293 cells cotransfected with pcmv-Flag-5-HT2aR and pcmv-Myc-GRPR plasmids. Double immunofluorescence staining for 5-HT2aR and GRPR revealed that the expression of the two receptors overlapped in HEK293 cells. As revealed by confocal microscopy and shown in **Figure 6**, 5-HT2aR and GRPR were colocalized in the cytoplasm and membrane of HEK293 cells, as revealed by confocal microscopy.

DISCUSSION

In this study, 4 weeks of CUMS was used to establish a classical model of depression in rats. The results indicated that fluoxetine treatment had an antidepressant-like effect in a CUMS model of depression in rats. Fluoxetine treatment decreased the levels of GRPR mRNA and protein in the hippocampus of stressed rats. Our coimmunoprecipitation results showed that 5-HT2aR and GRPR combine with each other *in vitro*. Additionally, immunofluorescence results revealed that the 5-HT2aR and GRPR were colocalized in both the cell membrane and cytoplasm. Our findings provide additional research ideas to explore the pathogenesis of targeting GRPR signaling in depression. Our data also supports that a functional interaction between GRPR and 5-HT2aR, were supported at the cellular level.

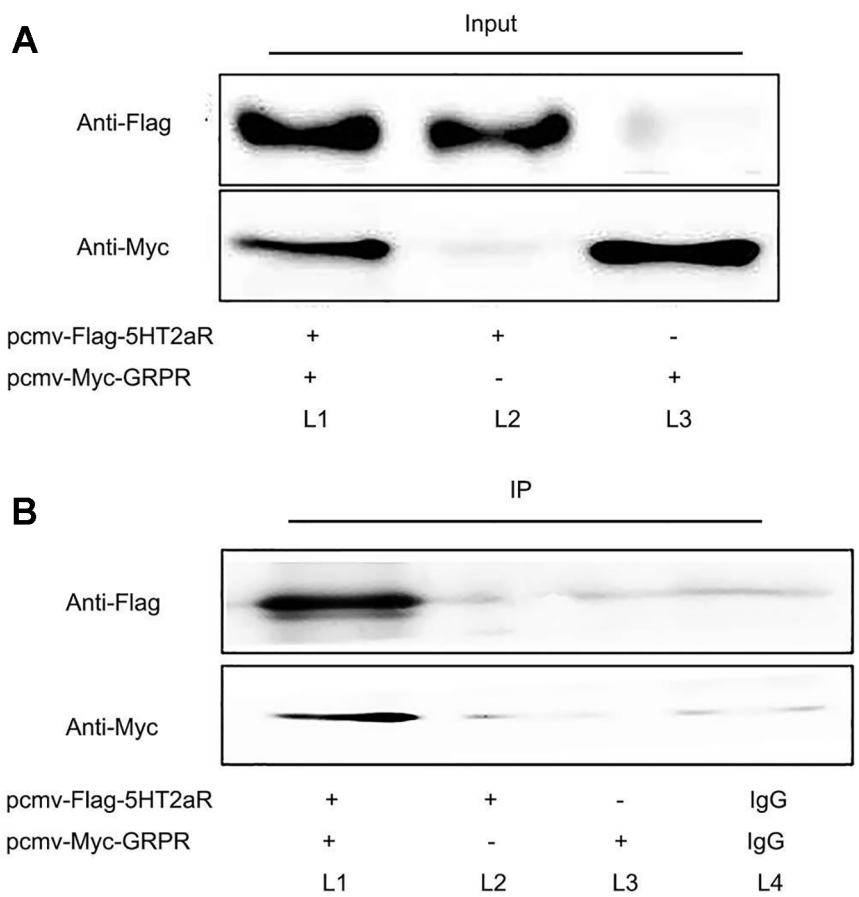

FIGURE 5 | Coimmunoprecipitation of 5-HT2aR and GRPR. Input **(A)** and IP **(B)**. The pcmv-Flag-5HT2aR and pcmv-Myc-GRPR plasmids were cotransfected into HEK293 cells (L1). The pcmv-Flag-5HT2aR plasmid was singly transfected into HEK293 cells (L2). The pcmv-Myc-GRPR plasmid was singly transfected into HEK293 cells (L3). IgG served as the negative control (L4).

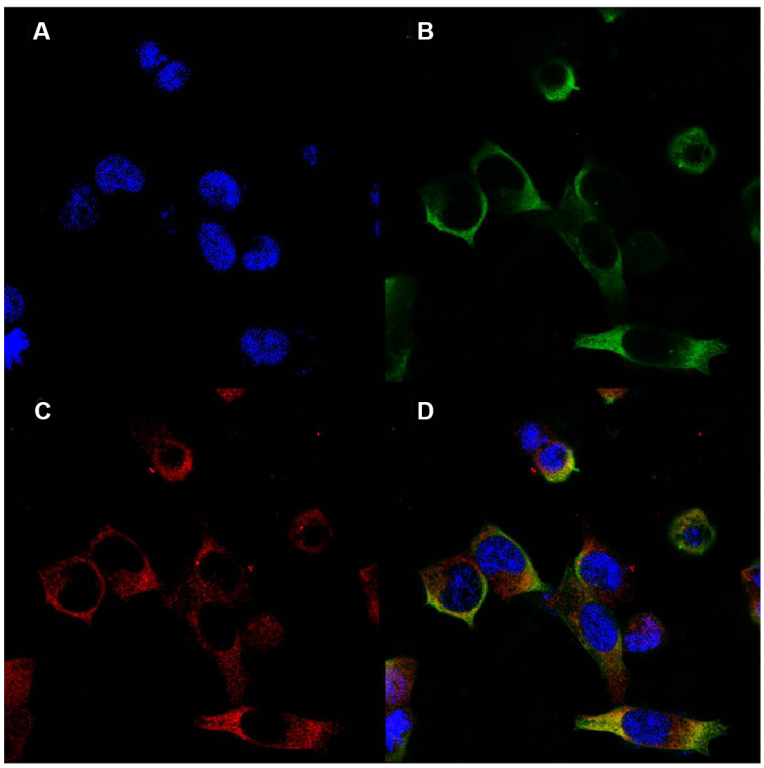

FIGURE 6 | Colocalization of 5-HT2aR and GRPR. Nucleus **(A)**; 5-HT2aR **(B)**; GRPR **(C)**; merge **(D)**.

CUMS is regarded as a valid animal model of depression-like behavior (25). In this study, a classical model of depression was successfully built by 4 weeks of CUMS. Anhedonia is one of the major symptoms of depression, and is manifested as a reduction in interest or pleasure in daily activities. Additionally, CUMS causes many other symptoms of depression, such as decreases in food/water intake, exploratory behaviors, and locomotor activity, and helplessness (26). A reduction in sucrose preference in stressed rats reflects anhedonia, and an increase in immobility time in the forced swimming test indicates a lower desire to escape, which may be similar to the helplessness symptom in depression (27). The lower body weight observed in the stressed rats suggests that CUMS induced a decrease in food/water intake. In the open field test, CUMS significantly reduced the distance moved, velocity, and rearing frequency of rats. These results indicated that stressed rats exhibited less activity and fewer exploratory behaviors in new environments (28). Fluoxetine treatment led to a significant improvement in body weight, sucrose preference, and distance traveled, rearing frequency, and velocity in the open field test, and a decrease in immobility time of the forced swimming test. These findings are in agreement with previous studies and suggest that fluoxetine has antidepressant-like effects.

In this experiment, we showed that the mRNA and protein expression of GRPR in the hypothalamus were significantly upregulated in CUMS rats and that the expression of GRPR was downregulated after fluoxetine treatment. GRP binds preferentially to GRPR and stimulates the release of adrenocorticotropic hormone (ACTH) and corticosterone, and increasing the activity of the HPA axis, evokes behaviors associated with stress (29). GRP and GRPR are present in several brain regions implicated in the stress response, including the amygdala, hippocampus, hypothalamus, and bed nucleus of the stria terminalis, as well as caudal brainstem structures such as the nucleus tractus solitarii, parabrachial nucleus, and locus coeruleus (30). In addition, studies have shown that the locomotor activity and non-aggressive social behaviors are increased in the GRPR-deficient mice (31). In this study, we further confirmed that the mRNA and protein expression of GRPR was increased in CUMS rats. The results also showed that chronic fluoxetine treatment restored the stress-induced increase in GRPR expression. However, the mechanisms by which fluoxetine treatment restores GRPR expression remain unclear.

Fluoxetine is an SSRI and exerts its pharmacological effects through the manipulation of the 5-HT system (32). Fluoxetine

selectively block 5-HT transporters, thereby increasing extracellular concentrations of 5-HT at the postsynaptic 5-HT receptors (33). In addition, fluoxetine treatment induces a complex array of neuropharmacological changes, such as a reduction in the density of 5-HT2aR (34). A Japanese cohort study suggested that 5-HT2aR may play an important role in the pathophysiology of the therapeutic response to SSRIs (35). In rats, chronic treatment with citalopram decreases the 5-HT2aR density in the brain cortex (36). Additionally, several studies have found that the CUMS-induced increase in 5-HT2aR expression can be decreased by fluoxetine administration (37, 38). Moreover, Qesseveur et al. explored the genetic variants of the 5-HT2aR gene that affect the therapeutic outcome of antidepressants. The results suggested that the genetic inactivation of the 5-HT 2A receptor may affect the antidepressant effects of SSRIs (39). These findings suggest that 5-HT2aR may be involved in the mechanism of action of antidepressant effect. In our study, we also found that chronic fluoxetine treatment decreased GRPR expression in the hypothalamus of rats. To explore the mechanism underlying the regulation of this response, we used HEK293 cells to explore the interactions between 5-HT2aR and GRPR. We used coimmunoprecipitation to identify the protein-protein interaction between 5-HT2aR and GRPR and used double immunofluorescence staining to study the colocalization of 5-HT2aR and GRPR *in vitro*. By performing coimmunoprecipitation and double immunofluorescence staining, we confirmed the interaction between 5-HT2aR and GRPR *in vitro*, which may provide new ideas for the treatment of depression.

One limitation is that our study was only performed *in vitro*, so it may not fully explain the possible mechanism of the interaction between these two receptors in the pathogenesis of depression. Therefore, it is necessary to confirm this conclusion in patients or animal models of depression. Another limitation is that we only explored the impacts of stress and fluoxetine treatment on GRPR expression. GRPR plays a physiological role by activating the PLC/PKC pathway, has a similar signaling pathway to 5-HT2aR. The molecular and cellular basis for GRPR-5-HT2aR cross-signaling may be an interesting research direction in this field.

In general, this study confirmed a change in GRPR expression in the rat hypothalamus after stress. Additionally, for the first time, this study provides experimental evidence of the interaction between 5-HT2aR and GRPR *in vitro*. These results highlight the involvement of GRPR in depression, and provide a novel biological target for the treatment of depression.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by The P.R. China legislation on the ethical care and use of laboratory animals, and the Institutional Animals Care Committee of Renmin Hospital of Wuhan University.

AUTHOR CONTRIBUTIONS

ZL and GW designed and supervised the study. DX carried out the experimental procedures and analyzed the date. ZL and DX interpreted results of experiments and drafted the manuscript. ZL revised the manuscript. All authors provided feedback on manuscript.

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Research on Supportive Psychosocial, Drug Treatment, and Health Education Intervention and Health Management Model of Community-Residing Elderly Adults With Late Life Depression in Liaoning Province: A Protocol

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Background: Late life depression (LLD), a common mental disorder, has become an increasingly acute public health concern with a quickly expanding geriatric population worldwide. To our knowledge, however, the incidence of LLD in northern cities in China has not been empirically investigated, and many elderly people with depressive moods and mild depressive symptoms have not been given sufficient attention.

Methods/Design: This is a multi-stage and prospective study. The first stage is a cross-sectional study, investigating the epidemiological characteristics of LLD in northern China and exploring the biological, psychological, and social risk factors for developing LLD based on a set of questionnaires from 6,800 community-residing elderly adults. The second stage involves statistical analysis, by constructing a risk factor model for LLD and analyzing their direct and indirect functional routes on the basis of structural equation modeling. The third stage is an experimental study a total of 60 elderly patients with LLD and their principle caregivers will be randomly assigned to control and trial groups. The trial group patients and caregivers will undergo supportive psychosocial, drug treatment, and health education (PDH) intervention, whereas the control group patients and caregivers will be treated routinely (treatment as routine, TAR, which includes drug treatment and health education). At the end of the intervention, depressive symptoms, quality of life, and the social and cognitive functioning of the patients in the two groups will be respectively assessed at a baseline and after 6, 9, and 12 months post-intervention by employing scales and questionnaires to analyze the effectiveness of the supportive PDH intervention measures in comparison with TAR. Ultimately, a supportive PDH intervention and health management model will be obtained by combining PDH intervention with mental health institutions, community health services, and aging families as the main line.

Discussion: This study will provide strong and suitable evidence for enhancing the integrated supportive PDH intervention and health management model of LLD patients among community-dwelling residents.

Ethics: This study has been approved by the Ethics and Research Committee of the First Affiliated Hospital of China Medical University (approval No. [2019] 2019-312-2).

Keywords: late life depression, community, risk factor, supportive PDH intervention, health management

INTRODUCTION

Late life depression (LLD) is complex, burdensome, and difficult to treat. Specifically, it refers to elderly people who, for the first time in their lives, meet criteria for major depressive disorder (MDD) or display clinically significant depressive symptoms such as functional disability, cognitive decline, increased risk of medical comorbidity (e.g., co-dementia), and even suicide in severe cases (1–4). Meanwhile, MDD in the elderly, as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV TR), has been a growing public health concern, and it is projected to be the second leading cause of disease by the year 2020 (5, 6). Epidemiological surveys reported the prevalence of MDD in elderly adults in different countries, such as the USA at 16% (7), England at 8.7% (8), and India at 24.1% (9), with considerable regional variation. Additionally, the results of a meta-analysis based on 28 studies and 76,432 subjects showed that the point, 12-month, and lifetime overall prevalence of MDD in elderly adults was, respectively, 2.7%, 2.3%, and 2.8% in China (10).

Evidence shows that successful antidepressant treatment is one of the most effective ways of reducing disability, preventing morbidity, and improving quality of life (QOL) in older patients (11). However, the response rate to an antidepressant trial with adequate dose and duration is often inadequate, and can be as low as 30%~40% in LLD patients (12). Moreover, studies have found that it is difficult to improve the cognition and personality traits of elderly patients with pharmacotherapy alone, and a number of adverse events (e.g., cardiovascular, gastrointestinal, and anticholinergic complications) are often associated with antidepressant use. Frequently, this leads to discontinuation, which contributes to a high recurrence rate and mortality (13, 14). Thus, various forms of non-pharmacological therapies with negligible side effects are being embraced by patients, such as meditation, motor therapy, cognitive behavioral therapy, interpersonal psychotherapy, reminiscence therapy, alternative therapies, and group or educational activities (15–19). These treatments are tailored to the abilities of elderly patients and target multiple contributing factors with the aim of alleviating loneliness and comorbid anxiety, enhancing social support, and improving QOL.

In Western countries in the 1970s, health management, an important topic in the field of healthcare, developed rapidly and was successfully implemented for chronic conditions such as hypertension, diabetes mellitus, and chronic obstructive pulmonary disease (20–22). By combining information technology, geriatric medicine, and community medicine, health management has been used to strengthen the management of chronic diseases in the elderly, improve their lifestyle, and reduce mortality rates from disease (23). In China, relevant documents promulgated by the Chinese State Council clearly stated that health management is a fundamental way to improve the health quality of the elderly, extend life expectancy, and achieve healthy aging. However, there are few studies on community-based health management of patients with LLD, even though it is imperative to develop health management services for the elderly in the field of gerontology.

According to literature review and analysis, we found that there are four main deficiencies of the existing research. First, due to a large elderly population base, rapid aging growth, uneven distribution of mental health resources, and insufficient public attention to the mental health of the elderly, under-recognition and under-treatment of depression appears to be severe in Liaoning which ranked first among 19 provinces and cities in terms of the proportion of the elderly population aged 65 and above in 2016. Second, research on interventions for LLD is limited. Previous studies have been mostly based on pharmacotherapy, psychosocial intervention, health education, or a combination of any two of these, but none, to our knowledge, have been conducted combining all aspects together. Third, research on the risk factors of LLD has been limited, and the methods used have not been sufficiently systematic. Previous studies were limited to investigating risk factors (24–26), but lacked any discussion of the internal relations among the various factors. As such, they were limited with regard to providing guidance based on their results and conclusions for the treatment and intervention of diseases. Fourth, there is a lack of systematization and wholeness in research on the intervention of LLD. Previous studies mostly focused on tests of intervention methods. Due to impoverished intervention resources, objectives, and effects, and intervention objects and their background environment, intervention measures often have poor extensibility and cannot be repeated to verify research results. Here, we will comprehensively analyze the risk factors leading to LLD in Liaoning province, and construct integrated supportive PDH intervention and health management model of LLD patients among community-dwelling residents, aiming to provide scientific evidence for the implementation of interventions and health management, as well as formulation of relative health policies.

OBJECTIVES

This study is designed to comprehensively assess the overall community mental health resources available to LLD patients, along with the prevalence, risk factors, and prevention and treatment status of LLD in Liaoning. By exploring and constructing the integration of psychosocial interventions, medical care, and health education with psychiatric hospitals, community health services (CHS), and aging families, this study can provide a reference for the practice of supportive intervention and health management, as well as guidance for formulating health policies in Liaoning. The aims of this study are as follows:

1. Analyze the epidemiological characteristics of LLD in Liaoning province.
2. Construct a risk factor model for LLD in Liaoning.
3. Conduct supportive psychosocial, drug treatment, and health education (PDH) interventions alongside treatment as routine (TAR) in a trial group and control group, respectively, to compare their effectiveness with regard to patients and their caregivers.
4. Establish a supportive PDH intervention and health management (SIHM) model for elderly patients with depression according to the local economic level, mental health resources, and demographics of Liaoning.

METHODS/DESIGN

Organizational Structure

Team members of the preliminary investigation will consist of six graduate students studying psychiatry, two clinical psychiatrists with experience in the fields of mental health, geriatrics, and medical psychology, and five CHS staff members from Liaoning province. Before implementing the questionnaire survey, graduate students and CHS staff will undergo unified training, to explain the specific purpose, content, significance, procedures, and principles of the investigation. Further, the team members will learn the definitions and scoring criteria of each item in the questionnaires and scales, communication skills for interacting with elderly patients and their caregivers, and possible problems and solutions that might be encountered. After sampling according to inclusion and exclusion criteria, the psychiatrists, accompanied by other team members, will enter selected communities, ask permission from community leaders, and complete the investigation in public activity areas or at elderly residences accompanied by the arranged community staff.

In addition, team members of the intervention will comprise graduate students in psychiatry, one clinical psychiatrist, and one psychologist employed to supervise and guide the intervention process. After team members work out a detailed intervention plan according to the purpose of the project and the characteristics of the research objects, the research team will organize experts in psychiatry, psychotherapeutics, social medicine, and geriatrics to review and approve the plan. Afterwards, the plan will be revised and improved based on expert advice before implementation. Then, in the course of carrying out the plan, the intervention team members will record any problems with the intervention process and immediately report back to the project leader for revisions before further implementation. The goal of such cycles is to ensure the effectiveness of the intervention.

Quality Control

In order to ensure the quality of the preliminary investigation of this project, we formulated the following measures. First, there will be unified training for researchers before data collection to reduce bias caused by differences among researchers. Second, when carrying out the questionnaire, the environment will be as quiet as possible, so as to facilitate the emotional stability of the research subjects and avoid adverse effects from the internal and external environment. Third, when collecting the questionnaires after completion, missing items will be promptly identified (without violating the principle of voluntariness) to ensure the accuracy and integrity of the survey data. Finally, data obtained from the survey will be input into statistical software for analysis by two investigators working together. At the same time, before statistical processing, SPSS descriptive statistics will be used to verify the recorded data, and errors will be corrected.

Similarly, the following three measures were developed to ensure the quality of the intervention. First, members of the intervention team will regularly report the concrete content of the intervention measures and the implementation process to the project leader and clinical psychiatrists. Second, a clinical psychiatrist will be invited to evaluate, guide, and correct the accuracy of the intervention measures and methods, along with any problems with the intervention and suitable solutions. Third, contingency plans will be developed and imparted to researchers in case of any emergencies while executing intervention measures.

Study Design

This is a multi-stage and prospective study. It includes the following three stages:

1. The first stage is a cross-sectional study, based on the baseline information from 6,800 community-dwelling elderly adults collected through a set of questionnaires. The aim of the first stage is to investigate the epidemiological characteristics of LLD in northern China, and to explore the biological, psychological, and social risk factors for developing LLD.
2. The second stage involves statistical analysis, with the aim of constructing a risk factor model for LLD and analyzing direct and indirect functional routes on the basis of structural equation modeling (SEM).
3. The third stage is an experimental study, comprising a 6-month supportive intervention period and a 6-month follow-up period. In order to ensure the participation of the subjects in this study and improve the effectiveness of the interventions, a total of 60 elderly patients with depression and their principle caregivers will be randomly and equally assigned to trial and control groups.

Trial group patients and caregivers will undergo supportive PDH interventions, and control group patients and caregivers

will receive TAR. Subsequently, depressive symptoms, QOL, and the social and cognitive functioning of the patients in the two groups will be respectively assessed at a baseline and after 6, 9, and 12 months post-intervention by using scales and questionnaires to analyze the effectiveness of the supportive PDH intervention measures. Ultimately, an SIHM model will be constructed by combining PDH intervention with mental health institutions, CHS, and aging families as the main line.

Participants

We plan to enroll the participants between December 2019 and December 2022. The goal is to recruit 6,800 community-residing elderly adults to complete the baseline investigation. Then, 60 LLD patients who meet more strict criteria, along with their principle caregivers, will receive 6 months of supportive PDH intervention followed by a 6-month follow-up period. The inclusion and exclusion criteria for all the respondents, patients with LLD, and caregivers are listed in **Table 1**.

Enrollment, Supportive PDH Intervention, and Follow-Up

1. The 6800 community-residing elderly adults will be screened and enrolled by the investigators according to the inclusion and exclusion criteria. To ensure the balance and representativeness of sample sources, we intend to adopt a multi-stage sampling method. At the first stage, four cites will be randomly selected in Liaoning province by randomized sortition. At the second stage, one district and one township will be randomly selected from each selected city. At the third stage, one urban community and one village community will be randomly selected from each selected district/township. At the fourth stage, elderly residents will be selected from each selected urban community and village community with a convenient sampling method.
2. The 60 community-residing elderly adults with LLD and their principle caregivers will be allocated to trial and control groups. In addition, we will ensure that there are no statistical differences between the two groups in term of the degree of depression, drug treatment programs, knowledge of mental health, geographic distribution, and other aspects before carrying out the interventions. The trial group of 30 elderly patients and their principle caregivers will receive the same content and frequency of individualized and group intervention for 6 months. By contrast, the 30 elderly patients in the control group and their principle caregivers will receive TAR, which are suitable for all MDD patients as routine. During this period, except for any additional treatment for comorbid physical diseases or short-lasting benzodiazepines for severe insomnia, other antipsychotic medications, systematic psychotherapy, and long-lasting benzodiazepines will not be allowed. The detailed content, forms, and methods of the supportive PDH intervention are listed in **Table 2**.

Considering that Shenyang, the capital of Liaoning, has the second-largest elderly population in the province, a multi-stage

TABLE 1 | Inclusion and exclusion criteria for community-residing elderly adults in rural and urban cites/villages in Liaoning province.

Research subjects	Inclusion criteria	Exclusion criteria
6,800 community-residing elderly adults	1. aged 60 years old or above at the time of enrollment; 2. permanent urban/rural community-dwelling residents (at least 5 years of residency) with urban/rural *hukou* status in Liaoning province; 3. comprehension, reading, and writing skills to independently complete the questionnaires/scales or complete them with the assistance of the researchers without obstacles; 4. voluntary participation and signed informed consent.	1. serious health concerns, such as acute infectious diseases, unstable cardiovascular disease, etc.; 2. lifetime substance or alcohol dependence; 3. high suicide risk; 4. met criteria for dementia (major neurocognitive impairment in DSM-5); 5. in the process of a depressive episode; 6. former permanent urban/rural community-dwelling resident that has been away for 1 year or more.
60-LLD patients	1. aged 60 years old or above at the time of enrollment; 2. permanent urban/rural community-dwelling residents (at least 5 years of residency) with urban/rural *hukou* status in Liaoning province; 3. meet diagnostic criteria for MDD without psychotic features based on the Chinese version of MINI according to DSM-IV TR (the core diagnostic criteria of the symptomatology and course of disease are consistent with DSM-5). 4. comprehension, reading, and writing skills to independently complete the questionnaires/scales or complete them with the assistance of the researchers without obstacles; 5. voluntary participation and signed informed consent.	1. 1) serious health concerns, such as acute infectious diseases, unstable cardiovascular disease, etc.; 2. lifetime substance or alcohol dependence; 3. high suicide risk; 4. met criteria for dementia (major neurocognitive impairment in DSM-5); 5. in the process of a depressive episode; 6. former permanent urban/rural community-dwelling resident that has been away for 1 year or more.
60-principle caregivers	1. assumes most of the responsibility of caring for the patient (e.g. daily care, accompanying the patient to medical treatment, etc.) 2. no communication barriers; 3. voluntary participation and signed informed consent.	1. caregiver who receives remuneration; 2. history of any psychiatric disorder; 3. suffers from diagnosed organic or psychiatric disease (e.g., depression); 4. refuses to provide authentic and reliable information to the research team.

LLD, late life depression; MINI, mini-international neuropsychiatric interview; DSM-IV TR, Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision; MDD, major depressive disorder.

TABLE 2 | Content, form and method of supportive PDH interventions.

	Supportive PDH interventions
Content	P: Supportive psychosocial intervention 1. Supportive therapy, including the application of listening, sympathy, comfort, and guidance, constructive and instructional language, asking patients to actively communicate with themselves and intervention team members. 2. Cognitive behavioral therapy, including the correction of abnormal cognition and behavior, and guiding patients to correctly face major stressful events in life or attitudes and treatment skills in the event of recurrence. 3. Social skills training, including self-care skills training, interpersonal skills training (e.g., positive communication with family members, and active participation in related group activities). 4. Mindfulness therapy, including breathing, mindfulness, and walking. 5. Interest cultivation, including encouraging patients in the trial group to participate in outdoor sports therapy and entertainment therapy (e.g., painting, carpentry, clay, knitting, chess, card games, etc.), and cultivating personal interests. 6. Group nostalgia therapy, including guiding patients to recall old classic songs, movies, important events, happy childhood events, and achievements made in life or at work. D: Supportive drug treatment 1. Patients will be guided to use drugs strictly in accordance with a safe and standardized system. 2. Principle caregivers will be normatively urged to provide supervision to improve the medication compliance of the patients. H: Supportive health education 1. Mental health education, including symptoms, early signs of recurrence and preventive measures of LLD. 2. Drug treatment health education, including knowledge about drugs (e.g., antidepressants), the importance of maintaining and taking medicine on time, the significance of pharmacotherapy for preventing recurrence and deterioration, and self-management skills for drug therapy. 3. Lifestyle health education, including establishing a reasonable and balanced diet, good sleep hygiene, quitting smoking, limiting alcohol, and adhering to a moderate exercise regime.
Form	P: Supportive psychosocial intervention 1. Rely on the platform of CHS to carry out group psychological intervention for patients. 2. Rely on the family, and have intervention team members work with the principle caregivers to provide psychological counseling and personalized treatment for patients. H: Supportive health education 1. Distribute manuals and pamphlets on the prevention and treatment of depression in the elderly. 2. Regularly carry out group discussions and lectures for patients and their principle caregivers. 3. Open a psychological consultation platform on the Internet. 4. Cooperate with CHS to establish an LLD rehabilitation self-help group and organize group activities regularly. (group therapy will be conducted once a month, and family and individual therapy will be conducted at least twice a month)
Method	1. Make physical examination appointments or temporary on-site appointments with patients and their principle caregivers. Collect patient health information and establish health management files. 2. Health assessment and prediction, with a detailed intervention plan. 3. Pre-intervention training. The project leader and main group members will conduct unified training for follow-up CHS members. The main content of the training will pertain to symptoms and condition assessments, medication and efficacy observations, physical and mental health recording, interviewing skills, psychosocial intervention methods, and home visits and telephone follow-up skills. 4. Intervention. The follow-up members should strictly follow the intervention outline formulated by the research group and adopt the supportive PDH method to complete the intervention. The project team will arrange fixed personnel to supervise and guide the members regularly. Overall, the intervention will last 6 months. 5. Follow-up. In the first 3 months after the intervention, a monthly home visit and a telephone follow-up will be conducted. A telephone follow-up will be conducted every 2 weeks for the next 3 months, where the follow-up period will last 6 months. During each follow-up, it is necessary to evaluate the health management status of the patients and any intervention goals that have been achieved, while devising new goals based on the actual situation, and providing corresponding individual counseling according to the patients' mastery of relevant knowledge, skills, and personal needs.

sampling method will be applied during this phase as well. At the first stage, one district and one township will be randomly selected by randomized sortition in Shenyang. At the second stage, one urban community and one village community will be randomly selected from each selected district/township. At the third stage, with the help of local community health workers, 60 elderly patients with LLD who meet the inclusion criteria will be selected from each selected urban community and village community with a convenient sampling method.

In addition, the 60 elderly patients in the trial and control groups will receive clinical visits at the baseline and after 6, 9, and 12 months post-intervention using scales and questionnaires. The primary treatment and intervention outcome evaluation index shows the change in the total Geriatric Depression Scale (GDS) score. A secondary index includes the change in the total Older People Quality Of Life (OPQOL) score, the 36-item short-form (SF-36) from the Medical Outcomes Study (MOS), the Social Disability Screening Schedule (SDSS), the Family APGAR Scale, the Montreal Cognitive Assessment (MoCA), the Knowledge-Attitude-Practice (KAP) for LLD, the General Self-Efficacy Scale (GSES), and the Irritational Beliefs Scale (IBS). That is, the effectiveness of the supportive PDH intervention will

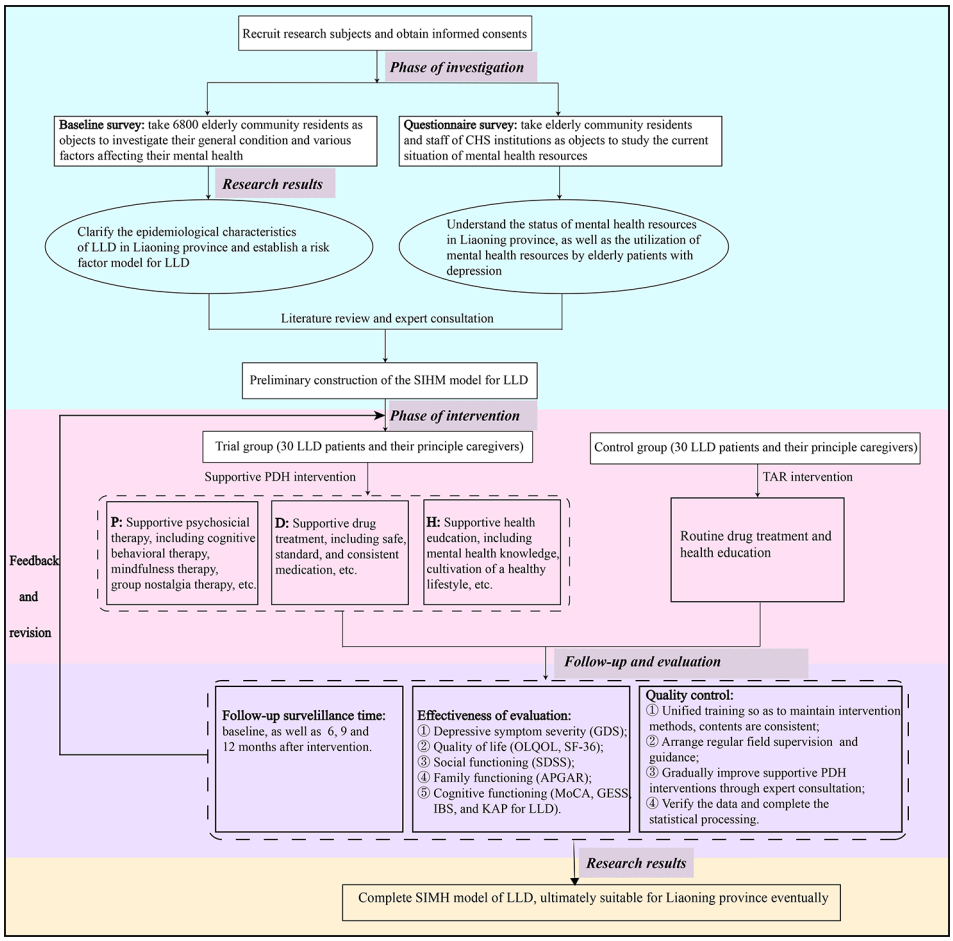

FIGURE 1 | The research flowchart.

be comprehensively evaluated in terms of depressive symptoms, QOL, and social, family, and cognitive functioning. See **Figure 1** for a detailed overview of the research procedure.

DATA COLLECTION

Screening, Demographics, Depressive Symptoms, Quality of Life, Social Functioning, Family Functioning, and Cognitive Functioning Data

At screening, the trained investigators will examine, gather, and record participant eligibility. The Chinese version of MINI (27) will be used to confirm DSM-IV TR criteria for MDD (28) and assess substance/alcohol abuse disorders and other potential exclusion criteria. After enrollment, to investigate the risk factors of LLD, data will be collected regarding the demographics, lifestyle, medical history, sleep quality, family history of psychiatric disease, LLD course, treatment and intervention process, concurrent treatment and intervention measures, social support, negative life events, activities of daily living, interests and hobbies, local mental health resources, and KAP for LLD, using a set of questionnaires that we will design. Other data, including coping style, personality traits, and the severity of depressive symptoms, will be assessed and acquired using standardized Chinese scales and questionnaires, respectively (29, 30).

Finally, to evaluate the effectiveness of supportive PDH intervention, follow-up data regarding improvements in depressive symptoms, QOL, and social, family, and cognitive functioning will be assessed and acquired using other standardized Chinese scales and questionnaires, respectively (29–31).

Sample Size, Power, and Effect Size

The primary goals of this study are to analyze the epidemiological characteristics and construct a risk model for LLD in Liaoning. The study design for this part is a cross-sectional study. Wang et al. reported the prevalence of MDD in elderly Chinese at 2.8%, where the ratio of urban to rural was 1.4:1 (10). That is, if the testing level α and the relative error ϵ are

set to 0.05 and 0.15P, 6179 community-residing elderly adults are expected to enroll, according to the following equation (32):

$$N = [Z^2_{a/2}P(1-P)]/\epsilon^2$$

Given that 10% of the data are missing, a total of 6,800 elderly residents (approximately 3414 urban residents and 3,386 rural residents) should be included.

The secondary goals of this study are to conduct and evaluate integrated supportive PDH interventions and TAR for the objects in the trial group and control group, respectively. The study design for this part is an experimental study, and the sample size can be determined by the change in the value of the mean and standard deviation of the depression score of elderly patients before and after intervention in a previous study (33). That is, if the testing level α and type-II error β are set as 0.05 and 0.10, respectively, the sample size of the trial group and control group will be roughly 25 in both cases, according to the following equation (32):

$$N_1 = N_2 = 2[\frac{(z_{\alpha/2} + z_\beta)\sigma}{\sigma}]^2$$

Given that 20% of the data are missing, each group should have a sample size of 30 LLD patients. Meanwhile, to facilitate the compliance of patients in the later intervention stages, and to ensure the effectiveness of the intervention, each patient's primary caregiver will be required to accompany the participant. In the later stages, we will further revise the sample size according to the preliminary experimental results from the intervention study.

The participants, method, assessment time, and data collection administrators are listed in **Table 3**.

Data Preprocessing and Statistical Analysis

The statistical analyses will be performed by employing the SPSS statistical package, version 20.0 (SPSS Inc., Chicago, IL, USA), and all evaluations of significance will be determined based on two-sided tests using the 0.05 level of statistical significance. In addition, missing values will be replaced by the column mean/median in this study. The details of the analytic approach are as follows.

1. Demographic and clinical characteristics, lifestyle patterns, medical history, history of alcohol and drug abuse or dependence, family history of mental illness, self-knowledge and treatment attitude, and the self-efficacy levels and depressive symptoms of the subjects will be described by frequency distribution, percentage, arithmetic mean, and standard deviation.
2. Comparison of the above variables between patients with or without LLD will be made using the independent-sample *t*-test, Mann–Whitney *U*-test, and the chi-squared test, as appropriate.
3. Correlation analysis, logistic regression (for binary variables), and multiple linear regression (ANCOVA for continuous variables) will be used to determine whether the variables can enter SEM, and to test the direct and indirect pathways among these hypothesized determinants.

TABLE 3 | Data collection at baseline and follow-up evaluation.

Domain	Measure	Participants	Method	Assessment time	Administrator
Informed consent	Informed consent	All participants [b]	Interview	Screening	Investigator
Eligibility	Inclusion/Exclusion	All participants [b]	Interview	Screening	Investigator
Psychiatric diagnoses	MINI	All participants [b]	Interview	Screening	Investigator
MDS for the elderly [a]	Team-designed questionnaire	All participants [b]	Interview	Baseline	Investigator
Coping style, personality traits	SCSQ, EPQ	All participants [b]	Interview	Baseline	Investigator
KAP for LLD	Team-designed questionnaire	All participants [b]	Interview	Baseline and every follow-up	Investigator
Local mental health resources and resources available to LLD patients	Team-designed questionnaire	All participants [b] and staff of CHS institutions	Interview	Baseline	Investigator
Treatment and intervention process, concurrent treatment and intervention measures	Team-designed questionnaire	All participants [b]	Interview	Baseline and every follow-up	Investigator
Depressive symptoms	GDS	60 LLD patients and their primary caregivers [c]	Interview	Baseline and every follow-up	Investigator
Quality of life	SF-36, OPQOL	60 LLD patients and their primary caregivers [c]	Interview	Baseline and every follow-up	Investigator
Social functioning	SDSS	60 LLD patients and their primary caregivers [c]	Interview	Baseline and every follow-up	Investigator
Family functioning	Family APGAR Scale	60 LLD patients and their primary caregivers [c]	Interview	Baseline and every follow-up	Investigator
Cognitive functioning	MoCA, GSES, IBS	60 LLD patients and their primary caregivers [c]	Interview	Baseline and every follow-up	Investigator

LLD, Late Life Depression; MINI, Mini-International Neuropsychiatric Interview; MDS, Minimum Data Set; SCSQ, Simplified Coping Style Questionnaire; EPQ, Eysenck Personality Questionnaire; KAP, Knowledge-Attitude-Practice; GDS, Geriatric Depression Scale; SF-36, The MOS item Short From Health Survey; OPQOL, Chinese Revised Version of Old People Quality Of Life Questionnaire; SDSS, Social Disability Screening Schedule; APGAR, Adaptability, Partnership, Growth, Affection, and Resolve; GSES, General Self-Efficacy Scale; IBS, Irritational Beliefs Scale; MoCA, Montreal Cognitive Assessment; CHS, Community health service.

[a]Content of MDS includes demographics, lifestyle, medical history, sleep quality, family history of psychiatric disease and LLD course, social support, negative life events, daily activities, interests, and hobbies.

[b]"All participants" refers to the 6,800 community-residing elderly adults.

[c]30 LLD patients and their primary caregivers in the trial group, and 30 LLD patients and caregivers in the control group.

4. Amos version 22.0 statistical software will be used for SEM of the risk factors for LLD.
5. GDS, SDSS, SF-36, OPQOL, Family APGAR, GSES, IBS, MoCA, and the related KAP for LLD in the elderly will be used as evaluation indices, and analysis of variance (ANOVA) will be applied to compare the results of the control and the trial groups before and after intervention.
6. Regression analysis will be used to analyze the influence of various factors on the effectiveness of the supportive PDH intervention.

Ethical Considerations

This study [approval no. (2019) 2019-312-2] was approved by the Ethics and Research Committee of the First Affiliated Hospital of China Medical University, which verified that the study will be performed in accordance with all ethical standards set forth by the committee.

Participants will be assured of the confidential nature of the study, and written informed consent will be obtained. All data collection will be anonymous and in accordance with the provision of Chinese law regulating patient autonomy, rights, and responsibilities in the field of clinical information and documentation.

DISCUSSION

Based on an analysis of the current clinical report results, there are many basic studies and investigations of the pathogenic factors of LLD. Investigations mainly focus on three groups of variables. First, researchers study the correlation between the characteristics (34, 35) (biological factors such as disease course, classification, demographics, etc.) of the disorders and their occurrence and development. Second, there is a focus on psychological factors (36, 37) (personality traits, attribution and coping style, self-esteem, etc.) and how they act on the occurrence and development of the disease. Third, researchers consider the relationship between social factors (38–40) (stressful life events, family environment, social support, marital status, economic income level, etc.) and the occurrence and development of the disease. To some extent, these studies have revealed the relationship between LLD and various influential factors, and they have identified the related variables that can predict the condition and outcome of LLD. However, few studies provide a new perspective for studying the process of integrating multiple influential research variables, while analyzing the path of interaction between variables and exploring the influence of various factors on the overall research. In our study, therefore, biological, psychological, and social factors will be integrated to analyze and predict LLD risk factors. In addition, the results will be incorporated into the system of occurrence and development of the disorder. Then, SEM of the risk factors for LLD will be constructed. Furthermore, in view of differences in the prevalence of LLD between rural and urban areas, participants will be recruited from both rural and urban sites for a broadly inclusive and representative elderly population that ensures the equilibrium of sample sources. In doing so, the results and conclusions of the study will be widely generalizable.

Given the different causes, courses, and clinical manifestations of LLD, the treatment response of antidepressants varies. Evidence from literature reviews on depressive disorders indicates that comprehensive psychosocial intervention can effectively make up for the shortcomings of single drug treatments, while improving therapeutic effect (41). Our study regards community-residing elderly adults with LLD as the research subjects, unlike previous studies, which were restricted to inpatients or discharged patients. Further, we consider supportive psychosocial, drug treatment, and health education interventions to be pertinent, based on the identified risk factors of LLD. The formulation of the content, forms, and methods of interventions will be repeatedly revised by the team members based on literature reviews, expert consultation, and preliminary experimental results. The effectiveness of intervention will be evaluated according to improvements in depressive symptoms, family functioning, social functioning, QOL, and KAP for LLD. An SIHM model for LLD will ultimately be obtained by referring to the connotations and theory of health management. In addition, strict quality control procedures, validated assessments by trained investigators, and appropriate statistical analyses will be designed and applied in this study.

There are three anticipated limitations to this study. First, data collection at the baseline and with follow-ups will be completed through questionnaires and scales sent to elderly residents and their primary caregivers. As such, the results may be affected by the cooperative attitude, understanding, and educational level of the respondents. Second, because questionnaire surveys are limited by the designed items and established form of question-and-answer, the researchers cannot fully understand the respondents' innermost feelings, thoughts, and opinions on some items beyond what is provided by the questionnaires and scales. For that, quantitative surveys and qualitative interviews might be combined in future studies to evaluate the effect of supportive PDH intervention more thoroughly. Third, the sample source of this study is limited to LLD patients in Liaoning province, so the promotion of the research results on supportive intervention and health management model for LLD patients in communities may be limited. By conducting parallel control studies in multiple regions and increasing the sample size and sources, it can be further validated and supported.

CONCLUSIONS

The prevalence and detection rate of LLD has gradually increased over the past several years, and LLD affects an increasing number of elderly people and their families. However, the disorder remains under-diagnosed and under-treated, especially in Liaoning province, China. To our knowledge, there is no relevant research on the prevalence, incidence, and characteristics of LLD in Liaoning province based on large sample surveys. Identifying the risk factors of LLD and constructing integrated supportive PDH intervention models will assist with the treatment and intervention effectiveness of LLD. The results of this study will provide strong and suitable evidence for constructing a risk factor model of LLD, and this will enhance the effectiveness of supportive PDH intervention for LLD

patients in rural and urban communities. Ultimately, this study will construct an SIMH model that integrates risk factor identification, LLD diagnosis, and a formulation and implementation of supportive PDH intervention, along with an evaluation of its effectiveness based on full consideration of the status of mental health resources in Liaoning, LLD characteristics, and the actual situation faced by elderly patients and their families.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics and Research Committee of the First Affiliated Hospital of China Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GZ designed and revised the paper. LD wrote the paper. XS contributed to the coordination between community health services institutions and ethics formalities. CF and CT were responsible for data collection and management.

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Identification of Key Genes and the Pathophysiology Associated With Major Depressive Disorder Patients Based on Integrated Bioinformatics Analysis

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Background: At present, laboratory blood tests to support major depressive disorder (MDD) diagnosis are not available. This study aimed to screen potential mRNAs for peripheral blood biomarkers and novel pathophysiology of MDD.

Methods: The present study utilized public data from two mRNA microarray datasets to analyze the hub genes changes related to MDD. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes (DEGs) were performed. Finally, some potential mRNA quality biomarkers for hub gene expression in blood were identified.

Results: A total of 25 significantly co-upregulated DEGs and 98 co-downregulated DEGs were obtained from two datasets. The pathway enrichment analyses showed that co-upregulated genes were significantly enriched in the regulation of cell-matrix adhesion and mitochondrial membrane permeability which were involved in the apoptotic process. Co-downregulated genes were mainly involved in the neutrophil activation which in turn was involved in the immune response, degranulation and cell-mediated immunity, positive regulation of immune response, the Toll-like receptor signaling pathway, and the NOD-like receptor signaling pathway. From the PPI network, 14 hub genes were obtained. Among them, the subnetworks of *PLCG1*, *BCL2A1*, *TLR8*, *FADD*, and *TLR4* screened out from our study have been shown to play a role in immune and inflammation responses.

Discussion: The potential molecular mechanisms that have been identified simultaneously include innate immunity, neuroinflammation, and neurotrophic factors for synapse function and development.

Keywords: major depressive disorder (MDD), Gene Expression Omnibus (GEO), hub genes, enrichment analysis, protein-protein interaction network (PPI)

INTRODUCTION

Major depressive disorder (MDD) is a highly disabling mental illness involving an imbalance in brain chemicals, and it majorly contributes to the global burden of disease (1). According to the World Health Organization, an estimated 350 million people of all ages suffer from depression disorder globally (2). In a systematic review, the summary estimate of the prevalence of depression or depressive symptoms among medical students was 27.2%, and that of suicidal ideation was 11.1% (3). A psychiatric disorder is not a sign of personal weakness or a character flaw, but it reveals an opposite result with a highly prevalent heritability that accounts for major psychological (4), physical (5), and social impairments (6). At present, the criteria for MDD diagnosis and treatment are based on various signs and symptoms that do not always fit into strict diagnostic categories, such as the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (7). All of the possible causes for a set of past experiences have to be examined, including personal private medical information and confidential material, which increases stigma and makes diagnosis even more difficult (8). Recent studies implicate that functional magnetic resonance imaging (fMRI) may provide successful diagnostic information in depression disorder classification (9, 10); however, objective criteria and gold standards in early diagnosis for patients with MDD remain to be elucidated (7, 11). Previously, the microarray technique was used for life science research purposes. Bioinformatics data-mining of gene and microarray technologies has widely been used for differential expression analysis to identify novel diagnostic and therapeutic biomarkers of diseases (12, 13).

Over the past decades, several biomarkers have been proposed for MDD (13–15), but at the moment none of these biomarkers reaches sufficient sensitivity and specificity to be implemented in clinical practice (14). Recently, many potential mechanism studies have demonstrated that multiple genes and cellular pathways participate in the occurrence and development of MDD (15) and other mental illnesses (16). Numerous researchers have found that the pathophysiology of depression results from changes in oxidative stress (17), immune system effects (18), and neuroinflammation (19) in the central nervous system (CNS) through cytokines, which regulate brain activities and emotions. To understand the molecular processes that control neuronal activity and arrive at an objective diagnosis, we tried to obtain novel indicators of possible molecular mechanisms and predict peripheral blood molecular biomarkers in MDD patients and attempted to provide potential therapeutic targets for this challenging disease.

In the present study, two mRNA microarray datasets with MDD and control groups were downloaded from Gene Expression Omnibus (GEO) and screened for differentially expressed genes (DEGs). Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis in the online Database of Enrichr were performed for the screened DEGs. Then, we established a protein–protein interaction (PPI) network based on the Search Tool for the Retrieval of Interacting Genes (STRING) database and Cytoscape software to identify hub genes related to MDD. Subsequently, the hub gene and miRNA-mRNA pair interactions were identified. This work will provide further insight into the pathophysiology of MDD development at the molecular level and explore the potential molecular targets for new interventional strategies.

METHODS

Microarray Data

In order to identify the genes expressed in MDD samples compared to normal tissues, after a careful review, two gene expression profiles (GSE76826 and GSE98793) were selected and downloaded from the Gene Expression Omnibus database (GEO, www.ncbi.nlm.nih.gov/geo/), which is a public functional genomics data repository of high-throughput gene expression data, chips, and microarrays.

The microarray dataset GSE76826 was deposited by Miyata et al. (20), and expression profiling arrays were generated using GPL17077 Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381 (Agilent Technologies, Inc., Palo Alto, CA). A total of 32 samples were utilized, including 10 samples of peripheral blood cells from patients with depression (MDD group), 10 samples of patients in remission, and 12 samples from healthy controls (control group). The samples of the MDD group and control group were selected for further analysis.

Additionally, the 192 gene expression profiles of the GSE98793 dataset by Leday et al. (21) were based on the GPL570 [HG-U133_Plus_2] platform using the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA). Blood samples of the dataset were collected from MDD ($N = 128$) and control patients ($N = 64$). We downloaded the raw CEL file and the probe annotation file. The probes were converted into the corresponding gene symbol according to the annotation information in the platform. All of the data were freely available online, and this study did not involve any experiment on humans or animals performed by any of the authors.

Data Pretreatment and Identification of DEGs

The raw microarray data of GSE98793 in CEL format were initially preprocessed into expression values through the Affy package (22) (http://www.bioconductor.org/packages/release/bioc/html/affy. html) in R software (version 3.5.2, https://www.r-project.org/), and then we used background correction, normalization, and summarization to create a robust multiarray average (RMA). The series matrix files of the GSE76826 dataset were the normalized log-expression values available for further analysis.

To characterize differentially expressed genes (DEGs), the control group and the MDD group were analyzed using the LIMMA (linear models for microarray data) package (23) in the R/Bioconductor platform. Benjamini–Hochberg's method was used to control the false discovery rate, and the adjusted P-value < 0.05 and $|\text{Log2 fold-change}| > 0.6$ were defined as the threshold. The Venn diagram was also constructed using the VennDiagram package (24) in R. All significant DEGs are shown in a volcano plot generated using R software.

Gene Ontology and KEGG Pathway Analysis

Gene Ontology (GO) analysis is a common and useful method for large-scale functional enrichment research. To further analyze the potential biological process (BP), molecular function (MF), and cellular component (CC), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the overlapping DEGs between two the groups was submitted to the online Database of Enrichr (http://amp.pharm.mssm.edu/Enrichr/) to conduct functional and pathway enrichment analysis in this study. Enrichr is a useful online tool for annotating genes (25–27), which provides the functionality to perform simultaneous GO and KEGG analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Protein-Protein Interaction (PPI) Network and Hub Gene Identification

To systematically analyze the biological functions of the obtained DEGs between the two groups, the DEGs identified previously were mapped into the online search tool STRING database (STRING, V11.0; https://string-db.org/) (28) that could predict the protein functional associations and protein-protein interactions (PPI). A combined score ≥ 0.4 of PPI pairs was considered significant. Then, the Cytoscape software (http://www.cytoscape.org/, version 3.7.1; Institute for Systems Biology, Seattle, WA, USA) (29) was used for constructing and visualizing the transcriptional regulatory network of common DEGs. To further identify key elements in the biological process (BP), the hub genes in the network defined as possessing a connective degree ≥ 3 were identified and visualized using the CentiScaPe v2.2 plugin (30), and the degree of each protein node using MCODE in Cytoscape was calculated. All the parameters were set as defaults.

Construction of the mRNA-miRNA Interaction Network

To construct and analyze the miRNA-mRNA regulatory network, we applied the online prediction tools TargetScan (Release 7.2; http://www.targetscan.org/vert_72/) (31) and miRTarBase (Release 7.0, http://mirtarbase.mbc.nctu.edu.tw) (32) to predict

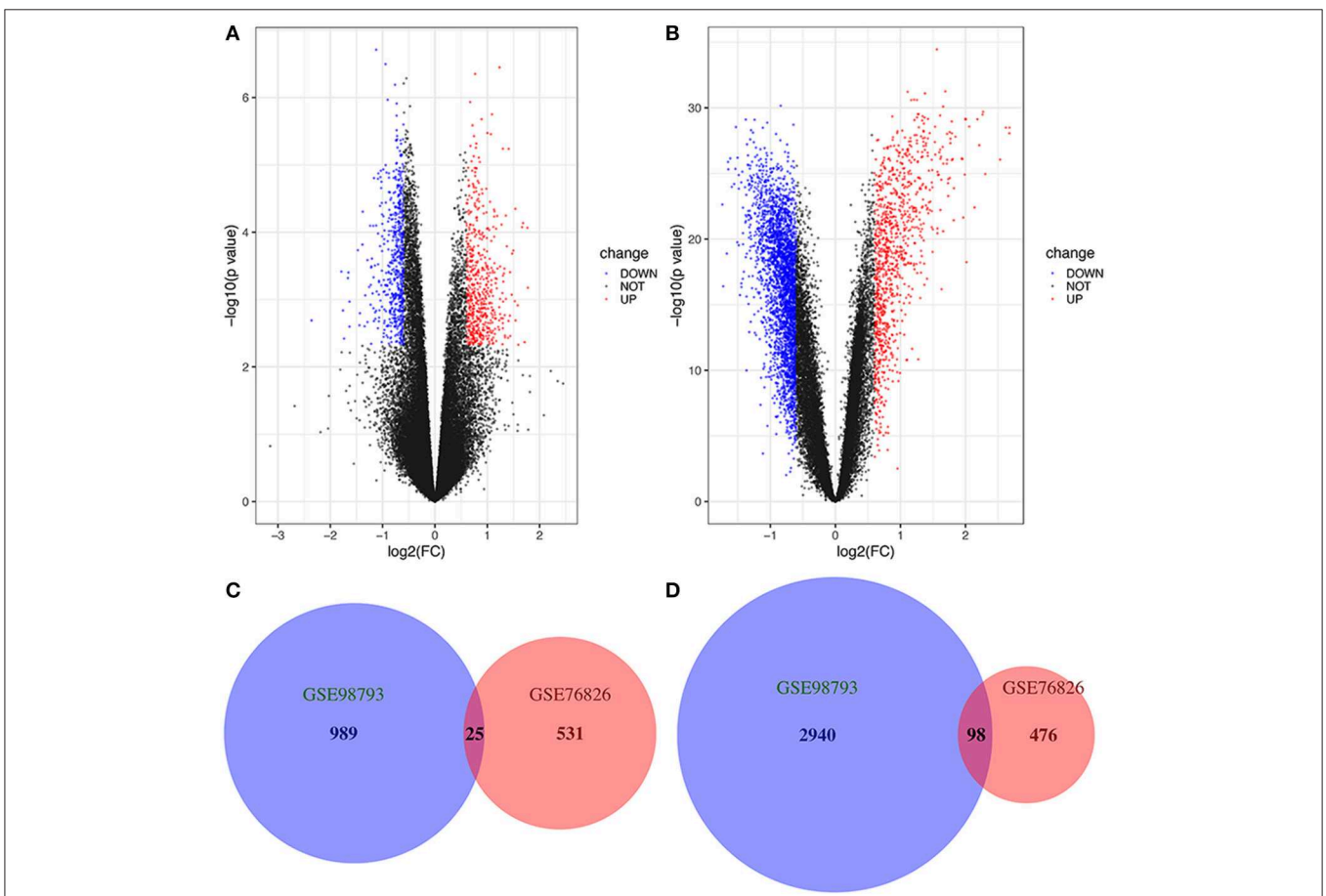

FIGURE 1 | Volcano plot and Venn diagram of DEGs in the mRNA expression profiling datasets. Volcano plots of DEGs in normal and MDD samples in the **(A)** GSE76826 and **(B)** GSE98793 datasets (FC, fold-change). Colors represent different genes: black nodes represent genes without significantly different expression, red nodes represent upregulated genes, and blue nodes represent downregulated genes. Venn diagrams illustrating the number of **(C)** upregulated and **(D)** downregulated genes in the two datasets. The intersection in red represents the DEGs that are common between the two datasets.

the possible target mRNAs. Those mRNA–miRNA pairs with inverse expression relationships were included for network construction. Finally, we used Cytoscape software to construct interaction networks of mRNAs and related miRNAs.

RESULTS

Identification of Differentially Expressed Genes

We studied two microarray MDD datasets (GSE76826 and GSE98793) from independent experiments to detect DEGs that were dysregulated in MDD samples compared to normal samples. In the GSE76826 gene chip, 1130 DEGs were identified; 556 genes were upregulated, and 574 genes were downregulated (**Figure 1A**). In addition to GSE98793, 4052 DEGs, including 1,014 upregulated genes and 3,038 downregulated genes, were identified (**Figure 1B**). The overlap analysis between the two datasets contained 25 significantly co-upregulated genes and 98 co-downregulated genes, as shown in the Venn diagram in R (**Figures 1C,D**). As a result, the number of co-downregulated DEGs is larger than the number of co-upregulated DEGs.

GO Functional and KEGG Pathway Enrichment Analysis of DEGs

To further investigate the functions and mechanisms of DEGs, GO and KEGG pathway enrichment analyses of upregulated and downregulated genes were performed in the online Enrichr database. According to the results of the enrichment analysis, a total of 337 GO terms and 27 pathways of DEGs (FDR < 0.05), including 269 biological processes (BPs), 36 cellular components (CCs), and 32 molecular functions (MFs), were obtained, and the top five of each items are presented (**Table 1**).

TABLE 1 | The top five GO terms in enrichment analyses of DEGs.

Category term	Description	Gene counts	*P*-value
Upregulated genes			
BP GO:0001952	Regulation of cell-matrix adhesion	2	0.001987916
BP GO:0031958	Corticosteroid receptor signaling pathway	1	0.008718559
BP GO:0042921	Glucocorticoid receptor signaling pathway	1	0.008718559
BP GO:0010839	Negative regulation of keratinocyte proliferation	1	0.009958094
BP GO:1902108	Regulation of mitochondrial membrane permeability involved in apoptotic process	1	0.009958094
CC GO:0005813	Centrosome	3	0.019316472
CC GO:0000242	Pericentriolar material	1	0.021047158
CC GO:0043292	Contractile fiber	1	0.033228487
CC GO:0030016	Myofibril	1	0.034438585
CC GO:0016607	Nuclear speck	2	0.052692171
MF GO:0005168	Neurotrophin TRKA receptor binding	1	0.008718559
MF GO:0016290	Palmitoyl-CoA hydrolase activity	1	0.008718559
MF GO:1990247	N6-methyladenosine-containing RNA binding	1	0.009958094
MF GO:0005167	Neurotrophin TRK receptor binding	1	0.011196142
MF GO:0004385	Guanylate kinase activity	1	0.017364119
Downregulated genes			
BP GO:0043312	Neutrophil degranulation	14	9.01080E-08
BP GO:0002283	Neutrophil activation involved in immune response	141	9.97699E-08
BP GO:0002446	Neutrophil-mediated immunity	14	1.10359E-07
BP GO:0050778	Positive regulation of immune response	5	7.30177E-06
BP GO:0032757	Positive regulation of interleukin-8 production	4	6.92217E-05
CC GO:0042581	Specific granule	8	1.26147E-06
CC GO:0101002	Ficolin-1-rich granule	8	3.56455E-06
CC GO:1904813	Ficolin-1-rich granule lumen	6	3.32349E-05
CC GO:0035579	Specific granule membrane	5	8.94684E-05
CC GO:0034774	Secretory granule lumen	8	1.70272E-04
MF GO:0005509	Calcium ion binding	8	8.00000E-05
MF GO:0046872	Metal ion binding	9	3.26444E-04
MF GO:0017110	Nucleoside-diphosphatase activity	2	2.08127E-03
MF GO:0016620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	2	0.007154400
MF GO:0032813	Tumor necrosis factor receptor superfamily binding	2	0.007702242

If there were more than five terms enriched in this category, the top five terms were selected according to P-value. BP, biological process; CC, cellular component; DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function.

The GO analysis results showed that for BP, upregulated DEGs were significantly enriched in glucocorticoid and corticosteroid receptor signaling pathways, regulation of cell-matrix adhesion, negative regulation of keratinocyte proliferation, and regulation of mitochondrial membrane permeability involved in apoptotic processes. Downregulated DEGs were significantly enriched in neutrophil-mediated immunity, degranulation, and activation involved in the immune response, positive regulation of the immune response and interleukin-8 production. Upregulated DEGs that were significantly enriched in CC, included centrosome, pericentriolar material, contractile fiber and myofibril. Downregulated DEGs that were significantly enriched in CCs included specific granule, specific granule membrane, secretory and ficolin-1-rich granule lumen. GO MF showed that the upregulated DEGs were significantly enriched in neurotrophin TRKA receptor binding, palmitoyl-CoA hydrolase activity, N6-methyladenosine-containing RNA binding, neurotrophin TRK receptor binding, and guanylate kinase activity. Downregulated DEGs were significantly enriched in calcium ion binding, metal ion binding, nucleoside-diphosphatase activity, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor, and tumor necrosis factor receptor superfamily binding. These results are comprehensively summarized (**Table 1**).

Moreover, 27 KEGG pathways were overrepresented in the DEGs. Only two upregulated DEGs, including the thyroid hormone signaling pathway and fatty acid elongation, and the 25 downregulated DEGs were significantly enriched in KEGG pathways, including measles, Toll-like receptor signaling pathway, complement and coagulation cascades, hepatitis B and influenza A, etc. The results obtained for the KEGG enrichment analyses are shown in **Figure 2**.

PPI Network Construction and Hub Gene Identification

To systematically analyze the biological functions of the obtained DEGs between the two groups, a PPI network of DEGs was constructed based on the STRING database and was visualized by Cytoscape (**Figure 3A**). In the PPI network, which has 54 nodes and 60 edges, it is well acknowledged that subnetwork analysis of genes plays important roles in integrated biological networks. Based on the results of the degree calculation using the cytoHubba plugin of Cytoscape, the most significant module was identified to have relatively high degrees in the regulatory network (**Figure 3B**).

The hub genes may play significant key roles in signal transduction during the progression of MDD, which were determined from the PPI network using the cytoHubba plugin (**Figure 3A**). A total of 14 genes were identified as hub genes. The gene symbols, full names, and implications of these hub genes are shown in **Table 2**.

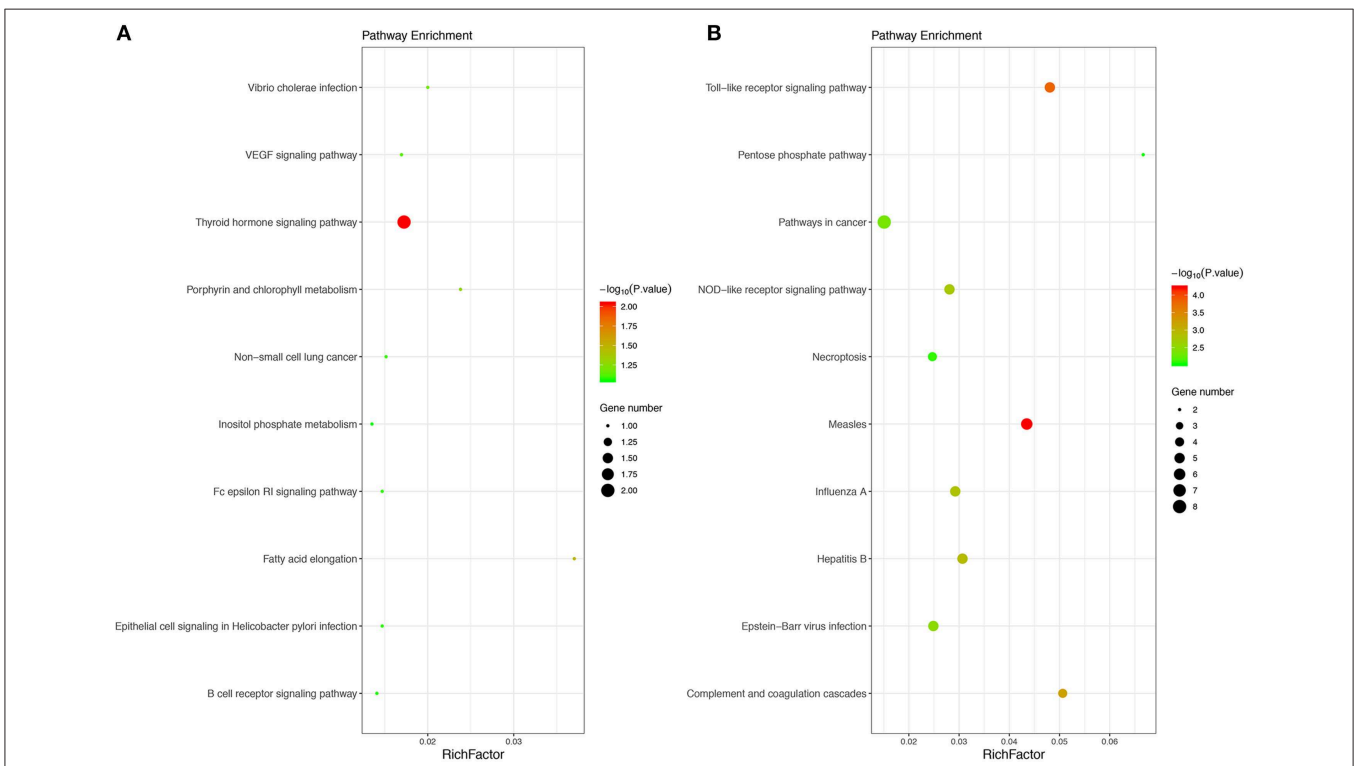

FIGURE 2 | The top 10 KEGG pathways of upregulated **(A)** and downregulated **(B)** enriched DEGs. The size of bubble shows the enrichment score, while colors indicate enrichment significance. KEGG, Kyoto Encyclopedia of Genes and Genomes.

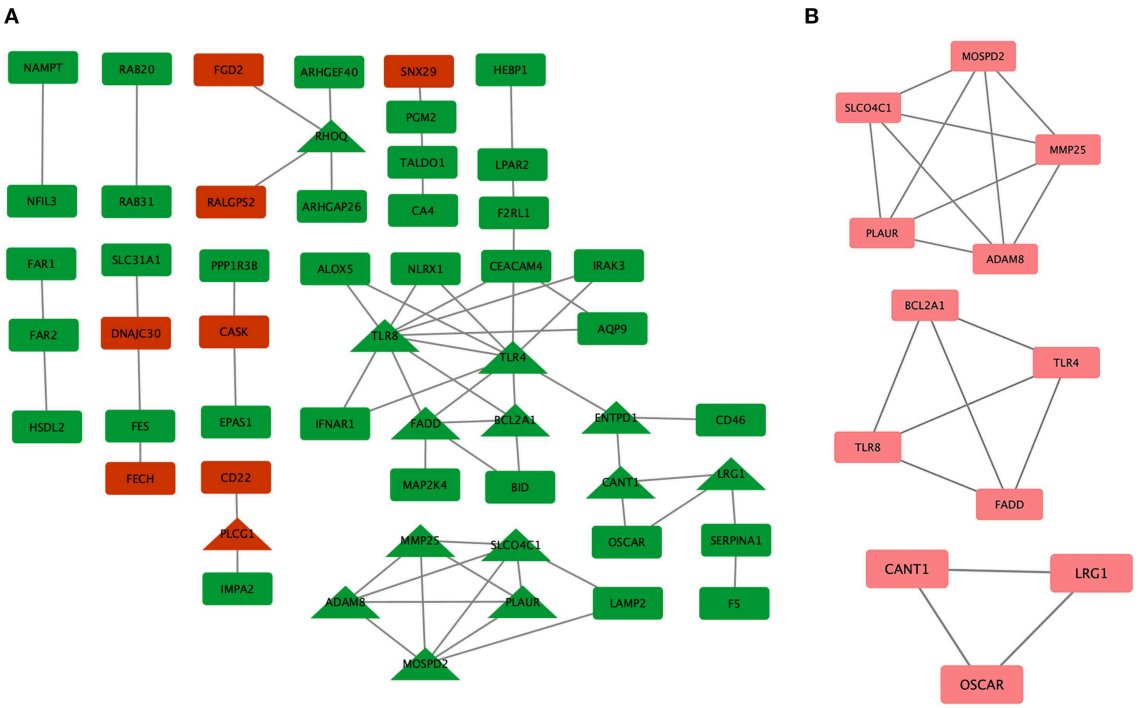

FIGURE 3 | Protein-protein interaction (PPI) network of differentially expressed genes (DEGs) in major depressive disorder (MDD) samples. **(A)** Triangular nodes represent hub genes; red nodes represent upregulated genes; green nodes represent downregulated genes. **(B)** The most significant module was obtained from the PPI network with 12 nodes and 19 edges.

Integrated Network Analysis of miRNA-mRNA Interactions

According to the hub genes identified previously, miRNA-target gene interaction pairs of reverse association were predicted by the miRTarBase and TargetScan databases, respectively. Based on the identified miRNA-mRNA pairs, we compared the interaction network containing 72 miRNA-mRNA pairs and visualized them with Cytoscape software. By comparing the targets of hub genes, PLCG1 was found to be a potential target of 20 miRNAs, including hsa-miR-218, hsa-miR-1, hsa-miR-30*, hsa-miR-320a, hsa-miR-200*, hsa-miR-331, hsa-miR-369, hsa-miR-429*, and hsa-miR-34*. Moreover, MOSPD2 and ENTPD1 were the potential targets of 8 miRNAs and 13 miRNAs, respectively. The miRNA-gene regulation network is presented in **Figure 4**.

DISCUSSION

Depression is a major human blight that has become a pervasive public health problem (3, 46). Despite the rising prevalence of MDD, we lack an understanding of the distinctive pathophysiology in contrast to many other brain disorders. At present, laboratory blood tests to support MDD diagnosis are not available, so diagnosing this disorder is more challenging than measuring height (47). Recently, however, genetic insights transformed a featureless landscape into one with real scientific toeholds (48). The rapidly developing and wide use of microarray technology has revealed thousands of genetic alterations during the progression of diseases, which may provide promising targets for the early diagnosis of mental illness (49). Thus, there is a great need to identify biomarkers and provide proof of principle for a translational approach to prioritize blood biomarkers of mood state in MDD samples. In the present study, we explored the crucial genes of blood biomarkers and pathways associated with MDD by bioinformatics methods. To achieve this, two mRNA microarray datasets were analyzed to obtain DEGs and hub genes between peripheral blood from patients with MDD and that from the control group. A total of 123 DEGs (25 co-upregulated genes and 98 co-downregulated genes) and 14 hub genes were identified between the two datasets. Then, the DEGs were subjected to functional and pathway enrichment analysis, and a PPI network was constructed and integrated network analysis of miRNA-mRNA interactions performed to enhance our understanding of the molecular mechanisms of MDD.

To analyze the functional and pathway enrichment of DEGs between the subject groups, significant GO BP terms and pathways were obtained, including corticosteroid and glucocorticoid receptor signaling pathways, regulation of cell-matrix adhesion, and mitochondrial membrane permeability involved in apoptotic processes in upregulated genes. Downregulated genes were associated with neutrophil activation involved in the immune response, degranulation and mediated immunity, positive regulation of immune response and interleukin-8 production. Because the neutrophil activation

TABLE 2 | Implications of the 14 hub genes.

Gene symbol	Full name	Implications
PLCG1	Phospholipase C gamma 1	PLCs control neuronal activity, which is important for synapse function and development. In addition, dysregulation of primary PLC signaling is linked to several brain disorders including schizophrenia, bipolar disorder and depression (33, 34).
RHOQ	Rho family member Q	Collybistin activation by RHOQ enhances postsynaptic gephyrin clustering and hippocampal GABAergic neurotransmission (35).
TLR8	Toll-like receptor 8	Antidepressants normalize elevated Toll-like receptor profiles in MDD (36).
TLR4	Toll-like receptor 4	The TLR4 signaling pathway may be a potential target for the anti-inflammatory treatment of depression (36, 37).
FADD	Fas associated via death domain	The neurochemical adaptations of brain FADD could play major role in counteracting the known activation of the mitochondrial apoptotic pathway in MDD (38).
BCL2A1	BCL2-related protein A1	BCL2 may play an important role in mediating the outcome of antidepressant treatment (39).
ENTPD1	Ectonucleoside triphosphate	Rodent studies suggest that ENTPD may be due to treatment diphosphohydrolase 1 with antipsychotics (40, 41).
CANT1	Calcium activated nucleotidase 1	The association between CANT1 and MDD has not been reported.
LRG1	Leucine-rich alpha-2-glycoprotein 1	The combination of increased LRG1 levels shows promise as a plasma-based diagnostic biomarker panel for detecting increased poststroke depression risk (42).
MMP25	Matrix metalloproteinase-25	The association between MMP25 and MDD has not been reported.
SLCO4C	Solute carrier organic anion	The association between SLCO4C1 and MDD has not been transporter family member 4C1 reported.
ADAM8	ADAM metallopeptidase domain 8	Possible involvement in extravasation of leukocytes (43).
MOSPD2	Motile sperm domain containing 2	Promotes migration of primary monocytes and neutrophils, in response to various chemokines (44).
PLAUR	Plasminogen activator, urokinase receptor	An element of the uPAR system and the molecules that collectively play a role in inflammation, tissue and axonal regeneration within the CNS (45).

MDD, major depressive disorder; CNS, central nervous system.

involved in the immune response appeared in the downregulated genes in the pathway analysis results, it could be involved in an important part of MDD. Surprisingly, we observed in the literature that depression is mostly correlated with both peripheral inflammatory processes and alterations in cellular immunity, mainly for cell-mediated immunity. To date, a large number of studies have demonstrated that depression has been associated with positive regulation of interleukin-8 production and immune response (18, 50, 51). Euteneuer et al. (52) revealed that patients with MDD exhibited higher neutrophil and monocyte counts and an increased neutrophil to lymphocyte ratio (NLR) than controls. They also found that lower anti-inflammatory activity was related to more severe somatic depressive symptoms. Although there have been few studies on the immune response and MDD, and it is still unknown how the immune response regulates the pathology of depression. According to our analysis results, we speculate that the cellular immunity system might take part in the progression of MDD.

Based on the KEGG pathway analysis, downregulated DEGs were enriched for the Toll-like receptor signaling pathway, complement and coagulation cascades, NOD-like receptor signaling pathway, hepatitis B, measles, and influenza A. Recent data have demonstrated that NOD-like receptor pyrin containing 3 (NLRP3) activation appears to bridge the gap between immune activation and metabolic danger signals or stress exposure, which are key factors in the pathogenesis of MDD and other psychiatric disorders. TLRs also seem to be present in humans, and recent studies showed that the mRNA expression of *TLR3* and *TLR4* was significantly increased in the dorsolateral prefrontal cortex (DLPFC) of depressed individuals compared with controls (53, 54). Further experiments at the transcription and protein expression levels suggest that *TLR3* and *TLR4* appear to be unique and important in brain functions (55). There is mostly evidence for Toll-like receptors (TLRs) in the brain that are associated with depression and suicide (53). Interestingly, the Clinical Practice Research Datalink (CPRD) study from the UK-based primary care database suggests that influenza A infections are associated with a moderately increased risk of developing depression (56).

A PPI network was constructed to investigate the interrelationship of the DEGs, and 14 hub genes were identified, including *RHOQ*, *TLR8*, *TLR4*, *FADD*, *BCL2A1*, *ENTPD1*, *CANT1*, *LRG1*, *MMP25*, *SLCO4C1*, *ADAM8*, *MOSPD2*, *PLAUR,* and *PLCG1*. In addition to *PLCG1*, all other genes were downregulated in the PPI network. Inflammation is not the only cause of depression and cannot explain its entire pathophysiology, but it is an important pathogenic factor that explains one possible mechanism of depression. The subnetwork of *PLCG1*, *BCL2A1*, *TLR8*, *FADD*, and *TLR4* screened out from our study has been shown to play a role in inflammation (36–39). *FADD* and *BCL2A1* were implicated in nonapoptotic cellular processes and emerged as new actors in innate immunity and inflammation. According to a previous study, the anti-inflammatory effects and TLR profiles are predictors of the response to antidepressant treatment in patients with MDD (36, 55). As a pattern recognition receptor, TLR4 has been shown to play a vital role in neuroinflammation. The TLR4-specific inhibitor Cli-095 markedly inhibited the upregulation of *TLR4* in the hippocampus and prefrontal cortex, and improved chronic unpredictable mild stress-induced depression-like behaviors in mice (57). Another study showed that stress significantly increased the expression of TLR4 and NF-κB in the hippocampus, and this phenomenon could be attenuated in TLR4 knockout mice (58). A growing body of research indicates that inflammation plays a critical role in the etiology and pathophysiology of depression.

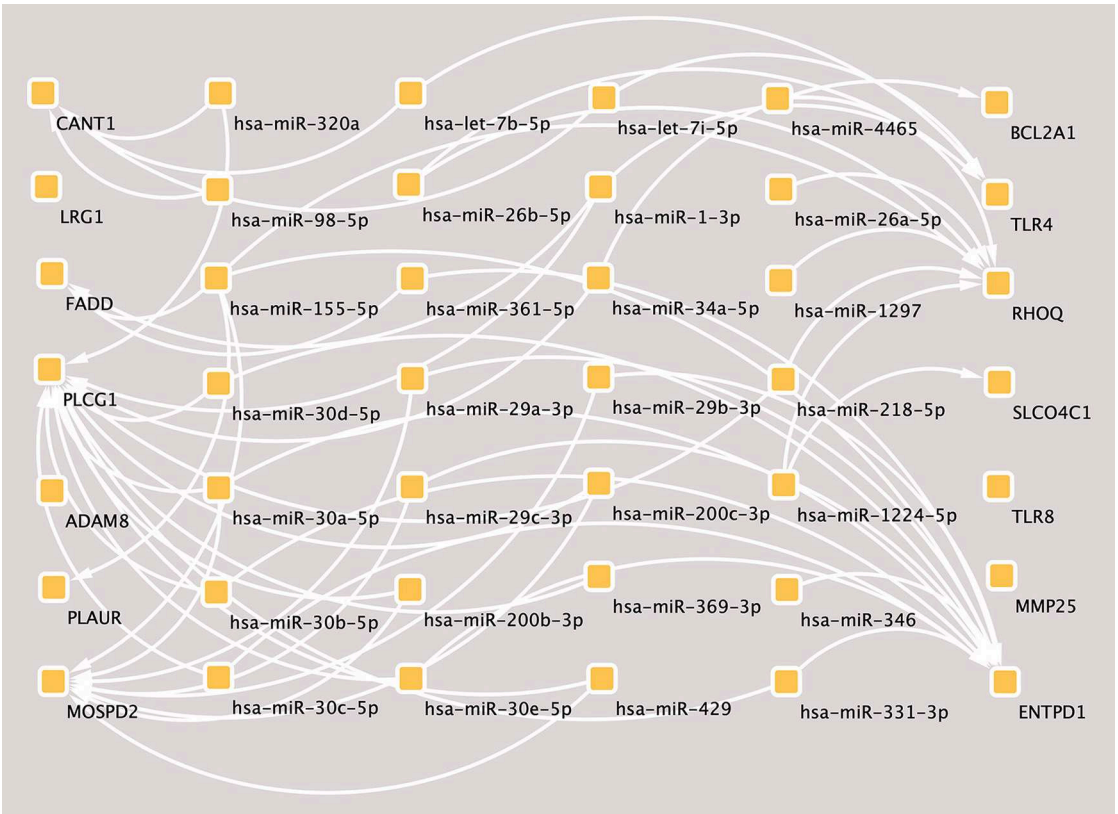

FIGURE 4 | The miRNA-mRNAs regulatory network in major depressive disorder (MDD). Solid lines indicate interaction associations between the miRNAs and mRNAs. miRNA, microRNA; mRNA, messenger RNA.

miRNAs are a group of endogenous non-coding RNA molecules that likely regulate ∼30% of human protein coding genes (59). According to the miRNA-mRNA binding data from online prediction tools, we identified genuine human miRNA-mRNA target pairs of MDD. In the present study, *PLCG1* was predicted to be a potential target of 20 differentially expressed miRNAs and was upregulated in MDD. Research has shown that BDNF-mediated PLCG1 signaling is required for the formation and function of inhibitory synapses, whereby the disruption of PLCG1 signaling in the hippocampus leads to such dysfunctions. Interestingly, a clinical study showed that 5-HT1A signaling through tyrosine kinase receptors activates PLC/protein kinase C (PKC) signaling, mediating the synaptogenesis and behavioral actions of anti-depressants (60). Furthermore, previous studies have demonstrated that PLAUR plays a role in inflammation, tissue regeneration and axonal regeneration within the central nervous system (CNS) (45). In the brain, the PLAUR/Rho system seems to promote axonal recovery following a synapse function injury (61), which may be a potential target for the development of therapeutic strategies. The binding of recombinant PLAUR activation of β1 integrin via low-density lipoprotein receptor-related protein-1 (LRP1) leads to activation of the Rho family small GTPase Rac1 and Rac1-induced axonal regeneration (62). Furthermore, the miRNA-mRNA target pair network identified that an integral membrane protein ecto-ATPase enzyme, belonging to the nucleoside triphosphate diphosphohydrolase family (ENTPD1), was potentially targeted by various differentially expressed miRNAs. There is evidence that long-term depression might be modulated by ATP and/or its dephosphorylated product adenosine, such as E-NTPDases (41), which might contribute to the neural basis for learning and memory mechanisms. Molecular and cellular studies have demonstrated that the expression of neurotrophic factors, particularly brain-derived neurotrophic factors, is important for synapse function and development (63, 64).

LIMITATIONS

The results of this study should be interpreted within the context of important limitations. First, our study utilized public data, but after screening of mRNA with clinical diagnostic and prognostic predictive value, it should be further explore the function of mRNA by *in vitro* and *in vivo* experiments. Second, the samples are from the peripheral blood cells of patients, so the associated analysis of miRNA/mRNAs in the brain regions with depression-related dysfunction may validate the data and strengthen the conclusion. Third, further validation studies could lead to additional insights into the disease process as well as the

validation and identification of additional functional biomarker candidates for improved clinical diagnostic of MDD patients.

CONCLUSION

In summary, this comprehensive bioinformatic analysis has identified numerous useful molecular targets for the future investigation of the mechanisms and selection of biomarkers for MDD. Some important biological processes and pathways, including the corticosteroid and glucocorticoid receptor signaling pathways, the Toll-like receptor signaling pathway, the NOD-like receptor signaling pathway, the neutrophil activation involved in the immune response, as well as the hub genes working in these processes, may provide novel insights into the development and progression of MDD. Furthermore, the potential molecular mechanisms that have been identified simultaneously include innate immunity, neuroinflammation, and neurotrophic factors for synapse function and development. In addition, further molecular biological experiments will be performed by our team to confirm the function of the identified genes in MDD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus database (GEO, www.ncbi.nlm.nih.gov/geo/): GSE76826 and GSE98793.

AUTHOR CONTRIBUTIONS

GZ and SX conceptualized and designed the article. ZZ, YZ, YW, JL, and JA analysed and interpreted the data. GZ drafted of the article. ZY, LS, and TS were responsible for critical revision of the article for important intellectual content. TS finally approved the article.

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The Insular Subregions Topological Characteristics of Patients With Bipolar Depressive Disorder

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The insular cortex appears to have a crucial role in emotional processing and cognitive control in bipolar disorder (BD). However, most previous studies focused on the entire insular region of BD, neglecting the topological profile of its subregions. Our study aimed to investigate its subregion topological characteristics using the resting-state functional connectivity (rsFC) in patients with BD on depression episode. The magnetic resonance imaging (MRI) data of 28 depressed BD patients and 28 age- and gender-matched healthy controls (HCs) were acquired. We observed that compared to HCs, depressed patients with BD exhibited significantly decreased rsFC between the right ventral anterior insula (vAI) and the left middle temporal gyrus/the right angular, the right dorsal anterior insula (dAI) and the left precuneus, as well as the right posterior insula and the right lingual gyrus. Furthermore, hyperconnectivity was observed between the left dAI and the left medial frontal gyrus, as well as right dAI and left superior temporal gyrus in BD depression. However, no significant group effect was observed between aberrant FC patterns and clinical variables. These findings revealed the functional connectivity patterns of insular subregions for the depressed BD patients, suggesting the potential neural substrate of insular subregions involved in depressive episode of BD. Hence, these results may provide a neural substrate for the potential treatment target of BD on depression episode.

Keywords: bipolar disorder, functional connectivity, resting-state magnetic resonance imaging, insular subregions, neural substrate

INTRODUCTION

Bipolar disorder (BD) is a chronic mental disease, characterized by alternating episodes between mania and depression (1). The high frequency and long duration of depressed symptoms is susceptible to psychosocial dysfunction and poor treatment, which further increase disease's burden, even the risk of suicide (2, 3). Therefore, it is a compelling need to investigate the mechanisms of depression in BD.

Accumulating evidence from neuroimaging studies have suggested that the insular cortex is critically involved in the pathogenesis of BD (4–6). Recent meta-analyses of studies using voxel-based morphometry revealed that patients with BD had aberrant structure and morphology in the insula (7, 8). Furthermore, one positron emission tomography (PET) study found that BD patients showed significantly higher binding rate of serotonin transporter in the insula (9).

Interestingly, the insula consists of several subregions, namely the ventral anterior insula (vAI), the dorsal anterior insula (dAI), and posterior insula (PI) (10), showing distinct histological characteristics (11). Meanwhile, this segmentation of insular cortex was confirmed by diffusion tensor imaging (DTI) data (12, 13). Previous studies found decreased volume of bilateral AI in BD patients (14, 15), which might be associated with abnormal emotional regulation in BD (16, 17). Besides, AI may provide transdiagnostic signatures to differentiate BD from major depressive disorder (MDD) (18). Taken together, the insular subregions may play distinctive roles on the pathogenesis of BD. Therefore, it will be meaningful to explore the topological profiles of insular subregions in BD.

On the other hand, the functional connectivity (FC) has been successfully applied for mapping complex neural circuits, reflecting the organization of brain networks. Numerous functional magnetic resonance imaging (fMRI) studies have demonstrated that BD patients showed abnormal FC patterns in some specific brain regions, such as between the pregenual anterior cingulate cortex (ACC) and amygdala/thalamus/pallidostriatum, respectively (19), as well as between amygdala and dorsal lateral prefrontal cortex (VLPFC) (20). Notably, the aberrant FC pattern between the insula and the PFC has also been observed in BD patients (21). Besides, previous study revealed that the aberrant FC between the AI and the inferior parietal lobule (IPL) of the executive control network (ECN) contributed to distinguishing dimension of emotion regulation between BD and MDD. Thus, the distinct FC patterns of insular subregions may provide potential neural substrate underlying emotion regulation dimension in BD patients.

In this study, we examined intrinsic FC of insular subregion in patients with BD. We hypothesized that depressed patients with BD would exhibit disrupted FC between insular subregions and some specific brain regions associated with emotion regulation. Furthermore, we hope to explore the relationship between the aberrant FC patterns of insular subregions and the symptom dimensions of BD on the episode.

METHODS

Participants

Twenty-eight patients with BD on depression episode were enrolled from outpatient departments at Shanghai Mental Health Center. The BD patients were diagnosed independently by two physicians based on the Structured Clinical Interviews for Diagnostic and Statistical Manual Fourth Edition (DSM-IV). Including criteria: having been diagnosed as BD with current depression episode, aged 18–60 years, being right-handed, and having more than 9 years of education. To reduce the risk of mood instability, participants with BD were allowed to continue medication treatment, such as lithium, atypical antipsychotics, anticonvulsants (e.g., valproate, lamotrigine, carbamazepine, or topiramate), and antidepressants. The 24-item Hamilton Rating Scale for Depression (HAMD) >20 (22) and the Young Mania Rating Scale (YMRS) <7 (23) were collected to assess the clinical symptoms of BD patients. Thirty age- and gender-matched healthy volunteers were recruited from local community by advertisement. Excluding criteria of all participants were as follows: having a history of Axis I or Axis II psychiatric disorders of DSM-IV, having a history of substance dependence or substance abuse within the 6 months prior to assessment, having a history of electroconvulsive therapy, suffering serious neurological or medical disorders (e.g., head trauma and epilepsy), and other MRI contraindications (e.g., pregnancy and breast-feeding).

The study was approved by the Investigational Review Board (IRB00002733—Shanghai Mental Health Center, China). All participants gave written informed consent after a full description of the aims and design of the study.

Image Acquisition

MRI raw data was acquired using Siemens 3.0 T MRI scanner in Shanghai Mental Health Center. High-resolution T1 images were acquired by the gradient recalled echo (GRE) sequence as the following parameters: repetition time (TR) = 2300 ms, echo time (TE) = 2.96 ms, field of view (FOV) = 24 × 24 cm^2, slice thickness = 1.0 mm, 192 slices, gap = 0.0 mm, voxel = 1.0 × 1.0 × 1.0 mm^3, matrix = 240 × 256, and scanning time = 9 min 14 s. Resting-state images were collected by echo planar imaging (EPI) sequence as the following parameters: TR/TE = 2000 ms/30 ms, FOV = 220 × 220 mm^2, slice thickness = 4.0 mm, 33 slices, gap = 0.6 mm, voxel = 3.4×3.4×4.0 mm^3, scanning time = 6 min 46 s, and 200 bolds. During the scanning, the participants were instructed to keep resting with their eyes closed.

Data Preprocessing

Resting-state fMRI images were preprocessed using a toolbox of Data Processing and Analysis for Brain Imaging (DPABI, http://rfmri.org/dpabi). The first 10 volumes from each subject were discarded for the stability of the initial magnetic resonance imaging signal. For each participant, fMRI scans were first realigned to correct for head motion. Exclusion criteria for excessive head motion were >2.5 mm and/or translation >2.5° rotation. The nuisance covariates (i.e., the six motion parameters, the first time derivations, signals of the global brain, cerebrospinal fluid, and white matter) were regressed out from the MRI data. The processed data were band-pass filtered by using a frequency range of 0.01–0.08 Hz. A two-step coregistration method were used to transform the regressed fMRI data into the Montreal Neurological Institute (MNI) space: first, each subject's structural images were coregistered with the mean realigned fMRI image; then the structural images were segmented into gray matter, white matter, and cerebrospinal fluid on the basis of transformation parameters that coregistered with the MNI T1-weighted template. Realigned

images were then normalized to the MNI space and resampled to $3 \times 3 \times 3$ mm^3 voxels. Finally, the images were smoothed with an 8-mm full width at half maximum (FWHM) Gaussian kernel. We also calculated frame-wise displacement (FD), which indexes the volume-to-volume changes in head position (24). There was no significant difference in mean FD ($T = 0.02$, $p = 0.87$) between BD patients (0.15 ± 0.09) and healthy controls (HCs; 0.16 ± 0.08).

Definition of Insular Subregions

The insular seed regions of interest (ROI) were defined using masks based on the previous study (25). Selection of target ROIs of the insular subregions were defined using max voxel locations as described in Deen et al. (25). Spherical ROI masks (3 mm diameter) were created for each of the target ROIs using the DPABI, with max voxel locations as reported in Deen et al. specified as center of sphere (**Table 1**).

Resting-State fMRI Analyses

Connectivity maps were obtained at the individual subject level for bilateral subregions within the insular seed regions by averaging the signal across all voxels in the ROI. Then, to calculate Pearson's correlation between the mean ROI time-series and the time-series from each whole brain acquired voxel. Correlation maps were converted to z-maps using Fisher's r-to-z transformation. Mean Fisher's z transformed values were extracted from target ROI masks using MarsBar and imported into SPSS (IBM, version 19.0) for analysis.

Seed-to-Voxel

A whole-brain approach was used to explore whole-brain FC anchored on bilateral insular subregions in BD and HCs. Seed-to-voxel analyses of the FC differences between the groups were performed separately using the two-sample *t*-test using DPABI, as the age, gender, education, and mean FD were covariates. The significant threshold was $p < 0.05$, and it was corrected for multiple comparisons with a Gaussian random field (GRF) correction. Once a significant FC difference between patients and controls ($p < 0.05$, voxel z value > 2.3, GRF corrected) was observed, multiple comparison corrections were performed to identify the surviving clusters.

Relationship Analysis Between Clinical Variables and FC Patterns

TABLE 1 | The MNI coordinates of the ROIs.

	The MNI coordinates		
	X	Y	Z
L_vAI	−33	13	−7
R_vAI	32	10	−6
L_dAI	−38	6	2
R_dAI	35	7	3
L_PI	−38	−6	5
R_PI	35	−11	6

L, left; R, right; vAI, ventral anterior insula; dAI, dorsal anterior insula; PI, posterior insula; MNI, Montreal Neurological Institute; ROIs, regions of interest.

We extracted the mean values of significantly aberrant FC patterns. Then, Pearson's partial correlations (two-tailed) were conducted between significantly aberrant FC and HAMD scores, controlling for age and gender. Notably, the HAMD scale was categorized into seven subscale factors based on its Chinese version, including anxiety/somatization, change of weight, cognitive dysfunction, atypical circadian rhythm, retardation, sleep disorder, and desperation.

RESULTS

Demographics and Clinical Characteristics

No significant differences were observed in gender (male/female: 14/14 vs. 18/10), age (31.79 ± 12.83 vs. 33.79 ± 9.95), and education (13.32 ± 3.37 vs. 13.64 ± 3.35) between BD patients and HCs (all $p > 0.05$). The detailed information was showed in **Table 2**.

Group Differences in Seed-Based Insular-Subregion Networks

Compared with HCs, the patients with BD had significantly decreased FC between the R_vAI and the left middle temporal gyrus ($T = -4.66$, $p < 0.05$, **Figure 1**), as well as the right angular ($T = -5.17$, $p < 0.05$, **Figure 1**). BD patients had increased FC than HCs between the L_dAI and the left medial frontal gyrus (MFG; $T = 5.51$, $p < 0.05$, **Figure 2**). While showing increased FC between the R_dAI and the left superior temporal gyrus (STG; $T = 4.19$, $p < 0.05$, **Figure 3**), BD patients had significantly decreased connectivity between the R_dAI and the left precuneus ($T = -4.88$, $p < 0.05$, **Figure 3**) than HCs. Patients with BD also had significantly decreased connectivity than HCs between the R_PI and the right lingual gyrus ($T = -4.41$, $p < 0.05$, **Figure 4**). The detailed information was observed in **Table 3**. These results survived even after correction for multiple comparisons ($p < 0.05$, voxel z value > 2.3, GRF corrected). No other group

TABLE 2 | Demographic and clinical characteristics of BD and HCs groups.

	BD (n = 28)	HCs (n = 28)	T/χ^2	*p*
Gender (M/F)	14/14	18/10	1.17[‡]	0.28
Age (years)	31.79 ± 12.83	33.79 ± 9.95	0.65[‡‡]	0.52
Education (years)	13.32 ± 3.37	13.64 ± 3.35	0.36[‡‡]	0.72
HAMD score	31.04 ± 7.92	–	–	–
Psychotropic medications, no.	23	–	–	–
Antidepressants	9	–	–	–
Lithium	7	–	–	–
Antiepileptic	10	–	–	–
Anxiolytics	4	–	–	–
Antipsychotics	13	–	–	–
Medication-free, no.	5	–	–	–

[‡]*Chi-square test for the gender distribution between BD and HCs groups.*
[‡‡]*Two-sample t-test for the group differences in both age and education.*
BD, bipolar disorder; HCs, healthy controls; HAMD, Hamilton Depression Rating Scale; M, male; F, female.

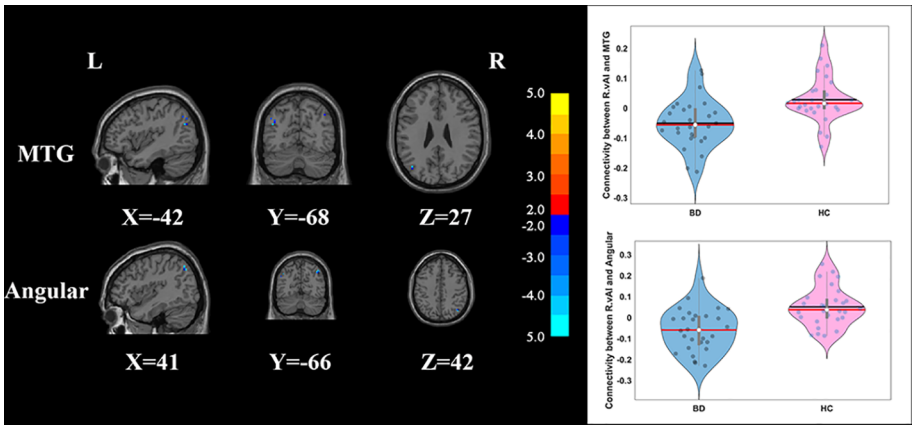

FIGURE 1 | Group differences of the whole-brain functional connectivity anchored in R_vAI: compared to HCs, BD patients showed significantly decreased functional connectivity between R_vAI and MTG, as well as R_vAI and angular (GRF corrected, $p < 0.05$, voxel Z value > 2.3). Blue indicates smaller values in BD. L, left; R, right; BD, bipolar disorder; HC, healthy control; R_vAI, right ventral anterior insula; MTG, middle temporal gyrus; GRF, Gaussian random field.

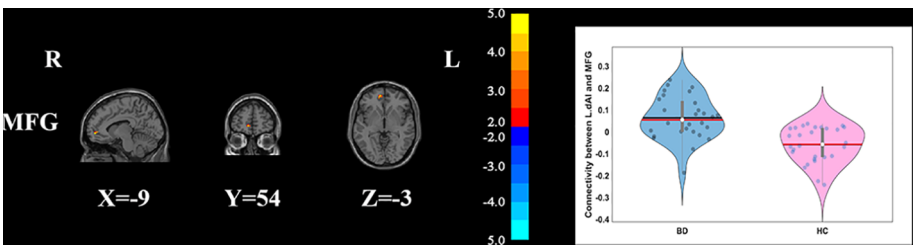

FIGURE 2 | Group differences of the whole-brain functional connectivity anchored in L_dAI: compared to HCs, BD patients had significantly higher functional connectivity between L_dAI and MFG (GRF corrected, $p < 0.05$, voxel Z value > 2.3). Red indicates larger values in BD. L, left; R, right; BD, bipolar disorder; HC, healthy control; L_dAI, left dorsal anterior insula; MFG, middle frontal gyrus; GRF, Gaussian random field.

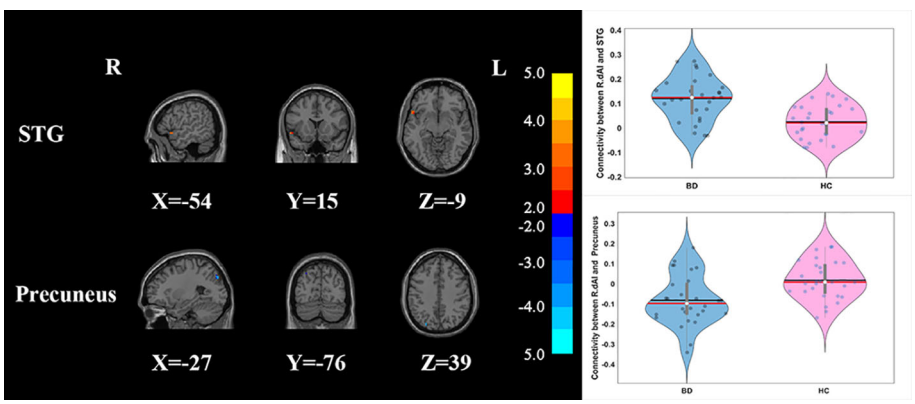

FIGURE 3 | Group differences of the whole-brain functional connectivity anchored in R_dAI: while showing decreased functional connectivity between R_dAI and precuneus, BD patients had significantly higher functional connectivity between R_dAI and STG compared to HCs (GRF corrected, $p < 0.05$, voxel Z value > 2.3). Blue indicates smaller values in BD and red indicates larger values in BD. L, left; R, right; BD, bipolar disorder; HC, healthy control; R_dAI, right dorsal anterior insula; STG, superior temporal gyrus; GRF, Gaussian random field.

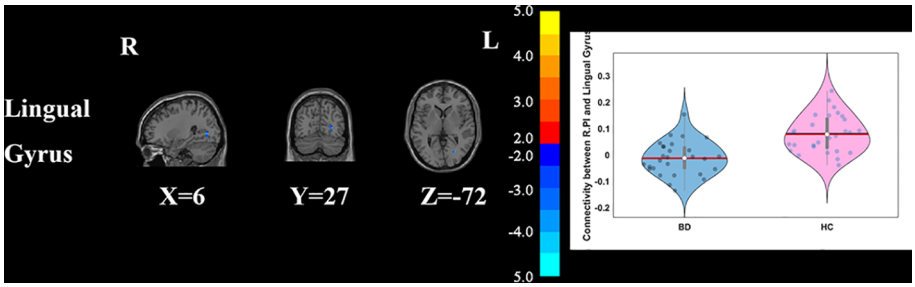

FIGURE 4 | Group differences of the whole brain functional connectivity anchored in R_PI: BD patients had significantly decreased functional connectivity between R_PI and lingual gyrus (GRF corrected, $p < 0.05$, voxel Z value > 2.3). Blue indicates smaller values in BD. L, left; R, right; BD, bipolar disorder; HC, healthy control; R_PI, right posterior insula; GRF, Gaussian random field.

TABLE 3 | Group differences in seed-based functional connectivity of the insular subregions.

Seed	Connected regions	L/R	Voxels	BA	MNI coordinates			*T*
					X	Y	Z	
R_vAI	Middle temporal gyrus	L	14	39	−42	−68	27	−4.66
	Angular	R	7	19	41	−66	42	−5.17
L_dAI	Medial frontal gyrus	L	12	29	−9	54	−3	5.51
R_dAI	Superior temporal gyrus	L	8	31	−54	15	−9	4.19
	Precuneus	L	7	6	−27	−76	39	−4.88
R_PI	Lingual gyrus	R	45	6	27	−72	3	−4.41

BA, Broadmann area; BD, bipolar disorder; HC, healthy controls; dAI, dorsal anterior insula; AI, ventral anterior insula; PI, posterior insula; MNI, Montreal Neurological Institute.

differences were observed by seeding the L_vAI or L_PI ($p > 0.05$, voxel z value > 2.3, GRF corrected).

Associations Between Insular Subregions Connectivity and Clinical Symptoms

We explored the relationships between these abnormal FC patterns of the insular subregions and clinical symptoms. However, no significant correlation was found between FC indexes and age, depression, or other clinical characteristics within BD group.

DISCUSSION

Using a seed-based ROI analyses, our study showed the aberrant FC between right vAI and left middle temporal gyrus, right vAI and right angular, left dAI and left MFG, right dAI and left STG, right dAI and left precunus, as well as right PI and lingula gyrus. Therefore, the present study provides evidence that the insular subregions have aberrant FC patterns in BD patients on depression episode.

Emerging evidence suggests that the insular cortex, as an integral hub of salience network (SN), plays a pivotal role in behavioral stimuli detection modulating the dynamic coordination between internal and extra-personal stimuli, and integrating information of diverse cognitive control, emotional processes (26–29). Previous findings showed that BD was associated with abnormal structure and function in specific subdivisions of the insula (30–32). Neuroimaging studies focusing on the resting-state FC (rsFC) of insular subdivisions revealed the discriminative ability of dysfunctional connectivity patterns of anterior insula for bipolar depression (18, 33). Hence, the aberrant profiles of insular subregions may provide a novel insight for the pathophysiology of BD depression.

Our finding showed increased FC between right AI and several specific brain regions, including the middle temporal gyrus and angular, which are known as nodes of the default network (DMN). These findings are consistent with previous studies of aberrant FC in BD (33–35). Ellard et al. (33) observed that compared to patients with unipolar depression and HCs, BD patients showed significantly aberrant FC between right AI and the IPL in DMN. The DMN might involve in self-referential mental process and social cognition (29, 31, 36, 37). Furthermore, it is reported that the DMN was associated with the symptom of BD patients, such as rumination. Lois and his colleague found decreased FC within the DMN in remitted BD patients (38). Converging evidence from neuroimaging studies using memory tasks indicated that the DMN involves in the retrieval processing of self-related episodic memory (39, 40). In consistent with previous findings, our findings revealed that abnormal intra-network between the SN and the DMN involved in BD on the depression episode (5).

Among insular subregions, the vAI is closest to limbic cortex showing extensive relationships with other cortical regions, while the dAI primarily is connected with dorsal ACC (dACC) along with other regions of control networks(41–43). Consistently, our results demonstrated increased FC between left vAI and MFG, STG, and

precuneus in BD depression. Furthermore, our study observed that depressed patients with BD had aberrant rsFC profiles anchored on dAI, including the hyperconnectivity with the MFG and the STG, and hypoconnectivity with the precuneus. The MFG and STG has been identified as key nodes in ECN involvement in goal-directed behavior and cognitive control (26, 44, 45). Previous studies have found the altered FC between dAI and the IPL in the ECN, which was related to impairments of perceived emotion control (33). Additionally, our study observed that BD patients had hypoconnectivity between dAI and precuneus. As a key node of DMN, the precuneus is important for self-reference processing (46), consciousness (47), integration of past and present information (48), and perspectives of social interaction (49). Young and his colleague found that BD patients showed increased hemodynamic activity in the anterior insula during positive memories recall of specific autobiographical memory (AM) tasks, while showing decreased activity in the precuneus during negative memories recall of AM tasks (50).

As a major hub of the SN, the AI serves as identifying the salient stimuli information and forwarding to higher cognitive regions (31). Furthermore, emerging evidence supports the idea that the AI might perceive regulatory control demands and facilitate dynamic switching between DMN and ECN (28, 29, 51). Interestingly, our results showed aberrant rsFC patterns of the AI, including hyperconnectivity with nodes of ECN and hypoconnectivity with nodes of DMN. These results indicated that the AI could integrate the abnormal affective and cognitive process in BD patients, and facilitate the switching between DMN and ECN (28, 29, 51).

As for the rsFC patterns of PI, we detected its dysconnectivity with the lingual gyrus within the visual recognition network, which may be involved in the perception of facial emotion stimuli (52–54). Neuroimaging studies using rsFC and DTI approaches have observed the abnormality of lingual gyrus in patients with BD (55, 56). Consistently, numerous task-based fMRI studies found abnormal activation of lingual gyrus in patients with BD during emotional face processing (57).

LIMITATION

Although our study provided substantial evidences showing abnormal FC between insular subregions and other brain areas, several limitations should be considered when interpreting our findings. First, our study reveals the potential mechanism of insular subregions' connectivity patterns underlying BD. However, the cross-sectional study may neglect the characterization of disease's development trajectory, and ignore the dynamic changes of brain function along with mental states. Second, the sample size of our study is modest, which may impose some restriction on the reliability and generality of our findings. Third, although we acknowledge the well-established relationship between abnormal rsFC patterns of insular subregions and clinical symptoms, we failed to replicate the significant correlation in our study. It may be due to the less sensitivity of HAMD scale for its variety of clinical symptoms in BD patients on depression episode. And lastly, most patients with BD were treated with lithium, antiepileptics, anxiolytics, or antidepressants, and even some with frequent polymedication at the time of MRI in the study. We further explored the possible effects of BD medication on insular subregions connectivity, and finally found an effect of antiepileptic on lingual regions (in the file of Supporting Information-2). As a consequence, further research is needed to assess the effects of psychotropic medications on BOLD signal with a relatively large sample to replicate our results in future study.

CONCLUSION

Our study found that BD patients on depression episode had abnormal FC among insular subregions and other brain regions, including the medial temporal gyrus, angular, MFG, STG, precuneus, and lingual gyrus involved in DMN, ECN, and the visual recognition network. Considering that these regions related to the emotional process and cognitive control, our findings provided substantial evidence of abnormal brain functional network of BD on depression episode.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shanghai Mental Health Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DP and TS designed and supervised the project. MQ, GL, HZ, and YH were responsible for the collection of participants. MQ and GL undertook the analysis of raw MRI data and the preparation of the manuscript. JW and SY gave the guide of the data analysis. All authors have participated in the revision of the finial manuscripts

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00253/full#supplementary-material

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State-Independent Microstructural White Matter Abnormalities in Major Depressive Disorder

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Background: Even with continuous antidepressant treatment, residual symptoms and the risk of relapse can persist in remitted major depressive disorder (MDD) patients. Hence, having a clear recognition of the persistent abnormalities of the underlying neural substrate in MDD through a longitudinal investigation is of great importance.

Methods: A total of 127 adult medication-free MDD patients with an acute depressive episode and 118 matched healthy controls (HCs) underwent diffusion tensor imaging. Over a 6-month treatment course, 62 remitted patients underwent a second scan. Remission was defined as a 24-item Hamilton Depression Rating Scale ($HAMD_{24}$) score ≤7 for at least two weeks. Diffusion tensor imaging was performed with a 3.0 T scanner. Differences in whole-brain fractional anisotropy (FA) between MDD patients and HCs were assessed by an independent *t*-test using gender, age, and education as covariates.

Results: Significant FA reductions in the left insula, left middle occipital gyrus, right thalamus, left pallidum and left precuneus were observed in current MDD (cMDD) patients compared with HCs. Moreover, significant FA reductions in the left insula were observed in remitted (rMDD) patients compared to HCs. However, no significant differences in FA values were found when comparing cMDD and rMDD patients.

Conclusions: The abnormalities in the insula showed state-independent characteristics, while the abnormalities in the middle occipital gyrus, thalamus, pallidum and precuneus seemed to be state-dependent impairments in MDD patients.

Keywords: major depressive disorder, diffusion tensor imaging, white matter microstructure, fractional anisotropy, state-independent

INTRODUCTION

Major depressive disorder (MDD) is a prevailing chronic mental disorder with 6.6% annual and 16.2% lifetime prevalence (1, 2). Over 30% of MDD cases develop as an unremitted depression with higher recurrence and function impairments compared to remitted depression despite trials of various antidepressant treatments (3–6). Notably, even in remitted individuals, obvious cognitive complaints, function impairments and the risk of relapse persist. These are a result of the persistence of the underpinning neural abnormalities that are unresolved with continuous antidepressant treatments (6).

Over the last several decades, numerous magnetic resonance imaging (MRI) studies have described the neural circuits that underpin MDD. Pooled functional MRI studies have found that frontal-limbic circuit dysfunctions are a key neural substrate in the pathophysiology of MDD (7–12). Diffusion of white matter, the infrastructure connecting cortical and subcortical regions, has been proposed as the basis of the structural connection alterations involved in MDD. Several structural MRI studies have identified widespread white matter abnormalities in MDD patients, mainly localized at the right frontal lobe, the left lateral occipital lobe, the genu of the corpus callosum (CC), the left anterior limb of the internal capsule (ALIC) and the left superior longitudinal fasciculus (SLF) (13–16).

Although cross-sectional studies have repeatedly reported white matter abnormalities in MDD patients, few studies have addressed white matter alterations over time with longitudinal studies. Carceller-Sindreu et al. found that white matter volume reduction in the prefrontal cortex in a small sample of patients with first-episode depression, which was normalized over a 2-year treatment course (17). Repple et al. examined the alterations of fractional anisotropy (FA), mean diffusivity, radial diffusivity and axial diffusivity in MDD patients throughout a 2-year treatment course. Patients with current depression showed higher mean diffusivity in the prefrontal lobe, which was dissipated at the remission phase (18). Based on these findings, white matter microstructural abnormalities seemed to be state-dependent alterations fluctuating with depression symptoms in the pathogenesis of MDD. Nonetheless, some other cross-sectional studies reported that patients with remitted MDD (rMDD) also show FA reductions in the amygdala and medial prefrontal cortex. Moreover, patients with rMDD show higher FA in multiple frontal-limbic brain areas, multiple posterior cingulate cortex regions and the insula than those subjects who fail to achieve remission (9, 19, 20). In this way, white matter microstructural abnormalities seem to be state-independent characteristics of MDD. Thus, the alterations of white matter microstructure in MDD may be a complex question, with mixed state-dependent and state-independent alterations co-occurring. However, evidence is scarce and incongruous.

To reveal possible state-dependent and state-independent white matter alterations, we conducted a large sample prospective study to investigate impairments and potential alterations over a 6-month treatment course using whole-brain FA analysis. Whole-brain FA analysis is a widely used diffusion tensor imaging (DTI) white matter data-analysis method that can measure the structural integrity of white matter areas and can be used to quantify the fiber orientation (21). It has been widely used for evaluating the disruption of white matter and the trajectory of white matter changes in MDD. Specifically, decreased FA has consistently been reported to be related to depression severity and illness duration in MDD, and a proposed DTI will be used to measure the trajectory of white matter microstructural alterations (13, 22). We hypothesized that prominent impairments would be observed in current MDD (cMDD) patients, and there would be state-independent alterations in rMDD with early-stage interventions.

METHODS

Participants

One hundred and twenty-seven patients with MDD who were experiencing a major depressive episode at the time of enrollment as assessed by the Structured Clinical Interview for DSM-IV (SCID-IV) were recruited from Zhumadian psychiatric hospital *via* consultant psychiatrists from 2013 to 2017. All patients had a 24-item Hamilton Depression Rating Scale ($HAMD_{24}$) score ≥20 and received no psychotropic medication within 2 weeks (6 weeks for fluoxetine) before recruitment. The exclusion criteria were: any other DSM-IV psychiatric disorder except for generalized anxiety disorder and social anxiety disorder; perinatal depression; history of head injury or neurological disorders; DSM-IV Substance Abuse Disorder or significant drug and/or alcohol use; color blindness. Demographic information was collected by a self-designed demographic information table. Illness history was collected by a structured clinical interview.

The control group consisted of one hundred and eighteen healthy volunteers recruited from communities in Zhumadian from 2013 to 2017. The exclusion criteria for the healthy controls were: a history of any psychiatric disorder or major physical disease, color blindness, pregnancy or breastfeeding, first-degree relatives with a history of psychiatric disorder, alcohol or drug abuse or dependence. Both the healthy volunteers and the patients had to be right-handed.

This study was approved by the ethics committee of the second Xiangya Hospital of Central South University on December 30th, 2012 and by the ethics committee of Zhumadian Psychiatric Hospital on January 9th, 2013, respectively. The number of IRB approval in the Second Xiangya Hospital was 238 and that in Zhumadian Psychiatric Hospital was 002. Written informed consent was obtained from all participants.

Treatment and Efficacy Assessment

All patients received a 6-month course of antidepressant treatment (either an SSRI or an SNRI) according to the advice of the patient's attending psychiatrist. Patients were assessed with $HAMD_{24}$ and HAMA at baseline, the end of the 0.5, 1st, 2nd, 3rd, 4th, 5th and 6th month during the follow-up process. Five experienced manic symptom onset during the 6-month treatment period. In the sixth month, 75 patients completed

the 6-month clinical assessment, while 52 patients failed. Clinical remission was defined as $HAMD_{24}$ scores ≤7 for at least two months and maintaining the low score ($HAMD_{24}$ ≤7) to the end of the sixth month. Among the 75 patients, 62 achieved clinical remission. Of the 62 remitted patients during the 6-month follow-up, 56 patients received an SSRI treatment and six patients received an SNRI treatment. DTI was acquired for all patients at baseline and for those who finished the follow-up at the end of the sixth month. Since the number of unremitted patients at the end of the sixth month is too small (n = 13), for follow-up data analysis, only the data of those who achieved remission were analyzed in this study. Eventually, 127 cMDD patients and 62 rMDD patients with intact DTI data were analyzed. In addition, 118 matched healthy controls (HCs) were also enrolled in this study.

Imaging Protocol

All participants were scanned using a 3.0T MR scanner (Signa HDxt MR, GE Healthcare, Milwaukee, WI). During scanning, foam pads and earbuds were used to reduce head motion and scanner noise respectively. Participants were required to keep still with their eyes closed. Diffusion-weighted images were obtained using a single-shot echo-planar imaging sequence according to the following parameters: repetition time (TR) = 13,000 ms; echo time (TE) = 85.9 ms; number of excitations (NEX) = 1, field of view (FOV) = 256 × 256 mm^2; matrix size = 128 × 128; slice thickness = 3 mm; 32 non-collinear diffusion directions with a b-value of 1,000 s/mm^2 and one additional volume without diffusion weighting (b = 0 s/mm^2) were acquired; and 50 transverse slices without gaps, covering the entire brain. We also acquired high-resolution 3D brain anatomical images using a T1-weighted BRAVO sequence according to the following parameters: TR = 6.8 ms, TE =2.5 ms, flip angle = 9°, slice gap = 0 mm, turnover time (TI) = 1,100 ms, NEX = 1, FOV = 256 × 256 mm^2, matrix size = 256 × 256, and 192 contiguous sagittal slices with slice thickness = 1 mm.

DTI Data Processing

Pipeline for analyzing Brain Diffusion images (PANDA) in FMRIB'S Software Library (FSL) 2 was used for image pre-processing [FMRIB's Software Library, pre-processing (FMRIB's Software Library, http://www.fmrib.ox.ac.uk/fsl)] (23). Images obtained in DICOM format were initially converted to ANALYZE format. The diffusion tensor images were corrected for distortions caused by head motion and eddy currents using affine registration in Eddy Current Correction. After completing these pre-processing steps, a diffusion tensor model was fit to each voxel using DTIFit to generate images of FA. Then, all participants' FA images were first nonlinearly aligned to the FA template in the MNI space3. Finally, the aligned FA images were averaged to create a mean FA image, and we used the mean FA image as the white matter mask for further statistical analysis.

Statistical Analysis

Demographic and clinical data are presented as the means ± standard deviations (SDs). Continuous variables were analyzed by two-sample *t*-tests, while categorical variables were analyzed using chi-square (χ^2) tests.

Using SPM12 software (http://www.fil.ion.ucl.ac.uk/spm/software/spm12), two-sample *t*-tests were implemented to establish abnormal FA values between the cMDD and HC groups on whole-brain FA. Regarding the abnormal clusters in cMDD as masks, two-sample *t*-tests were also implemented to establish abnormal FA values between the rMDD and HC groups. In addition, paired-samples *t*-tests were also implemented in rMDD group between at baseline and the end of the six-month follow-up. Gender, age and education were controlled as covariables in the above statistical analyses.

RESULTS

Demographic and Clinical Characteristics

Demographic and clinical characteristics are presented in **Table 1**. There were no statistically significant differences between these three groups regarding age, gender and education. Additionally, there were no statistically significant differences between cMDD and rMDD regarding onset age, total illness duration, current illness duration, the number of episodes and $HAMD_{24}$ at baseline. There were no significant differences at baseline

TABLE 1 | The demographic and clinical characteristics of cMDD (n = 127), rMDD (n = 62), and HC group (n = 118).

	Current Depression (BS) Group Mean (SD) n = 127	Remitted Group (FL) Mean (SD) n = 62	Healthy Control Group Mean (SD) n = 118	Statistical tests BS v. HC	Statistical tests FL v. HC
Age (years)	35.39 ± 9.18	36.26 ± 9.16	35.01 ± 8.86	t = 0.334 p = 0.739	t = 0.889 p = 0.375
Gender (Male/Female)	58/69	25/37	53/65	$\chi2$ = 0.014 p = 0.906	$\chi2$ = 0.349 p = 0.555
Education (years)	10.35 ± 3.35	10.35 ± 3.53	10.65 ± 3.25	t = –0.707 p = 0.480	t = –0.567 p = 0.572
Onset age	32.09 ± 9.11	32.60 ± 8.66	–	–	–
Current length (months)	3.32 ± 2.92	3.18 ± 2.36	–	–	–
Total length (months)	42.46 ± 52.51	46.66 ± 59.23	–	–	–
Frequency	2.06 ± 1.37	2.16 ± 1.53	–	–	–
$HAMD_{24}$	31.48 ± 7.58	2.42 ± 2.41	–	–	–

BS, baseline; FL, follow-up; HC, healthy controls.

between follow-ups and dropouts in gender, age, education, onset age, total illness duration, current illness duration and the number of episodes (not presented).

White Matter Abnormalities in cMDD Patients

The left insula, left middle occipital gyrus, right thalamus, left pallidum and left precuneus showed significant FA value reductions in the cMDD group compared with HCs ($p < 0.001$, uncorrected, cluster extend voxels = 10). There's no region of FA value increase in the cMDD group as compared with the HC group. Detailed anatomical regions are shown in **Table 2** and **Figure 1**. In addition, there was no significant difference in FA values between dropouts and included individuals at baseline.

White Matter Abnormalities in rMDD Patients

Regarding the decreased clusters in cMDD as masks, we found the rMDD group also showed significant FA value reductions in the left insula as compared with HCs ($p < 0.001$, uncorrected, cluster extend voxels = 10). Detailed anatomical regions are shown in **Table 3** and **Figure 2**.

White Matter Alterations Between cMDD and rMDD Patients

No significant difference in FA value was observed between the baseline and follow-up scans in rMDD group ($p < 0.05$, uncorrected).

DISCUSSION

The present longitudinal study investigated white matter alterations in a relatively large sample of MDD patients over a 6-month antidepressant treatment course. Our results revealed significant FA value reductions in the left insula, left middle occipital gyrus, right thalamus, left pallidum and left precuneus in cMDD relative to HCs. After 6 months of antidepressant treatment, significant FA value reductions were still observed in the left insula in rMDD patients when compared to HCs.

TABLE 2 | Significantly decreased FA clusters between cMDD and HC group.

	MNI coordinates (x y z)	Cluster size (voxels)	BA	AAL	Z-scores
cMDD < HC	–30 32 10	11	45 (L)	Insula	3.72
	–40 –62 8	10	19 (L)	Middle Occipital gyrus	3.86
	4 0 –2	25	Thalamus 50 (R)	Thalamus	3.90
	–16 6 0	14	GlobPal 51 (L)	Pallidum	3.75
	–22 –50 46	16	7 (L)	Precuneus	3.73

BA, Brodmann area; MNI, Montreal Neurological Institute. The MNI coordinate is for the peak voxel of the respective clusters.

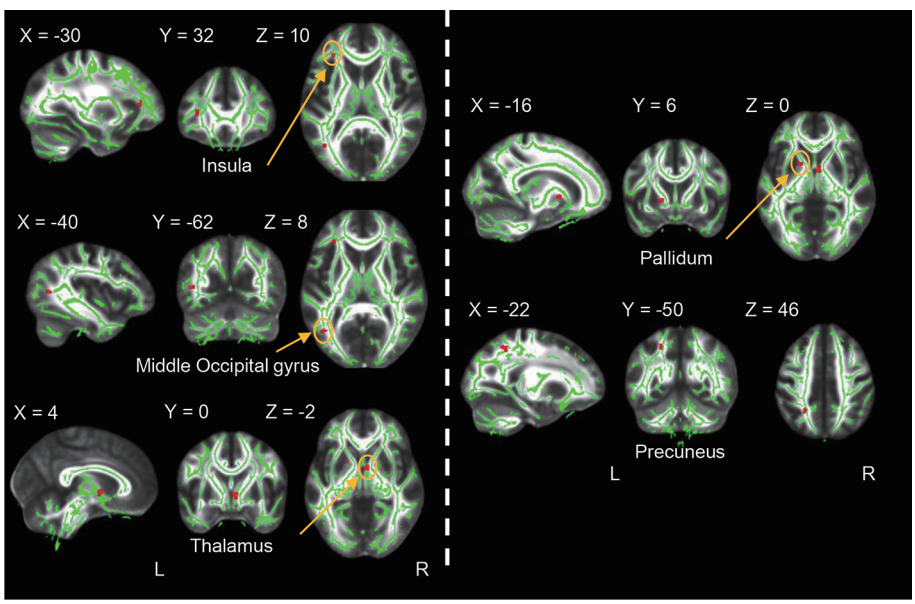

FIGURE 1 | Differences between the cMDD and HC groups in whole-brain FA analysis. The cMDD group showed reduced FA value in the left insula, left middle occipital gyrus, right thalamus, left pallidum and left precuneus as compared with the HC group ($p < 0.001$, uncorrected, cluster extend voxels = 10).

TABLE 3 | Significantly decreased FA clusters between the rMDD and HC group.

	MNI coordinates (x,y,z)	Cluster size (voxels)	BA	AAL	Z-scores
rMDD < HC	–30 32 10	28	45 (L)	Insula	3.75

BA, Brodmann area; MNI, Montreal Neurological Institute. The MNI coordinate is for the peak voxel of the respective clusters.

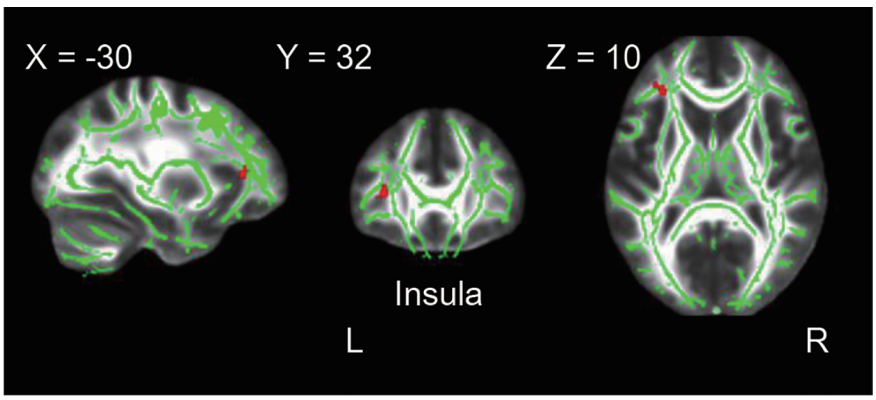

FIGURE 2 | Difference between the rMDD and HC groups in whole-brain FA analysis. The rMDD group showed reduced FA value in the left insula as compared with the HC group ($p < 0.001$, uncorrected, cluster extend voxels = 10).

The primary finding of the present study was that the white matter abnormalities in the left insula persisted throughout illness, from acute episode to remission, showing a state-independent character. The existence of state independence and trait impairment is associated with worse clinical outcomes, poorer working ability and more severe social function decline and higher rates of recurrence (24). Among clinical symptoms, several possible state-independent impairments of depression have been reported, especially the well-acknowledged sustained attention and executive function (25, 26). The FA reductions in the left insula showed as a state-independent impairment in the present study, revealing the possibility that the dysfunction of the insula potentially underlies this special state-independent or set of trait impairments (e.g., recurrent or persistent cognitive impairments).

The insula contains extensive anatomical connections to cortical and limbic regions, mainly including the prefrontal, anterior temporal, visual, and auditory cortices and the thalamus. These regions play a key role in emotional and cognitive processing (27, 28). Numerous studies have demonstrated that the functional activity and connectivity of the insula are perturbed in MDD patients, especially the dysfunction in integrating bottom-up and top-down information in emotional and cognitive processing (29–32). In addition, greater levels of maladaptive rumination, anxiety and hopelessness have also been reported because of the dysfunction of the insula and the fronto-insular network (33). After antidepressant treatment, functional reductions have also been reported in the insula (34). Consistent with the results of functional connective studies, we found FA reductions in the left insula in the episode phase and even in the sustained remission phase. Previous studies have also reported decreased FA in the left insula in young MDD patients and elderly unremitted patients when compared to remitted individuals (19). Our findings provide more direct evidence that white matter abnormalities in the insula not only in the episode phase but also persisting to the remission phase in adult MDD patients, despite a 6-month antidepressant treatment regimen further verified the crucial role of the insula in the neural circuitry of depression both in function and structure.

Another important finding of the present study was that significant FA reductions were found in the left middle occipital gyrus, right thalamus, left pallidum and left precuneus in current MDD patients but not in the remission state. Consistent with clinical symptoms, the alterations of FA in these regions were reversed by the antidepressant treatment. We tend to believe that these changes possess obvious state-dependent characteristics. All these regions are important components of frontal-subcortical circuits, which have been proposed as crucial circuits that modulate both affective and cognitive performance. The thalamus has always been regarded as an intermediate node between different subcortical areas and the cerebral cortex, connecting with key regions in the frontal-subcortical circuits, including the insula, orbitofrontal, cingulate, amygdala and dorsolateral prefrontal. Converging evidence suggested that volumetric abnormalities and dysfunction of the thalamus are present in both depressed young and elderly adults with obvious affective symptoms (35–37). White matter abnormalities of the thalamus have also been reported in depressed patients when compared to control groups (38). Our study provides a compelling supplement for the evidence of white matter abnormalities in currently depressed patients that would be

reversed by a longitudinal effective treatment. Another important functional region of the frontal-subcortical circuits, the pallidum, has been reported to have white matter abnormalities in depressed patients, corresponding to executive function impairment. This region also showed state-dependent features of white matter abnormalities in the present study (39). The precuneus, a key node of the default-mode network, plays a central role in visuospatial imagery, episodic memory retrieval and self-processing operations (40). Accumulating evidence suggests that the precuneus has an important role in the neuropathology of depression (41–43). Consistent with previous studies, FA reduction in the precuneus was also observed in current MDD patients when compared to healthy controls. Furthermore, our previous study showed significant grey matter volume changes in the middle occipital gyrus, thalamus, precuneus and frontal gyrus in nonrefractory depressive disorder patients (34), also providing favorable evidence that these regions in the frontal-subcortical circuits of MDD patients would have synergetic functional and structural impairments.

The strength of this study is that all MDD patients enrolled were antidepressant-free. Antidepressant exposure alleviated the emotional disturbance and exerted a neurotrophic effect, including increased expression of neurotrophic factors and neuron remodeling (44, 45). All antidepressant-free MDD patients in acute episodes with no interference from antidepressants are of great importance in identifying the primary white matter abnormalities. The main limitations of this study should also be acknowledged. First, some patients dropped out during the 6-month treatment course due to unavoidable reasons (such as moving to seek employment, severe gastrointestinal reactions, etc.). However, there were no differences in the demographic and clinical characteristics between remitted follow-ups and dropouts. The relatively high drop-out rate also resulted in a small number of unremitted patients at the end of the 6-month follow-up, limiting our ability to analyze the differences between patients with different prognosis. Second, we conducted a paired *t*-test to compare the current MDD group and the remitted group, but no significant difference between these two groups was found. We may suppose that even when MDD patients achieved remission, the degree of FA reduction reversal was not sufficient to reach a significant difference compared to the current depressed sample. Third, we did not obtain 6-month follow-up scans in the controls to compare the magnitude of change during the follow-up period. This potential confounding factor should be considered in future studies. Finally, what we have done reveals possible state-dependent and state-independent white matter abnormalities; to further identify additional potential state-dependent and state-independent impairments, more clinical assessments, neuropsychological tests and social function rating scales are needed in future studies.

The present study investigated the trajectory of white matter abnormality changes in unmedicated MDD patients over a 6-month antidepressant exposure. The insula, a crucial region that modulates both affective and cognitive performance, showed the characteristics of state-independent impairment, while the middle occipital gyrus, thalamus, pallidum and precuneus, important nodes of the frontal-subcortical circuits, all showed white matter abnormalities in MDD patients and seemed to show state-dependent impairments that fluctuate with the depressive symptoms. Further studies should place more emphasis on the association between neurophysiological mechanisms and clinical symptoms to confirm the reliability of these state-dependent and independent impairments in depression, eventually leading to better treatment selections and clinical outcomes.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committees of the Second Xiangya Hospital of Central South University and the Zhumadian Psychiatric Hospital. Written informed consent was obtained from all participants. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QD: collected data, conducted the statistical analysis, drafted the manuscript, edited and submitted the manuscript. JL, XL, JS, LZh, MW, HG, FZ, DY, HL, WG: collected data, reviewed and revised the manuscript. YZ: conceptualized and designed the study. BL, LZe, YF: statistical analysis, critically reviewed, edited and revised the manuscript; DH: critically reviewed and revised the manuscript. LL: conceptualized and designed the study, collected data, critically reviewed and revised the manuscript. All authors have approved the final version of this manuscript.

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Safety and Tolerability of Both Arm Ischemic Conditioning in Patients With Major Depression: A Proof of Concept Study

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Purpose: A substantial proportion of patients with major depressive disorder (MDD) does not respond or cannot tolerate to currently available treatments. This study was to assess the safety and tolerability of Remote Limb Ischemic Preconditioning (RLIPC) as an adjunctive therapy in patients with MDD.

Patients and Methods: Enrolled patients underwent RLIPC, five cycles of simultaneous bilateral arm ischemia, 5 min and followed by reperfusion of each cycle, and once daily for eight consecutive weeks. Depression and anxiety severity, and quality of life were assessed every 2 weeks. Descriptive analysis was used for safety and tolerability data.

Results: Thirty-seven participants completed at least one RLIPC. Twenty-four of them (64.9%) completed the study. Twelve patients prematurely discontinued the study due to poor adherence, and one due to a mild side effect. The changes in HRSD-17, GAD-7 and QOL-6 total scores from baseline to the endpoint were significant from the end of second week treatment onwards. The responder and remission rates were 59.46% (22/37) and 54.05% (20/37) at the endpoint, respectively.

Conclusion: RLIPC was safe and well tolerated, and may be effective in reducing depressive symptoms in patients with MDD. Large studies are warranted to test its efficacy and safety as monotherapy or adjunctive therapy in the treatment of MDD.

Keywords: major depressive disorder, remote ischemic preconditioning, safety, tolerability, effectiveness

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INTRODUCTION

Major depressive disorder (MDD) is one of the most common mental disorders in the general population and a major public health problem across the world. It is associated with high rates of morbidity and mortality (1). According to the World Health Organization, MDD affects approximately 4.4% of the world's population, has high occupational and economic impact, and will become the second leading globe burden of disease by 2020 (2). In China, the latest national epidemiological survey found that the 12-month and life time prevalence of depressive disorders was 3.6 and 6.8%, and MDD was 2.1 and 3.4% respectively (3).

Although a number of effective treatments are available, a substantial proportion of patients with MDD does not respond or cannot tolerated to the first-line antidepressants (4). In the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trials, only about one-half of patients responded, and one-third of remitted with the first level of antidepressant treatments (5, 6). After failing an antidepressant for a current depressive episode, switching to a different antidepressant in the same class or a different class, or augmenting with an antidepressant, mood stabilizer, or antipsychotics is a common practice. Non-pharmacological augmentation such as repetitive transcranial magnetic stimulation (rTMS) has become available to manage inadequate response to the initial treatment of an antidepressant(s) (7–9). Although, rTMS does not have severe side effect, a 5 day per week schedule for a minimal 4–6 weeks treatment to determine its benefit is cumbersome for those who have already struggled socially and financially. More convenient and inexpensive approaches with fewer short- and long-term side effect are worthy of exploration. Remote ischemic preconditioning (RIPC) appears to fit this profile.

RIPC consists of several cycles of brief and nonlethal limb ischemia (10, 11). With a series of transient ischemic episodes, it can activate multiple endogenous protective mechanisms to attenuate ischemic injury and alleviate dysfunction to distant organs due to acute reduction of blood supply (12). RIPC may also have neuroprotective effects through a variety of potential mechanisms, including regulation of specific neural pathway and signal transduction, inhibition of inflammatory and immune responses, decrease of oxidative stress and calcium overload, attenuation of glutamate excitotoxicity, and suppression of apoptosis (10–14). Its neuroprotective effect was observed in participants with symptomatic intracranial arterial stenosis (ICAS) and carotid artery stenosis (CAS), which could reduce the new brain lesions and the impairment of neural circuits and connectivity, and decrease the risk of various psychiatric or neurological conditions (e.g., cognitive decline, dementia and depression) (15–17). A recent study found that RIPC could prevent the progression of white matter hyperintensities and ameliorate headache, dizziness, sleeping disorder and cognitive impairment in elderly patients (83.5 ± 2.3 years) who had intracranial atherosclerotic stenosis (18).

Although the neurobiology of MDD remains unknown, the involvement of neurotrophic factors, inflammatory cytokines, the hypothalamus–pituitary–adrenal axis, and glutamate receptors in the pathophysiology of depression have been reported (19, 20), which was overlapped and consistent with the potential mechanisms of RIPC. RIPC has effect on a variety of changes in peripheral and central nervous system, which may reverse the neurobiological changes and improve depressive or related symptoms (e.g., cognition, sleep, and physical symptoms) (16, 18). Therefore, we conducted a proof of concept study to assess the safety and tolerability of Remote Limb Ischemic Preconditioning (RLIPC) as an adjunctive therapy in patients with MDD who were treated with an antidepressant for a current depressive episode.

MATERIALS AND METHODS

Subjects

This study was a single-arm, open-label, prospective, multicenter clinical trial. The study protocol was approved by the Institutional Review Board of Hongkou District Mental Health Center (Shanghai, China). Written informed consents were obtained from each participant before any study-related procedure was performed.

All patients were inpatients from Hongkou District Mental Health Center of Shanghai, Shulan (Hangzhou) Hospital of Zhejiang Province, and Wuxi Mental Health Center of Jiangsu Province. The study was conducted from the January of 2018 to the June of 2019. Patients who met the following criteria were enrolled into the study: 1) age of 18–65 years old; 2) diagnosed with MDD according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5); 3) a score of ≥14 on the 17- item Hamilton Rating Scale for Depression (HRSD-17) at the screening visit; 4) had the capacity for the informed consent for the study.

Patients with a history of manic or hypo-manic episode were excluded from this study. Patients were also excluded if they had any of the following physical diseases: 1) Extracranial and intracranial arterial stenosis verified with structural MRI, and other intracranial abnormalities such as infection, tumor and bleeding; 2) Known bleeding disorders or platelet counts <100 * 10^9/L, history of retinal and visceral hemorrhage, or on thrombolytic therapy; 3) Refractory hypertension defined as systolic pressure >180 mmHg or diastolic pressure >110 mmHg with evidence-based treatments; 4) Severe renal failure define as the rate of creatinine clearance <0.6 ml/s or serum creatinine >265 umol/l; 5) severe hepatic failure defined as serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) three times higher than the upper normal limit; 6) History of heart attack or a diagnosis of coronary artery disease; 7) Peripheral vascular disease of the upper extremity such as severe subclavian artery stenosis verified with MRI; or 8) Soft damage, vascular injury or fracture in the upper extremities. In addition, female patients who were pregnant, planning to be pregnant, or breast feeding during the study period were also excluded.

Antidepressant Treatment

For patients who were taking an antidepressant(s) for two weeks or more and met the inclusion criteria without any exclusion criteria, the RLIPC was added to the ongoing treatment regimen. For patients who did not take any medication or took an antidepressant(s) for less than two weeks for their depression at screening, an antidepressant was initiated and titrated up to a therapeutic dose within 2 weeks. For those who tolerated with the newly initiated antidepressant and continued meeting the inclusion criteria, the RLIPC was added. The initiation of any other psychotropic medication including typical/atypical antipsychotic agents, mood stabilizers, anticonvulsants, and stimulants, psychotherapy and physical treatment (rTMS and ECT) were not permitted during the study period. If necessary, short half-life hypnotics of benzodiazepines or non-benzodiazepines could be prescribed to manage anxiety, agitation and insomnia.

Implementation of RLIPC

The RLIPC application consisted of five cycles of simultaneous bilateral upper arm ischemia through a complete blocking of the arterial and venous blood flow to the forearms. Each cycle lasted for 5 min and followed by reperfusion for 5 min (16). An electric autocontrol device with cuffs was used to produce a pressure of 200 mmHg for ischemic response. The device Ischemic Precondition Training Instrument IPC-906 was provided by Beijing Renqiao Cardio-cerebrovascular Disease Prevention Research Nantong Co., Ltd, China. The RLIPC process could be stopped at any time if the patient experienced any intolerable discomfort or side effect. The enrolled patients underwent RLIPC once daily for eight consecutive weeks under the supervision and guidance of nurses and research assistants.

Safety Monitoring

Treatment-emergent adverse event was assessed and recorded at every treatment, including inability to tolerate RLIPC that leads to the discontinuation from the study and objective signs of tissue or neurovascular injury resulting from RLIPC procedure (16). The severity of an adverse event was determined by a research staff member. The forms of adverse event (AE) and serious adverse event (SAE) reporting were completed in according to the requirement from Institutional Review Board of Hongkou District Mental Health Center (Shanghai, China). Clinical laboratory tests including complete blood count, electrolytes, liver and kidney function tests, urine pregnancy test (if applicable), and ECG examination were performed at baseline and at the end of study.

Efficacy Assessment

The HRSD-17 was administered by the trained psychiatrists and psychologists. The Generalized Anxiety Disorder Scales 7-item (GAD-7) and quality of life 6-item (QOL-6) were completed by the patients. All these assessments were performed at baseline, week 2, week 4, week 6, and week 8.

Statistical Analysis

Demographic characteristics, and the rate of AE, SAE, response and remission were analyzed with descriptive statistics. The safety population included all patients who received at least one RLIPC treatment. The changes from baseline to endpoint in the total scores of HRSD-17, GAD-7 and QOL-6 were analyzed with ANOVA for repeatedly measured data based on the intent-to-treat sample. Paired t-test was also used to analyze the difference in changes in HRSD-17, GAD-7 and QOL-6 between baseline and following up visits. Depression response and remission were defined as a 50% decrease in the HRSD-17 total score from baseline to the endpoint and a HRSD-17 total score ≤7 at the endpoint, respectively. The last observation carried forward (LOCF) strategy was used to impute missing values of efficacy assessment for those subjects who did not complete the study. All statistical analyses were carried out by using SPSS 25.0 software program. Criterion for statistical significance was set at $\alpha = 0.05$.

RESULTS

Demographics and Baseline Clinical Characteristics

Forty patients who met the criteria of inclusion and did not meet any criterion of exclusion were included in the study after a screening assessment. Thirty-seven participants who completed at least one RLIPC procedure were included for the analysis. These patients were more likely to be female (n = 30, 81.1%), married (n = 27, 73.0%), and employed (n = 17, 45.9%) (**Table 1**). The education, age, and baseline severity scores of HRSD-17, GAD-7 and QOL-6 at study entry were also shown in **Table 1**. All patients were Han Chinese.

Disposition

During the 8-week study period, 64.9% (24/37) patients completed the study and twelve patients prematurely discontinued the study

TABLE 1 | Demographics and clinical characteristics of enrolled patients with depression.

Characteristic	N	%
Race (Han)	37	100
Gender		
Male	7	18.9
Female	30	81.1
Marriage		
Unmarried	7	18.9
Married	27	73.0
Divorced and Widowed	3	8.1
Occupation		
Employed	17	45.9
Retired	11	29.7
Unemployed	3	8.1
Student	6	16.2
	Mean	*SD*
Education (years)	11.51	4.58
Age (years)	42.54	15.98
Symptom severity at baseline		
HRSD-17	20.51	8.50
GAD-7	10.11	4.94
QOL-6	15.00	3.33

HRSD-17, 17-item Hamilton Rating Scale for Depression; GAD-7, Generalized Anxiety Disorder Scales 7-item; QOL-6, Quality of Life 6-item.

due to poor adherence defined as continuous interruption for more than two days in a row at any time (n = 5) or to be discharged (n = 7). Among those patients who prematurely discontinued, four patients discontinued at the second weekend, seven patients at the fourth weekend and, one patient at the eighth weekend, respectively.

Adverse Events

Of 37 patients enrolled in the study, only one patient had a mild side effect with numbness of limbs and petechiae of skin under the pressure cuff area during the first two weeks of RLIPC. The numbness of limbs and petechiae of skin disappeared one week after stopping the RLIPC, and the patient discontinued the study.

Efficacy Data

The decrease in HRSD-17 and GAD-7 total scores from baseline to the endpoint were significant, 20.51 ± 8.50 vs. 9.84 ± 8.00 ($P < 0.001$) and 10.11 ± 4.94 vs. 4.46 ± 3.33 ($P < 0.001$), respectively (**Table 2**). The increase in total scores of QOL-6 from baseline and the endpoint were also significant, 15.00 ± 3.33 vs. 19.14 ± 2.78 ($P < 0.001$) (**Table 2**). The significant changes in the total scores of HRSD-17, GAD-7 and QOL-6 occurred from the end of second week treatment onwards (**Figure 1**). The responder and remission rates were 59.46% (22/37, 95% CI as 43.40–74.25%) and 54.05% (20/37, 95% CI as 38.13–69.43%), respectively.

DISCUSSION

This proof of concept study has shown that RLIPC was safe and well tolerated in patients with MDD. Therefore, large, randomized, placebo or active-controlled, monotherapy or adjunctive to an antidepressant studies are needed to explore its efficacy in different stages of the treatment of MDD. Since this procedure can be done at home with minimal side effect, this approach especially appeals to those who are sensitive to medications and do not have cardiovascular diseases and bleeding-prone diathesis.

TABLE 2 | Efficacy outcomes of adjunctive remote limb ischemic preconditioning to antidepressants in patients with depression (N = 37).

Measure	Mean	SD	F	P
HRSD-17				
Baseline	20.51	8.50	21.08	<0.001
Endpoint[a]	9.84	8.00		
Change	−10.68	6.84		
GAD-7				
Baseline	10.11	4.94	12.83	<0.001
Endpoint[a]	4.46	3.33		
Change	−5.65	4.72		
QOL-6				
Baseline	15.00	3.33	11.04	<0.001
Endpoint[a]	19.14	2.78		
Change	4.14	3.82		
	N	*NO*	*YES*	*% (95% CI)*
Response[b]	37	15	22	59.46 (43.40, 74.25)
Remission[c]	37	17	20	54.05 (38.13, 69.43)

[a]*Using last observation carried forward strategy for missing data.*
[b]*Response defined as a 50% decrease in the HRSD-17 total score from baseline to endpoint.*
[c]*Remission defined as a HRSD-17 total score ≤7 at the endpoint.*
HRSD-17, 17-item Hamilton Rating Scale for Depression; GAD-7, Generalized Anxiety Disorder Scales 7-item; QOL-6, Quality of Life 6-item.

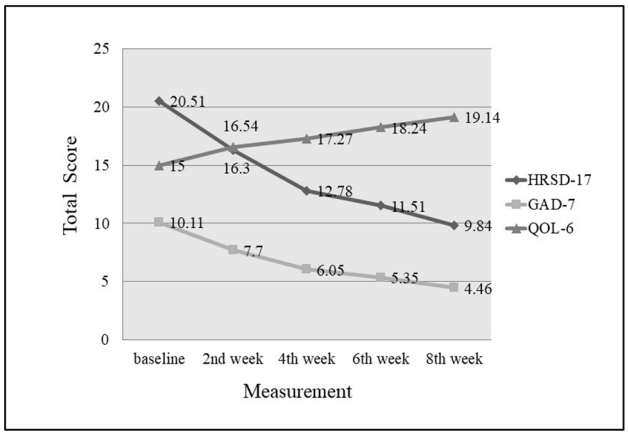

FIGURE 1 | Changes of HRSD-17, GAD-7 and QOL-6 total score from baseline to endpoint. Compared with baseline, total scores of HRSD-17 and GAD-7 decreased significantly, and QOL-6 increased significantly by the end of second week ($P < 0.001$).

The long-term safety of RLIPC was demonstrated in patients with severe CAS (16) and patients with ICAS (21). RLIPC had no significant effect on the heart rate, oxygenation index, and mean flow velocity in patients with both ICAS and healthy volunteers (15). Only one patient discontinued the present study because of numbness of limbs and petechiae of skin, which was lower than that reported in CAS patients (9.5%) (16). The seven patients prematurely discontinued the study because they were discharged home. These patients might not prematurely discontinue the study if they stayed in the hospital. Without considering these seven patients, about 16% of patients prematurely discontinued who did not undergo the procedure per the treatment protocol, which was higher than those in CAS patients (RLIPC group 1.6% and sham RLIPC group 4.8%) (16). The discrepancy could be interpreted as MDD patients had poorer treatment compliance to RLIPC than CAS patients. In routine outpatient settings, up to one in five individuals may not be adherent to the prescribed antidepressants (22, 23). About one third of patients with depression could drop out from short-term neurostimulation treatment such as transcranial direct current stimulation (tDCS) and repetitive transcranial magnetic stimulation (rTMS) (24). Since a maximum of 20% of missing sessions was considered reasonable for adherence, our study suggests that RLIPC in MDD can be a reasonable method for treatment adherence.

In terms of efficacy, antidepressant combined with RLIPC significantly reduced depressive and anxious symptoms and improved the quality of life in patients with MDD as early as at the end of second week after an add-on therapy of RLIPC. By that time, all enrolled patients had taken an antidepressant(s) for 4 weeks or more. Thus, it is difficult to clarify the possible add-on effect and onset time of RLIPC. However, a remission

rate of close to 60% was twice as high as that of after an antidepressant monotherapy in effectiveness clinical trials (25, 26). A possible explanation was that the inpatients were enrolled in this study that might have a higher remission rate than outpatients with MDD. Clearly, it is necessary to investigate whether the earlier response and a higher remission rate in this study were due to a true effect of adjunctive treatment of RLIPC to an antidepressant(s) or an effect of different study settings. Although the placebo effect of such a device used in ischemia patients was not significant compared the sham group with control group (16), which is necessary to be further verified in different population.

LIMITATION

This study was limited due to open label, small sample size, only inpatients, and lack of clear definition of treatment stages. The additional limitation include: 1) it lack of enough information of drug use, thus may difficult to stratify the effect of drug or the add-on effect of RLIPC; 2) It also lack of data of placebo effect of such a device in the patients with MDD. However, this study achieved its goal by demonstrating the safety and tolerability, and possible efficacy in MDD with RLIPC.

CONCLUSIONS

RLIPC, a simple and noninvasive therapy, appeared to be safe and well-tolerated in patients with MDD. Large studies including inpatients and outpatients with clearly defined treatment stages are warranted to test its efficacy and safety as monotherapy or adjunctive therapy in the treatment of MDD. Such an endeavor may expand our current available tools to manage MDD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Hongkou District Mental Health Center (Shanghai, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZW and XL contributed to design, analysis and interpretation of data, and drafting the manuscript. KG and MR contributed to conception, design, and revising the manuscript. NL, JL, BY, JS, LH, and YF contributed the subjects' enrollment and the clinical assessments. XJ is one of the inventors of the electric autocontrol device that has been patented in China (ZL 2007 1 017670L 0) who supplied freely all devices in this study. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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DNA Methylation of the t-PA Gene Differs Between Various Immune Cell Subtypes Isolated From Depressed Patients Receiving Electroconvulsive Therapy

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Background: Major depressive disorder (MDD) represents a tremendous health threat to the world's population. Electroconvulsive therapy (ECT) is the most effective treatment option for refractory MDD patients. Ample evidence suggests brain-derived neurotrophic factor (BDNF) to play a crucial role in ECT's mode of action. Tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) are involved in BDNF production.

Hypothesis: The DNA methylation of gene regions encoding for t-PA and PAI-1 might be a suitable biomarker for ECT response prediction.

Methods: We withdrew blood from two cohorts of treatment-resistant MDD patients receiving ECT. In the first cohort (n = 59), blood was collected at baseline only. To evaluate DNA methylation changes throughout the treatment course, we acquired a second group (n = 28) and took blood samples at multiple time points. DNA isolated from whole blood and defined immune cell subtypes (B cells, monocytes, natural killer cells, and T cells) served for epigenetic analyses.

Results: Mixed linear models (corrected for multiple testing by Sidak's post-hoc test) revealed (1) no detectable baseline blood DNA methylation differences between ECT remitters (n = 33) and non-remitters (n = 53) in the regions analyzed, but (2) a significant difference in t-PA's DNA methylation between the investigated immune cell subtypes instead ($p < 0.00001$). This difference remained stable throughout the treatment course, showed no acute changes after ECT, and was independent of clinical remission.

Conclusion: DNA methylation of both proteins seems to play a minor role in ECT's mechanisms. Generally, we recommend using defined immune cell subtypes (instead of whole blood only) for DNA methylation analyses.

Keywords: brain-derived neurotrophic factor, tissue-type plasminogen activator, depression, immunology, DNA methylation, electroconvulsive therapy remission

INTRODUCTION

According to the World Health Organization, major depressive disorder (MDD) can, nowadays, primarily be accounted for approximately 800,000 suicide deaths per year. With >322 million individuals affected, it belongs to the most prevalent mental disorders worldwide (1). Unfortunately, the urge for appropriate therapy is being challenged by the incomplete knowledge of its etiology and pathomechanism.

"Genes load the gun and environment pulls the trigger"—an urban metaphor evolving in the last two decades—emphasizes on the interplay of nature and nurture for disease development: susceptibility to certain disorders depends on the genetic constitution, but additional environmental factors (e.g., stress, abuse or neglect) are often necessary to trigger disease onset (2–4). The latter effect is mediated by epigenetic mechanisms, including DNA/RNA methylation changes or histone modifications, that are capable of changing protein expression (4–8). Regarding MDD, no robust risk genes have been identified so far (9, 10), but various experiments underline the influence of maternal care on DNA methylation, stress reactivity, and anxiety-related behavior in mice (11). In line with these findings, Fuchikami et al. were able to distinguish healthy subjects from depressed patients by DNA methylation analysis of gene regions encoding for BDNF (brain-derived neurotrophic factor) (12)—a protein playing a pivotal role in various neuropsychiatric diseases, including depression (13–15).

Despite the huge variety of antidepressants that are available on the market, 30% to 50% of MDD patients fail to achieve complete remission (16, 17). Electroconvulsive therapy (ECT) has been stated to be the most powerful alternative in the acute treatment of refractory depression (18). A mounting body of evidence suggests ECT to modulate the immune system (19–21), and to boost peripheral neurotrophin expression (22–24). Accordingly, lowered BDNF serum levels of depressed patients (15) were restored after ECT (25). In this context, Sartorius and his group proposed blood-borne BDNF to contribute to parenchymal BDNF (26), and further animal experiments showed peripherally administered neurotrophins to promote neurogenesis and neuroplasticity in the brain (27), probably by mediating direct neuronal support (28) or modulating microglial functions (29). Impaired neuroplasticity [e.g., decreased hippocampal volumes (30), diminished dendritic ramification, or reduced numbers of neurons and oligodendrocytes (31, 32)] are a hallmark of MDD patients or at least of a subgroup thereof. ECT's proposed pro-neurogenic properties are, therefore, considered to be of therapeutic relevance (25, 33, 34).

The enzyme t-PA (tissue-type plasminogen activator) and its inhibitor PAI-1 (plasminogen activator inhibitor-1) are implicated in the cleavage of extracellular pro-BDNF and are thus important for BDNF production (35, 36). Both proteins have additional roles in the regulation of the immune system, as in macrophage migration (37) and NF-κB activation (38). Interestingly, depressed patients show declined t-PA serum levels (39) and raised PAI-1 activity (40) when compared to healthy controls. Further evidence for their involvement in MDD arises from animal experiments that found low t-PA measures in the CNS (correlating with high levels of PAI-1) to be associated with anxiety- and depressive-like behavior (41). Additionally, pro-BDNF and t-PA concentrations were upregulated in the rat hippocampus—followed by an increase of mature BDNF—after treating rodents with electroconvulsive shocks, the animal model of ECT; intriguingly, this effect was not seen upon imipramine treatment (42). Although both proteins seem to be a link between multiple factors that are highly relevant for MDD (43, 44), their epigenetics have not been investigated in this context yet.

We are the first ones to analyze the DNA methylation of gene regions encoding for t-PA (*PLAT*) and PAI-1 (*SERPINE1*) in depressed patients undergoing a course of ECT. Importantly, previous studies in the biomarker field of psychiatric epigenetics analyzed—if merely blood was taken—the DNA methylation of the diverse mixture of immune cells only. Instead of investigating different immune cell subtypes separately, these studies tend to draw conclusions that highly depend on immune cell counts and their changes. To address this problem, we isolate peripheral blood mononuclear cells (PBMCs) from our patients and sort defined immune cell subpopulations (i.e., B cells, monocytes, natural killer (NK) cells, and T cells) using flow cytometry. Analyzing the DNA methylation of these subsets separately enables us to unravel whether there is a particular immune cell subtype that takes the lead regarding clinical outcome, thereby allowing us to get a deeper insight into ECT's therapeutic effects. By these means, we pursue (1) to characterize ECT's general influence on DNA methylation of defined target regions reported to be implicated in MDD, (2) to illuminate the relevance of DNA methylation at baseline or ECT-associated epigenetic changes for clinical remission, and (3) to explore the inaccuracy of DNA methylation measurements obtained from whole blood only. We hypothesize ECT to cause changes in the epigenetics of defined immune cell subtypes in target regions encoding for t-PA and PAI-1, and this effect to be of therapeutic relevance. We further suggest the DNA methylation of these regions to serve as a possible biomarker for ECT response prediction.

MATERIAL AND METHODS

Study Design

Our prospective study included two different cohorts of refractory MDD patients: the 1st cohort (also referred to as cross-sectional cohort in the following; n = 67) was acquired at the Department of Psychiatry and Psychotherapy of the Charité Berlin (Germany), the 2nd one (=longitudinal cohort; n = 30) at the Department of Psychiatry, Social Psychiatry and Psychotherapy of the Hannover Medical School (Germany). The study was approved by the Ethics Committee of the Charité (EK-224-05c) and the Hannover Medical School (2842-2015) and followed the ethical principles of the Declaration of Helsinki (1964), including its later amendments. All participants gave written informed consent before study inclusion.

Patients

MDD was diagnosed according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) and disease severity assessed before and after the 1st, 4th, the last, and the maintenance ECTs by using either the Hamilton Rating Scale for Depression (HAM-D), or the Montgomery-Åsberg Depression Rating Scale (MADRS) and the Beck's Depression Inventory (BDI-II). The Mini-Mental State Examination (MMSE) was conducted at the same time points. A decline in HAM-D or MADRS scores of ≥50% was interpreted as response, and values of ≤7 (HAM-D) or ≤10 (MADRS) as remission. Non-responsiveness to two state-of-the-art antidepressants (after two weeks of treatment with adequate dosages, respectively) was classified as treatment-resistance. Patients diseased with an infectious, autoimmune, or schizophrenic disorder were excluded. Further exclusion criteria were heightened levels of CRP (C-reactive protein) or pronounced leukocytosis.

ECT Application and Sample Collection

As it was standard clinical practice, ECT was applied three times weekly for up to four weeks. Ultra-brief pulse devices [Mecta 5000Q and Thymatron® System IV (Somatic LLC)] served for right unilateral electrical stimulation. Seizure threshold was assessed upon the first ECT session (age-based method), and the stimulus intensity increased if the seizure was insufficient (i.e., showing an EEG seizure activity below 30 sec or a motoric response shorter than 20 sec). Bilateral ECT was considered if the patient did not show any improvement after two consecutive weeks of treatment. Anesthesia was obtained with propofol or etomidate (cross-sectional cohort) or with methohexital and remifentanil (longitudinal cohort). Succinylcholine served for muscle relaxation in all study participants. Fasting blood samples were withdrawn (1) in the cross-sectional cohort: directly before starting the therapy and (2) in the longitudinal group (which served for the evaluation of DNA methylation changes throughout the full time course of ECT): before (8–10 a.m.) and 15 min after the 1st, the 4th, and the last ECT. Four follow up time points (before and after the 1st and 4th maintenance ECT) were included. Blood samples were collected in 2K EDTA-Gel S-Monovettes® (SARSTEDT AG & Co. KG) and stored at 4°C until further processing (maximum of 3 h).

Sample Processing

Blood

The blood from the cross-sectional cohort was directly used for DNA isolation. EDTA-blood from the longitudinal cohort served for PBMC isolation by density centrifugation as described elsewhere (45). Following the recommendations of Mallone et al. (46), some changes have been applied to the latter protocol; a complete description of the procedure is provided in the **Supplementary Material**.

PBMCs - Thawing, Staining, and Flow Cytometry

Frozen PBMCs were thawed based on a protocol reported elsewhere (47). Changes have been made to the latter procedure; a detailed explanation is given in the **Supplements**. After thawing, cells were stained as instructed by BioLegend's staining protocol[1] using 10% human serum in FACS buffer [=1% BSA (SIGMA-ALDRICH Co.) and 1 mM UltraPure™ EDTA (Invitrogen AG) in PBS (w/o Ca^{2+} and Mg^{2+}; Biochrom GmbH)] as a blocking solution, 7-AAD (1:100, BioLegend) as a viability marker and six different antibodies for the identification of defined immune cell subtypes. Immune cell populations of interest were subsequently sorted using a BD FACSAria™ Fusion flow cytometer and the BD FACSDiva™ 8.0.1. Software (Becton, Dickinson Biosciences). Sorted cells were centrifuged (900*g*, 5 min, 4°C), the supernatant discarded and kept in 500 µl RNAprotect Cell Reagent (QIAGEN N.V.) until further processing (up to one week). Importantly, cells were kept at 4°C and treated under sterile conditions throughout the whole process (i.e., staining, sorting, storing). Further information regarding the procedure is provided in the **Supplementary Material** (**Table S1**). The sorting layout is depicted in **Figure S1**.

DNA Isolation

Genomic DNA Isolation From Whole Blood

For genomic DNA (gDNA) isolation, aliquoted blood samples were thawed at 4°C overnight and 200 µl of each sample incubated with 15 µl dissolved Proteinase K (NucleoMag® Blood 200 µl Kit, Macherey-Nagel) for 1 h (RT). The following procedure was performed as instructed by the NucleoMag® Blood 200 µl Kit manual using an automated program designed for the Biomek® NXp pipet robot (Beckman Coulter GmbH). Isolated gDNA was stored at −80°C until further processing or directly used for bisulfite conversion.

Genomic DNA Isolation From PBMCs

gDNA of PBMCs and sorted immune cell subtypes was isolated using the AllPrep DNA/RNA 96 Kit (QIAGEN N.V.). Minor changes have been made to the recommended procedure; a detailed description is to be found in the **Supplements**.

[1] BioLegend. Cell Surface Flow Cytometry Staining Protocol 2017: https://www.biolegend.com/protocols/cell-surface-flow-cytometry-staining-protocol/4283/ last accessed:02.10.19; 5.22 p.m.

Bisulfite Sequencing

Bisulfite Conversion

Bisulfite conversion of gDNA—extracted from blood, PBMCs, and sorted cells—was performed following the protocol of the EpiTect 96 Bisulfite Kit (QIAGEN N.V.). Converted DNA was stored at −20°C until further processing or directly used for DNA amplification.

DNA Amplification

Primers for fragments of interest (**Supplementary Material, Table S2**) were established using different types of software [Methyl Primer Express V1.0 (Applied Biosystems™), Geneious Pro 5.6.7. (Biomatters Ltd)] and online tools {Ensembl[2], SNPCheck V3[3], NetPrimer[4], Metabion Biocalculator[5], Match 1.0 [BIOBASE GmbH (48)][6]}. Several research reports (49–61) were used as a rationale to define the genomic features we were primarily interested in. Correct fragment size was confirmed by gel electrophoresis using a 1% agarose gel (PEQLAB Biotechnologie GmbH). To amplify patient samples, 1 to 3 µl of bisulfite-converted DNA (of a sample each) was mixed with 5 µl HotStarTaq® Master Mix, 1.2 to 3.2 µl RNase free water (both QIAGEN N.V.) and 0.4 µl of the forward and reverse primer (Metabion GmbH), respectively. For amplification, the established PCR programs (see **Supplementary Material, Table S3**) were used, and the resulting products kept at 4°C to 12°C until clean up. Automated clean up (Biomek® NXp pipet robot) of the amplified fragments followed the instructions of the Agencourt AMPureXP Kit (Beckman Coulter GmbH) using CleanPCR magnetic beads (Clean NA). Eluted DNA was kept at 4°C until sequencing (up to two weeks).

Sanger Sequencing

DNA concentration of the amplified samples was measured with DeNovix DS-11 FX+ (DeNovix Inc.). A maximum of 30 ng bisulfite-converted DNA (per sample) was mixed with 2 µl Big Dye® Sequencing Buffer, 0.5 µl Big Dye® Terminator v.3.1 (both Applied Biosystems™), 0.6 µl forward or reverse primer of the respective fragment and a various amount (6.9 µl minus volume of DNA sample needed) of RNase free water per sample each. After PCR (see **Table S4**), fragments were automatically washed using the Biomek® NXp pipet robot and CleanDTR magnetic beads (CleanNA) following the protocol of the Agencourt® CleanSEQ® Kit (Beckman Coulter GmbH). 15 µl of HI-DI™ Formamide (Applied Biosystems™) was added to each sample, and the sequences analyzed by using a HITACHI 3500xL Genetic Analyzer and the 3500 Series Data Collection Software 2 (both Applied Biosystems™). The quality of the obtained sequences was assessed using the Sequence Scanner 2 Software (Applied Biosystems™). Only sequences above the threshold of >20 (QV) and a continuous read length close to the expected fragment size were included in the statistical analysis. All t-PA fragments that are listed in **Table S2** were sequenced in the cross-sectional cohort, but—to analyze the DNA methylation changes throughout the treatment course—only fragments showing significant results were repeated in the longitudinal cohort (fragment t-PA 2,4,5). In this context, blood-DNA of all time points was sequenced, whereas DNA isolated from immune cells was used from the 1st and last ECT only. Although PAI-1's DNA methylation was showing a very low inter-individual variance, one fragment (PAI-1 2), which serves as a binding site for multiple transcription factors, was repeatedly sequenced in the blood samples of the longitudinal cohort to obtain a larger group size for subsequent DNA methylation analyses.

Data Processing

The Epigenetic Sequencing Methylation Analysis Software [ESME (62), Epigenomics AG] served for DNA methylation analyses. Statistical analyses were performed with IBM SPSS Statistics 25 (IBM Germany GmbH), and data presented with SPSS and GraphPad Prism 8 (Graph Pad Inc.).

Statistics

Patients' demographics were normally distributed. Fisher's exact tests and T tests were used for the analysis of clinical baseline differences between ECT remitters and non-remitters. Bisulfite sequencing yielded methylation values of 39 single CpG sites within the t-PA gene and 35 CpGs within the PAI-1 gene. Before analysis, initial quality checks for the exclusion of potentially unreliable measurements were performed: CpGs with an inter-individual variance below 1% were excluded, leaving 35 CpG sites for the analysis of t-PA. DNA methylation variance of almost all CpGs (32 of 35) within the sequenced PAI-1 gene regions was lower than 1%, but for the purpose of completeness, we decided to include the DNA methylation data in our statistics nevertheless. Furthermore, study participants or CpGs with more than 10% missing values were additionally excluded, leaving a total of 87 patients (cross-sectional cohort: n = 59; longitudinal cohort: n = 28) for final statistics. Methylation data of all sample types (i.e., blood, PBMCs, and defined immune cell subsets) were tested for correlation (Pearson's correlation test) with clinical baseline parameters in the whole cohort as well as in ECT remitters/non-remitters only. Mixed linear models (residual maximum likelihood approach) were performed computing (baseline) methylation as the dependent variable. CpG position was entered as repeated measurement assuming a scaled identity of covariates. Estimated marginal means were calculated for the groups and compared by Sidak's post-hoc test. Parameter estimates were calculated for all factors and manually inspected. Model fits were compared using the −2loglikelihood ratio. Age, gender, and BMI were considered as covariates. Results are depicted as mean ± SD (standard deviation) or ± SE (standard error). Due to the explorative character of our approach, a nominal p-value of ≤0.05 (two-tailed) has been classified as significant (and has thus been the basis for the interpretation of our results), although Type 1 error correction has only been applied to the mixed linear models. For the purpose of

[2] Ensembl: www.ensembl.org last accessed:02.10.2019, 6.29 p.m.
[3] SNPCheck V3: https://genetools.org/SNPCheck/snpcheck.htm last accessed:02.10.2019, 6.31 p.m.
[4] NetPrimer: http://www.premierbiosoft.com/netprimer/ last accessed:02.10.2019, 6.33 p.m.
[5] Metabion Biocalculator: http://www.metabion.com/support-and-solution/biocalculator/ last accessed:02.10.2019, 6.34 p.m.
[6] Match 1.0 Public: http://gene-regulation.com/ last accessed:02.10.2019, 6.54 p.m.

completeness, the adjusted p-value is additionally indicated (wherever applicable), but in the respective tables only.

RESULTS

Clinical Baseline Characteristics

Baseline characteristics of patients are presented in **Table 1** (pooled cohort), and **Supplementary Material, Tables S5**, and **S6** (both cohorts separately). Prior to ECT treatment, patients were suffering from a moderate to severe depression, as the average psychometric test score was 28.5 (± 2.0; 19–34; HAM-D) and 31.7 (± 9.6; 12–45; MADRS). Among all study participants (n = 87), 33 individuals remitted upon ECT; 53 responded. 13 patients had minimally elevated levels for leukocytes (reaching values of up to 14.0 × 10^3/µl), but no signs of infection (i.e., heightened CRP). Participants were medicated while being treated with ECT. In this regard, 28 patients were taking benzodiazepines on a regular basis, with lorazepam being (besides oxazepam, bromazepam, zolpidem, and zopiclone) one of the most commonly prescribed ones (n = 24).

Association of DNA Methylation With Clinical Baseline Characteristics

Analyzing the correlation between the DNA methylation and the patients' clinical baseline characteristics (n = 87), revealed an association between the DNA methylation of total t-PA (r = −0.228, p = 0.033) and the MHRE region (r = −0.229, p = 0.033) with age. A correlation between the number of leukocytes and the mean DNA methylation of PAI-1 has further been found (r = −0.223, p = 0.047; **Supplementary Material, Table S7** and **Figure S2**).

DNA Methylation in Dependence on ECT Remission

Blood—Baseline

Baseline DNA methylation of defined t-PA gene regions differed between ECT outcome groups in the cross-sectional cohort analyzed (**Figure 1A**); these fragments have thus been chosen for further DNA methylation analyses (**Figure 1B, C**). In this regard, DNA methylation of these fragments did not differ between ECT remitters (n = 33) and non-remitters (n = 53) when both cohorts (n = 87) were investigated (**Figure 1C**, **Supplementary Material**, **Table 2**). There were no significant DNA methylation differences within the PAI-1 gene (**Supplementary Material, Figure S3**).

Immune Cells—Baseline

The DNA methylation of defined t-PA regions showed significant differences between ECT remitters (n = 10) and non-remitters (n = 11) in NK cells, T cells, as well as in the whole population of PBMCs (but not in B cells or monocytes; see **Table 2** and **Supplementary Material, Table S8**). Major differences in t-PA's DNA methylation were further found when the different sample types were analyzed (calculated for the whole cohort but being present also in ECT remitters and non-remitters; see **Figure 2** and **Supplementary Material, Table S9** for a detailed depiction). The highest contrast was found in the DNA methylation of the CREB binding site when T cells and monocytes were compared. These differences were present throughout the whole course of ECT as there was neither an interaction between remission nor between the sample type with the measured time points. DNA methylation of PAI-1 was measured in blood only.

DNA Methylation During the Course of ECT

The DNA methylation of defined t-PA gene regions changed upon a course of ECT. Regarding ECT's acute effect (=comparing the methylation status before and after a single ECT session), DNA methylation of CREB (with or without its adjacent binding site) was found to be altered in whole blood [CREB: p = 0.030, mean methylation(± SE): before = 0.365(± 0.010), after = 0.396 (± 0.010)] and in T cells (CREB+CTF/NF1: p = 0.019, before = 0.825(± 0.008), after = 0.799(± 0.008)). The latter CpGs seem to be further involved in ECT's long-term effect: in whole blood, DNA methylation of CREB+CTF/NF1 was found to be changed

TABLE 1 | Clinical baseline characteristics of patients.

		Whole cohort (n = 87)	Remitters (n = 33)	Non-remitters (n = 53)
Demographics				
Age in years, mean (± SD; range)		51.9 (± 16.6; 20–80)	56.0 (± 14.6; 29–80)	49.7 (± 17.5; 20–80)
Gender, n (%)	female	45 (51.7%)	17 (51.5%)	27 (50.9%)
	male	42 (48.3%)	16 (48.5%)	26 (49.1%)
Psychometric characteristics				
Age at diagnosis in years, mean (± SD; range)		32.9 (± 15.5; 12–78)	33.9 (± 14.6; 12–65)	32.2 (± 16.3; 14–78)
Current episode in weeks, mean (± SD; range)		24.4 (± 20.8; 3–124)	19.6 (± 22.2; 3–124)	27.1 (± 19.4; 6–96)
Psychotic symptoms, n (%)	Yes	14 (16.1%)	4 (12.1%)	10 (18.9%)
Medication				
Antidepressant drugs, n (%)	Yes	79 (95.2%)	31 (96.9%)	47 (94.0%)
Benzodiazepines, n (%)	Yes	28 (33.7%)	12 (37.5%)	16 (32.0%)
Antipsychotic drugs, n (%)	Yes	45 (54.2%)	21 (65.6%)	23 (46.0%)
Clinical parameters				
Leukocytes in ×10^3/µl, mean (± SD; range)		7.8 (± 2.3; 3.3–14.0)	7.6 (± 2.3; 3.8–12.4)	8.0 (± 2.4; 3.3–14.0)

Clinical baseline characteristics of refractory MDD patients treated with ECT (whole group vs. ECT remitters and non-remitters), presented as mean (± standard deviation (SD); range (=minimum–maximum) or quantity (absolute and percentual, n (%)). Clinical parameters were equally distributed between ECT remitters and non-remitters.

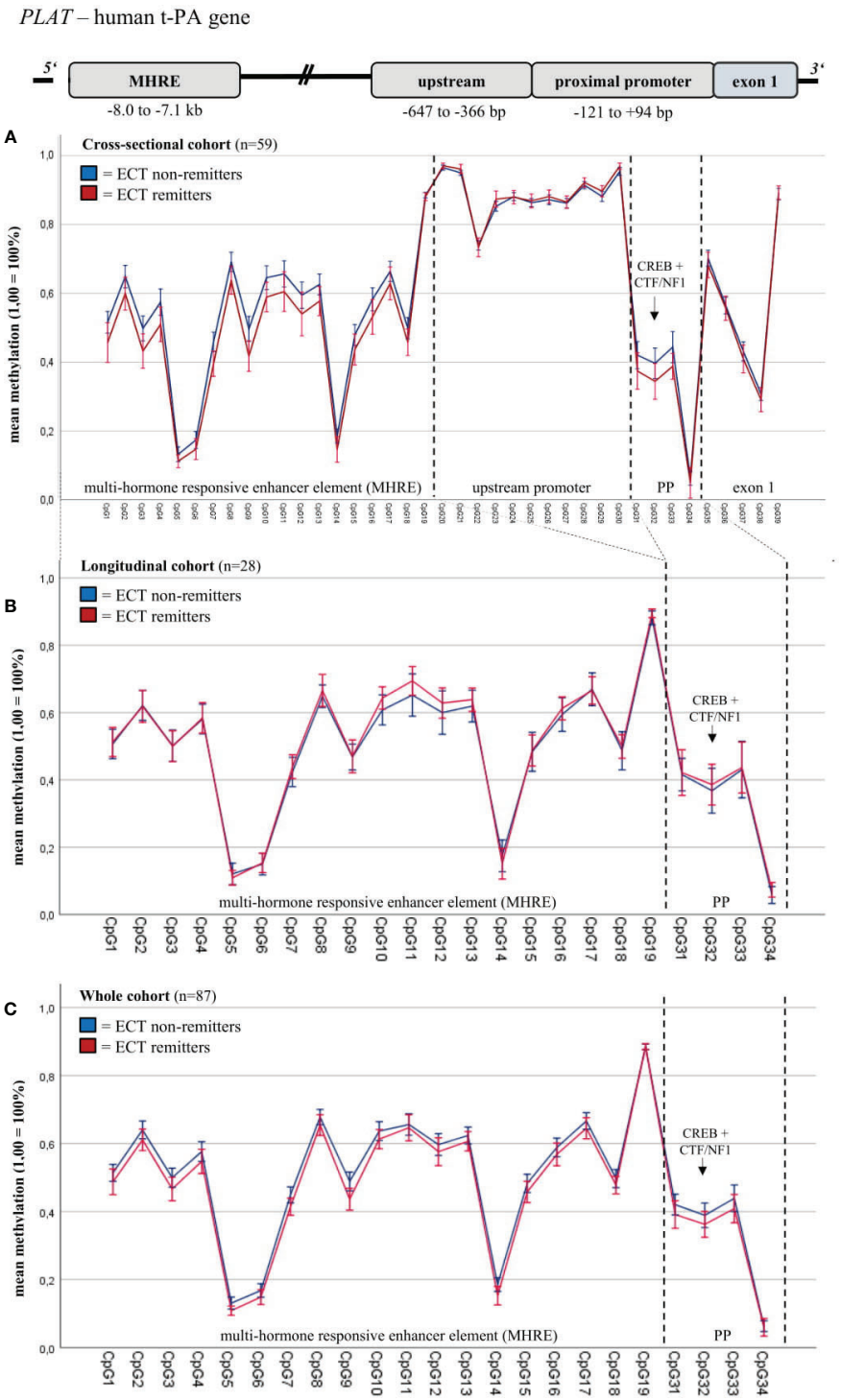

FIGURE 1 | Blood baseline DNA methylation of t-PA in ECT remitters and non-remitters. Mixed linear models (including Sidak's correction) revealed differences in the blood baseline DNA methylation of total t-PA (including fragment t-PA 2, t-PA 4, t-PA 5; see **Supplementary Material, Table S2**) and the MHRE region between ECT remitters and non-remitters in the cross-sectional cohort **(A)**; these fragments have thus been chosen for further DNA methylation analyses (**B**: longitudinal cohort, **C**: merged cohort). In this regard, DNA methylation of these fragments did not differ between ECT remitters (n = 33) and non-remitters (n = 53) when both cohorts (n = 87) were investigated **(C)**. MHRE, multi-hormone responsive enhancer; CREB, cAMP response element-binding protein; CTF/NF1, CCAAT box-binding transcription factor/nuclear factor 1; PP, proximal promoter.

TABLE 2 | DNA methylation of defined t-PA and PAI-1 gene regions in ECT remitters and non-remitters.

Region	Baseline	Whole time course of ECT			
	Blood (n = 87) (R/NR (± SE), 95% CI)	Blood (n = 28) (R/NR (± SE), 95% CI)	PBMC (n = 21) (R/NR (± SE), 95% CI)	NK cells (n = 21) (R/NR (± SE), 95% CI)	T cells (n = 21) (R/NR (± SE), 95% CI)
Total t-PA	0.455/0.472 (± 0.008/0.006) 0.440–0.470/0.460–0.483	0.479/0.459(± 0.005/0.006)** 0.469–0.488/0.447–0.471	0.647/0.622(± 0.008/0.009)* 0.632–0.663/0.605–0.640	0.615/0.592(± 0.009/0.010) 0.598–0.633/0.573–0.611	0.780/0.766(± 0.007/0.008) 0.766–0.794/0.751–0.781
MHRE	0.487/0.502 (± 0.008/0.006) 0.472–0.503/0.489–0.514	0.518/0.490(± 0.005/0.007)*** 0.508–0.527/0.477–0.503	0.690/0.654(± 0.008/0.009)** 0.675–0.706/0.637–0.671	0.655/0.621(± 0.009/0.010)* 0.636–0.673/0.601–0.641	0.804/0.781(± 0.007/0.008)* 0.790–0.817/0.766–0.796
CREB	0.381/0.407 (± 0.014/0.012) 0.353–0.409/0.384–0.430	0.375/0.393(± 0.009/0.012) 0.358–0.392/0.371–0.416	0.557/0.558(± 0.016/0.018) 0.526–0.589/0.521–0.594	0.559/0.573(± 0.014/0.015) 0.531–0.587/0.543–0.603	0.835/0.847(± 0.008/0.008) 0.819–0.851/0.830–0.864
CREB + CTF/NF1	0.393/0.417 (± 0.012/0.010) 0.369–0.416/0.398–0.436	0.383/0.405(± 0.007/0.010) 0.370–0.424/0.370–0.397	0.553/0.568(± 0.013/0.015) 0.528–0.579/0.538–0.597	0.534/0.565(± 0.012/0.013) 0.512–0.557/0.540–0.590	0.801/0.824(± 0.008/0.009) 0.785–0.817/0.807–0.841
Total PAI-1	0.156/0.142 (± 0.015/0.012) 0.127–0.186/0.118–0.165	0.164/0.152(± 0.009/0.013) 0.146–0.183/0.127–0.176	Measured in blood only	Measured in blood only	Measured in blood only

The DNA methylation of defined t-PA gene regions differed between ECT remitters (R) and non-remitters (NR) in various sample types analyzed (i.e., the blood, the whole population of PBMCs (peripheral blood mononuclear cells), natural killer (NK) cells, and T cells). Results were calculated by mixed linear models (including Sidak's correction) and are presented as mean [± standard error (SE)] and 95% confidence interval (CI). Total t-PA methylation includes three fragments (t-PA 2, t-PA 4, and t-PA 5), total PAI-1 methylation only one (PAI-1 2). For further information regarding the analyzed fragments, see ***Table S2*** *in the Supplements. MHRE, multi-hormone responsive enhancer, CREB, cAMP response element-binding protein, CTF/NF1, CCAAT box-binding transcription factor/nuclear factor 1. *p < 0.05, **p < 0.001, ***p < 0.0001.*

following a whole course of ECT (p = 0.002), but no consistent pattern in methylation changes (in terms of a consistent acute or long-term effect) was prevalent when comparing the DNA methylation values of each ECT session. In monocytes, DNA methylation of CREB and CREB+CTF/NF1 was found to be increased after treatment completion (CREB: p = 0.028, 1st ECT = 0.064(± 0.010), last ECT = 0.098(± 0.011); CREB+CTF/NF1: p = 0.023, 1st ECT = 0.109(± 0.012), last ECT: 0.150(± 0.014)). Importantly, these effects seem to be independent of clinical remission (see **Supplementary Material, Tables S10** and **S11**, and **Figure S4** for more details). DNA methylation of PAI-1 (measured in blood only) was left unaffected by ECT.

DISCUSSION

According to our statistics, ECT remitters and non-remitters did not differ in their baseline t-PA or PAI-1 DNA methylation, rejecting thereby our hypothesis of both genes to be suitable biomarkers of ECT responsiveness. However, major differences in the DNA methylation throughout the analyzed t-PA regions were found when the different immune cell subtypes (B cells, NK cells, monocytes, and T cells) were compared. These differences remained stable throughout the whole treatment course and were independent of clinical outcome.

The pattern of DNA methylation (not to be mistaken for the methylation rate) was similar in nearly all sample types with a relatively low mean methylation in the proximal promoter region, a highly methylated upstream promoter (measured in the blood of the cross-sectional cohort only), and an MHRE element showing mean methylation values somewhere in between these two stretches. The same pattern has been reported in various other cell types [i.e., astrocytes, fibroblasts, and HUVECs (human umbilical vein endothelial cells)] (50), though its relevance for t-PA expression has not been entirely clarified yet. A study of Dunoyer-Geindre et al. (published in 2011) suggested t-PA transcription to be inhibited in case of t-PA promoter methylation, most likely due to insufficient binding of various transcription factors (i.e., CREB, CTF/NF1, and Sp1) having their motifs within this region (49, 53). The same group investigated the influence of MHRE methylation—an enhancer element that is located −7.3 kbp upstream of tPA's first exon and that comprises several responsive units for glucocorticoids, mineralocorticoids, or androgens, for example (51)—on t-PA expression and proposed the MHRE region to be of neglectable relevance for t-PA production. Instead, they again confirmed their previous findings by showing an unmethylated proximal promoter region to be particularly required for efficient t-PA expression (50). However, transfection experiments reported the contrary and revealed the enhancer element (together with its hormonal regulators) to influence t-PA transcription, probably mediated by changes in open chromatin formation (51). This group has not investigated the effect of DNA methylation in particular, but more support for the notion of epigenetics being involved comes from Magnusson et al., who observed a decline of DNA methylation in the MHRE region to cause a concomitant

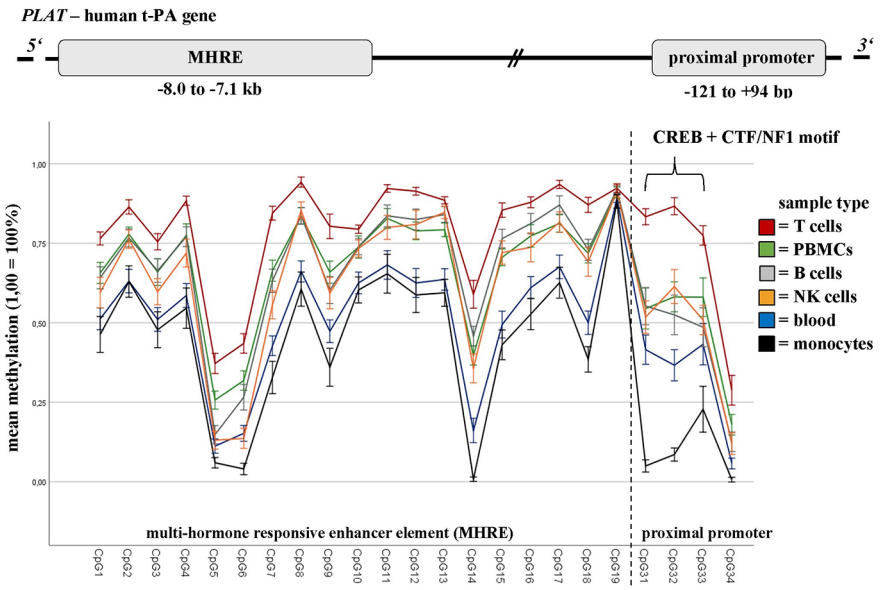

FIGURE 2 | Baseline DNA methylation differences of t-PA in blood and defined immune cell subtypes. DNA methylation rates of defined t-PA gene regions (i.e., the MHRE element (CpG1–CpG18), the CREB binding site (CpG31+32) and the CTF/NF1 motif (CpG33)) differ greatly between the sample types analyzed (namely whole blood (n = 28), peripheral blood mononuclear cells (PBMCs; n = 21), and defined immune cell subsets (n = 21)), collected from refractory MDD patients undergoing a course of ECT. Results are presented as mean (1,0 = 100%), bars are indicating the standard error. MHRE, multi-hormone responsive enhancer; CREB, cAMP response element-binding protein; CTF/NF1, CCAAT box-binding transcription factor/nuclear factor 1.

increase in t-PA production in cultured human endothelial cells (52).

The inconsistency between these findings hinders a clear interpretation of the methylation pattern found in our cells, though it would suggest monocytes (among those cells analyzed) to be predominantly involved in t-PA expression. Intriguingly, monocytes had also the lowest CpG methylation at the CREB binding site—a motif repeatedly shown to be involved in depression by mediating several effects down- and upstream of BDNF (63, 64). Nonetheless, t-PA's DNA methylation remained remarkably stable throughout the course of ECT, irrespective of the sample type analyzed, showing only marginal changes (partially with inconsistent patterns) that can be considered as negligible. Because DNA methylation was further showing only subtle differences between ECT remitters and non-remitters in sorted immune cell subpopulations, we must assume that the DNA methylation of t-PA is only of minor importance (if at all) considering ECT's therapeutic effects. Nevertheless, one should keep in mind that we only investigated the DNA methylation of major immune cells subtypes, leaving out subsets that are quantitatively small in numbers (like T regulatory cells or non-classical monocytes, for instance). Since we observed major differences between the CpG methylation of different immune cell subtypes, we highly recommend DNA methylation analyses to be performed in defined cell subsets and not in whole blood only. Especially if we consider leukocytes to be highly adaptive and plastic (65) (at least to some extent)—changing their composition in dependence of age (66), smoking behavior (67), BMI (68), health status (69, 70), and exercise (71), for example—one must conclude whole blood DNA methylation to be influenced by hardly controllable errors, especially in smaller group sizes.

The relatively small group size is also a limitation of our pilot study. Further, the resolution of our DNA methylation data is restricted by the sequencing technique used: while Sanger sequencing is sufficient to call large methylation differences among distinct cell types (**Figure 2**), other methods (as next-generation sequencing) are preferable to capture small DNA methylation changes. Particularly the results regarding the comparison of ECT remitters/non-remitters and the different time points of ECT treatment, must be, therefore, interpreted with caution. Another circumstance troubling our approach is the medication (including the anesthetics and muscle relaxants) that the patients received while being treated with ECT. As pharmaceuticals can potentially impact DNA methylation, particularly anesthetics were further suggested to mediate antidepressant effects that are comparable to ECT treatment (72). Besides drug intake, DNA methylation changes were also associated with age (73), gender (74), and BMI (75). These three factors were, therefore, considered as covariates.

CONCLUSION

Epigenetics of t-PA and PAI-1 seem to be of minor importance considering ECT's therapeutic effects and are not suitable as biomarkers for ECT response prediction. However, t-PA's DNA methylation differed greatly between the different immune cell

subsets analyzed. This result leads to the recommendation of DNA methylation analyses to be performed in various immune cell subtypes and not in whole blood only, especially if the cohort is small and the confounding factors hardly to control.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study involving human participants were reviewed and approved by the Ethics Committee of the Charité (EK-224-05c) and the Hannover Medical School (2842-2015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NM: Establishing and conducting the experiments described in the *Material and Methods* section (excluding the clinical part). Processing data, including their analysis. Writing the paper, including the **Supplementary Material**, KJ: Providing enormous support in all experiment-related issues, MBaj: Acquisition and treatment of the cross-sectional cohort (providing clinical data and blood samples), HM and CP: Recruitment and treatment of the longitudinal cohort (providing clinical data and blood samples), MBal: Giving advice regarding flow cytometry (choice of antibodies and general procedure). Designing the layout for flow cytometry and sorting the PBMCs. AK: Support with the processing of blood, SB: Substantial contribution to the conception of the work and acquisition of patients. Revising the study for important intellectual content, HF: Substantial contribution to the conception and design of the work, the acquisition of patient groups, and the analysis and interpretation of data. Drafting the work and revising it critically for important intellectual content. AN: Supervising the study. Essential contribution to the conception and design of the work, the acquisition of patient groups, and the analysis and interpretation of data. Drafting the work and revising it for important intellectual content. Strong guidance regarding the writing of the paper, and the presentation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00571/full#supplementary-material

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MINI REVIEW
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Heart Rate Variability for the Prediction of Treatment Response in Major Depressive Disorder

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Major depressive disorder (MDD) is one of the disabling diseases in the world-wide, and known to increase cardiac morbidity and mortality. Therefore, previous studies related heart rate variability (HRV) have been conducted to evaluate and diagnose MDD, and to predict treatment outcomes in patient with MDD. We reviewed extensively on the previous peer-reviewed publications associated with this issue, using Pub-Med. In this review article, we introduce the basic concept of HRV and HRV measures, and present several important findings associated with diagnosis and treatment prediction in MDD with using HRV parameters. Furthermore, we discuss the possible underlying mechanism of this phenomenon, and suggest several considerations for the future research.

Keywords: heart rate variability, major depressive disorder, antidepressant treatment, treatment, diagnosis

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INTRODUCTION

Major depressive disorder (MDD) is one of the most disabling conditions, featured by depressive episodes lasting at least two weeks, over changes in mood, cognition, and vegetative symptoms (1). However, because MDD is a heterogenous condition, and patients with MDD exhibit multiple variable symptoms, which make the correct diagnosis difficult (2). Furthermore, although antidepressant medication has been considered as the first-line treatment for MDD, only 50% of patients are non-responsive to initial treatment, and it is difficult to predict future responsiveness of MDD at the time of beginning treatment (3). Therefore, it is necessary to develop a more reliable method to diagnose MDD and predict treatment responsivity in MDD patients.

Numerous research findings have proven that major depressive disorder (MDD) is strongly associated with elevated risk for the development and progression of cardiovascular diseases (4–13). Autonomic nervous system (ANS) dysfunction is considered one of the pathways linking MDD and negative CVD outcomes (14). Heart rate variability (HRV), levels of variability of the heart beat-to-beat interval over time, has been known to provide an index of ANS functioning including the sympathetic and parasympathetic system (15). In this brief review, we aim to describe a clinical overview of the HRV parameters, methodologic issues, and HRV research which found an association between HRV parameters and MDD diagnosis, and between baseline HRV parameters and MDD treatment responsivity.

MATERIALS AND METHODS

We performed a brief review of major publication on the diagnosis for MDD with using HRV use, and predictive value of HRV parameters for treatment response, especially in patients with MDD. A structured literature search was conducted from the PubMed data base until March 2020 (with no publication data limitations) (Arksey and O'Malley, 2005). Search terms and databases were determined in consultation with a health science librarian at Korea University and Samsung Medical Center. Relevant articles which were identified using the following keywords: "heart rate variability" and "major depressive disorder" and "diagnosis"; "heart rate variability" and "major depressive disorder" and "treatment"; "heart rate variability" and "major depressive disorder" and "treatment response"; "heart rate variability" and "depression" and "remission". The retrieved title and abstracts were investigated for relevance for two reviewers (Kwan Woo Choi [KWC], and Hong Jin Jeon [HJJ]) using the following inclusion criteria: 1) the study focused on heart rate variability as the main outcome; 2) the study mainly focused on the diagnosis or treatment response of major depressive disorder (MDD); 3) the population of the study targeted adult people, who are older than 18 years old; 4) the article is written in English. In reviewing abstracts, citations were excluded from the review using the following criteria: 1) the study does not deal with specific HRV parameters; 2) the study was not written in English. The initial database search returned 155 database citations. 155 abstracts were selected to review for inclusion in the scoping review and 27citations qualified for full paper review. Following full paper review, seven articles were excluded as they did not meet inclusion criteria. The final set of 13 studies included (**Figure 1**).

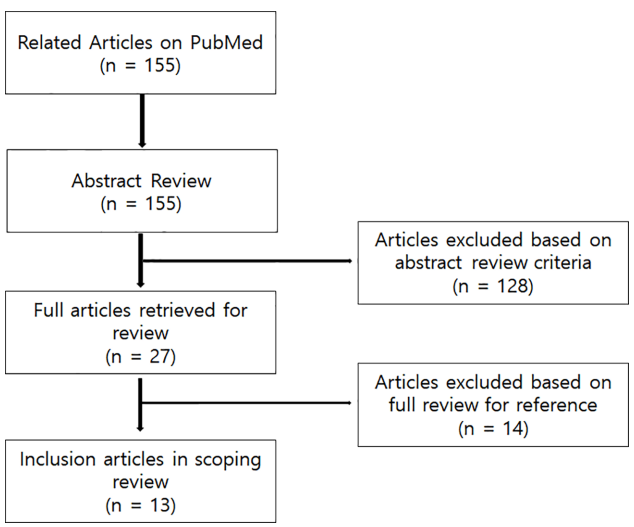

FIGURE 1 | Flow chart of review selection process.

HEART RATE VARIABILITY PARAMETERS

HRV is defined as fluctuation of the heart beat interval over time (16). Since the heart is dually innervated by the sympathetic and parasympathetic branches of the autonomous nervous system (ANS), subtle moment-to-moment changes in heart rate (HR) are qualitative indicators of ANS function (17). According to the reliable international guidelines, HRV parameters could be divided into two domains; frequency domain (spectral analysis) and time domain (non-spectral analysis) (**Table 1**) (15, 18).

Time Domain Measures

Time domain HRV features are calculated with simple mathematical methods to measure the amount of variability present in a specific time period in a continuous ECG signal (19). These parameters are based on the time series of R to R interval (RRI) from the ECG signals. The standard deviation of average normal to normal (NN) intervals (SDNN), the root mean square of successive differences (RMSSD), and the percentage of absolute differences in successive NN values greater than 50ms (pNN50) are widely utilized as time-domain HRV indicators. SDNN is known to reflect both sympathetic and parasympathetic functioning, whereas RMSSD and pNN50 are related to parasympathetic functioning (20–22).

Frequency Domain Measures

Frequency domain provides an assessment of vagal modulation of the RRI, extracted from the ECG. Frequency domain is mostly commonly acquired by fast Fourier transformation to separate RRI into characteristic very low frequency (VLF, 0.003–0.04 Hz), low frequency (LF, 0.04–0.15 Hz), and high frequency (HF, 0.15–0.4 Hz) band (20). Spectral measures are acquired over different time intervals (approximately 2.5 to 15 min), depending on the frequency being analyzed (20). According to previous studies, LF is influenced by both sympathetic and parasympathetic activities, and HF is affected by mostly parasympathetic activities (23, 24). LF/HF ratio is ratio of LF and HF, and it implicates the sympathetic predominance compared to parasympathetic activities (21, 22).

TABLE 1 | A brief description of the most relevant measures of heart rate variability.

Parameters	Description
Time domain	
SDNN	SD of the normal to normal intervals
RMSSD	Square root of the mean squared difference between successive RRs
NN50	The numbers of successive RR intervals that differ by more than 50ms
pNN50	The percentage of NN50
Frequency domain	
HF	Power band encompassing 0.15–0.4 Hz range
LF	Power band encompassing 0.04–0.15 Hz range
VLF	Power band encompassing 0.003–0.04 Hz range
LF/HF	The ratio between LF and HF

HF, high frequency; LF, low frequency; VLF, very low frequency.

HRV FOR THE DIAGNOSIS OF MDD

MDD is assoicated with the increased risk of cardiovascular morbidity and mortality (4, 5), and also known to be associated with reduced HRV (25, 26). For these reasons, there have been numerous studies to find the neurobiological biomarkers of MDD related to HRV parameters (**Table 2**).

Increased LF/HF Ratio

Consistently, there have been many reports which showed an increased LF/HF ratio in patients with MDD compared to HCs (25–28). According to the previous meta-analysis by Kemp et al. which compared 673 depressed patients and 407 healthy controls (HCs) with using 18 articles (25), depressed patients without cardiovascular disease showed reduced time domain HRV, HF HRV, and increased LF/HF ratio than HCs. Udupa et al. also found that 40 patients with MDD showed a more increased LF/HF ratio than 40 age matched HCs (27). More recently, Choi et al. found that patients with MDD showed an elevated LF/HF ratio especially after the stress induction compared HCs (28). The LF/HF ratio is associated with sympathetic predominance (34), which could be related to the increased sympathetic modulation or disrupted ANS modulation in MDD.

Low HF

As well as LF/HF ratio, decreased HF has also been one of the consistent HRV parameters which were significantly associated with MDD (25, 29, 30, 35, 36). In one meta-analysis, patients with MDD had lower resting levels of HRV than HCs (25). According the large-scale prospective Netherland Study of Depression and Anxiety (NESDA), Licht et al. also showed that remitted and current MDD patients had a lower HF compared to HCs, although they concluded the association appeared to be mainly associated with the effect of antidepressants (36). Rottenberg et al. also found significantly reduced HF in patients with MDD, however the overall effect size was relatively small according to their meta-analysis (29). In reviewing previous reports, HF could be particularly related with anxious depression. Reduction in HF might have significant relations with anxiety according to neurovisceral integration (NVI) model studies (37, 38). Moreover, various anxiety disorders were associated with reduced HRV (39, 40). Some investigators found that low HF in MDD is driven or exacerbated by co-occurring anxiety (26, 31, 41, 42). HF is associated with the parasympathetic tone (43, 44). Relatively high HF is known to reflect adaptive functioning, and neural activity in the prefrontal cortex related to emotional, cognitive, and autonomic regulation (38, 44). Further studies will be needed to evaluate the relationship between HF and MDD.

TABLE 2 | Heart rate variability for the diagnosis of major depressive disorder.

Reduced HRV	Kemp et al. (25), Kemp and Quintana, 2013
Increased LF/HF ratio	Udupa et al. (27), Kemp et al. (25), Kemp et al. (26), Choi et al. (28)
Lower HF	Rottenberg (29, 30), Licht et al. (31), Kemp et al. (25)
Lower pNN50	Wang et al. (32), Ha et al. (33), Choi et al. (28)

HF, high frequency; pNN50, the percentage of absolute differences in successive NN values greater than 50 ms.

Low pNN50

The pNN50 is known to be associated with HF, which reflects the activity level of the parasympathetic nervous system (15, 28). There have been several reports which showed reduced pNN50 in patients with MDD compared to HCs (28, 32, 33). Especially, Ha et al. showed that reduced in pNN50 in the medication-naïve, and newly diagnosed with elderly MDD patients who were older than 60 years old (33). Choi et al. also revealed a lower value of pNN50 compared to HCs at the stress phase, and recovery phase (28).

BASELINE HRV PARAMETERS FOR THE PREDICTION OF TREATMENT RESPONSE IN MDD

Antidepressants have been utilized as a front-line treatment of MDD, whereas only one-third to one-half MDD patients who take a complete initial course of antidepressants achieve remission (45, 46). Whereas there are consistent findings that tricyclic antidepressants reduce HRV, it is controversial whether selective serotonin reuptake inhibitors (SSRIs) alters HRV (25, 47). Although there have been studies which found increases in HRV or stability after successful antidepressant treatment in patients with MDD (48, 49), there has been paucity of research which found specific baseline HRV parameters, which could predict treatment responsivity in patients with MDD. **Table 3** summarizes previous HRV findings associated with MDD.

Previously, in response to the emotional stimulus, baseline changes in LF and LF/HF ratio were positively associated with the decrease level in MDD symptoms during fluoxetine treatment (50). More recently, Jain et al. found that baseline VLF was negatively correlated with symptom improvement in depression (51). Shapiro et al. showed that remitters in MDD had significantly more increased HF, and decreased LF than non-remitters during yoga treatment (52). Choi et al. found that delta LF/HF ratio (Stress phase–Rest phase), and delta pNN50 (Stress

TABLE 3 | Heart rate variability for the prediction of better or worse treatment response in MDD.

HRV measures	Related studies
Baseline LF, LF/HF response to the emotional stimuli	Fraguas et al. (50) (Fluoxetine treatment)
Baseline VLF	Jain et al. (51)
Higher HF, and Lower LF	Shapiro et al., (52) (Yoga treatment)
Higher Delta (Stress–Rest phase) LF/HF ratio, pNN50	Choi et al. (28)
Higher baseline HF	Kircanski et al. (31) (Anxious depression)

HF, high frequency; LF, low frequency; VLF, very low frequency; pNN50, the percentage of absolute differences in successive NN values greater than 50 ms.

phase–Rest phase) were significantly positively associated with treatment response (after 12 weeks) in patients with MDD (28). Regarding types of MDD, Kircanski et al. recently showed that patients with higher HRV, or HF had better treatment outcomes especially in anxious depression (31). However, in non-anxious depression, patients with lower HRV had better outcomes (31). Their study implicates that there might be subtype-specific treatment biomarkers in patients with MDD. A similar study. attempted to differentiate treatment response group from non-response group using EEG and HRV (53). However, the researchers did not predict treatment responsivity in depression using HRV parameters, while they could predict treatment outcome in MDD only using the EEG parameter (53). Despite focusing on PTSD diagnosis other than MDD, Minassian et al. showed that high LF/HF ratio (>6.7) before deployment was significantly associated with post-deployment post-traumatic stress disorder (PTSD) in active-duty marines (54).

POSSIBLE IMPLICATION

Disrupted autonomic function may be regarded as a serious pathophysiological candidate for elevated risk of cardiovascular mortality in patients with MDD. Thayer and Lane suggested a neurovisceral integration (NVI) model in the context of emotional regulation (55). According to the NVI model, decreased activation of the central autonomic network (CAN) may affect the decreased level of HRV. CAN is known to be the constellation of brain areas responsible for the neurobiological and physiological regulation of affect and attendant behaviors. According to the NVI modes, the CAN modulates the neuroendocrine, visceromotor, and even behavioral systems (37, 56). Furthermore, the CAN has connection with the sinoatrial node of the heart ***via*** the stellate ganglion through vagus nerve (57). Therefore, HRV is a widely utilized biomarkers of CAN regulatory functioning and considered an informative indicator of brain–body integration, and concomitant health or pathological states (58, 59). CAN is known to consist with the anterior cingulate cortex, insular cortex, ventromedial prefrontal cortex, and the various subcortical structures such as amygdala, hypothalamus, periaqueductal gray matter, parabrachial plexus, and etc. (55–57). Both direct and indirect links between frontal cortex and autonomic motor circuits have been known to be responsible for both the sympathetic and parasympathetic effects on the heart (21, 37, 56). Previous brain imaging studies found that brain regions such as right superior prefrontal, right dorsolateral prefrontal, right dorsolateral prefrontal and left rostral anterior cingulate cortices showed significantly functional decrease concomitantly with decreased HRV (37, 60–62). According to the Thayer and Lane, prefrontal top-down inhibitory and regulatory processes might influence on subcortical emotion regulation centers (37). MDD can be related to the prefrontal hypoactivation and the loss of inhibitory neural functioning with poor affective information processing and regulation (21, 37, 55, 56, 63). Prefrontal hypoactivity might be associated with altered cardiac function in MDD patients, specifically for treatment non-responders.

METHODOLOGIC CONSIDERATION

Although HRV is a non-invasive, pain free, economic and simple technique and one of the easily accessible modalities measuring ANS profiles (18), it is important to consider several important potential confounding factors for the future research.

Time of Measurement

Due to circadian variation in autonomic cardiac function and HRV (64, 65), it should be recommended to evaluate HRV parameters at about the same time of the day. Furthermore, participants should be recommended to have a normal sleep routine, no intense physical training, and no alcohol the day before the measurement (66–70).

Demographic Factors: Age, Gender, Alcohol Use, Smoking and Body Weight

According to the previous research, HRV decreases with aging (71), or HRV parameters changes with a trend toward a decrease in autonomous cardiac function (72, 73). HRV parameters are also known to have different profiles between male and female population (71, 72, 74). In the recent meta-analysis, Koenig and Thayer showed that females had a significantly lower mean RR interval, lower SDNN, lower LF power, lower LF/HF ratio and greater HF power, which implied more increased parasympathetic activity than males (74). Alcohol use is also associated with altered HRV parameters (69, 70). According to the meta-analytic study by Quintana et al., alcohol dependence patients showed reduced HRV compared to nondependent controls (69). On the contrary, the researchers found that habitual, and moderate alcohol drinkers showed increased levels of HF compared to nonhabitual drinkers in their other original study (70). It might be associated with a J-shaped curve that moderate alcohol use is related to a protective effect compared to alcohol dependence or abstinence (69, 70). Smoking is also associated with reduced HRV levels according to previous studies (75, 76). Recent studies also reported that even e-cigarette use decreased HF component, and increased LF and LF/HF ratio compared to controls (77, 78). Weight, height, and waist-to-hip ratio are also considered as potential confounding factors (79). Yi et al. recently showed that waist-to-hip ratio was more strongly correlated with HRV indices and more likely predict reduced HRV compared to body mass index (BMI), and percentage of body fat mass. However, although the previous study indicated no correlation between HRV and BMI (80), BMI should be considered as one of the confounding factors since it is still controversial (79, 81). Therefore, above-mentioned demographic factors should be considered to conduct future research related to HRV.

Antidepressant Medication

Previous studies suggest HRV alterations related to antidepressant medication. According to 2010 Kemp et al.'s

meta-analysis, they showed that tricyclic antidepressant (TCA) decreased HRV whereas SSRI nefazedone, and mirtazapine did not have any significant effect on HRV (25). On the contrary, their large-scale longitudinal study showed that SSRI, and serotonin and norepinephrine reuptake inhibitors decreased HRV parameters (82). More recently, without TCA and clozapine, there were no significant effect on HRV parameters associated with SSRI treatment (83). Futures studies will be needed to clarify relationships between specific treatment regimen and HRV parameters.

CONCLUSION

In conclusion, there have been several attempts to diagnose MDD, and to predict treatment responsiveness in patients with MDD with using baseline HRV parameters. We should consider methodological issues and potential confounding factors to examine relationships between MDD and HRV parameters. Furthermore, it will be needed to have larger sample size, prospective and longitudinal study design, and related other regimen such as neuroimaging, inflammatory markers, and so on for the more refined future research.

AUTHOR CONTRIBUTIONS

KC and HJ: Writing and reviewing the whole manuscript.

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Gender Differences in Associated and Predictive Factors of Anxiety and Depression in People With Epilepsy

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Purpose: Comorbid anxiety and depression in people with epilepsy (PWE) are highly prevalent and contribute to low quality of life (QOL) and may even lead to poor outcomes of epilepsy. Among the various factors that affect these negative emotional comorbidities, possible gender differences remain poorly understood and are often neglected. This research aimed to determine whether there are discrepancies in the incidence and influence factors of anxiety and depression between men and women with epilepsy in a hospital in northwest China.

Methods: A total of 158 adult PWE (female: N = 65; 41.1%) completed self-report questionnaires, including the Self-rating Anxiety Scale (SAS), the Self-rating Depression Scale (SDS), the Chinese version of the Quality of Life in Epilepsy-31 (QOLIE-31) inventory and the Pittsburgh Sleep Quality Inventory (PSQI). The comparison between male and female PWE was made by regression analysis.

Results: For the prevalence of anxiety and depression in PWE, no gender difference was found in this study. However, the moderating factors of psychiatric comorbidities were significantly different between men and women: male PWE with comorbid anxiety were more likely to be affected by sleep quality, while anxiety symptoms in female PWE were closely associated with the frequency of seizures. Education years and QOL social function were significant indicators of depression in male PWE but not in female PWE. The important and common predictor for anxiety and depressive symptoms in PWE was QOL energy/fatigue, with male patients being more affected.

Conclusion: For the PWE included in this study, the incidence of comorbid anxiety and depression in PWE was similar for men and women, but the moderating factors affecting comorbid anxiety and depressive disorders differed between genders: male PWE were more likely to be affected by psychosocial factors, while female PWE were more influenced by epilepsy itself. This exploration suggests that gender-specific health care should be considered in epilepsy therapy to improve the psychiatric condition and QOL of PWE, and different treatments should be conducted for male and female PWE to prevent negative emotional comorbidities.

Keywords: epilepsy, anxiety, depression, psychiatric comorbidity, gender differences, risk factors

INTRODUCTION

Anxiety and depression as high-rate psychiatric comorbidities are more common in people with epilepsy (PWE) (prevalence: 20%–50%) than in the general population (prevalence: 7%–20%) (1–5)]. The relationship between epilepsy and psychiatric disturbance is bidirectional; specifically, PWE are more prone to develop psychiatric comorbidities, while patients with certain primary psychiatric disorders are at higher risk of developing epilepsy (6). This bidirectional relation may be explained by the common pathogenesis of both epilepsy and psychiatric disorders (7). Psychiatric comorbidities, especially depression and anxiety, impact seizure disorders and the lives of PWE. To improve quality of life and promote efficiency in the treatment of epilepsy, screening for anxiety and depression and seeking for possible causes should become a necessary clinical routine. Unfortunately, psychiatric comorbidities often go unrecognized and untreated in PWE (8).

Although studies have found that women usually experience higher levels of anxiety and depression than men in the general population (9, 10), little is known about the gender differences in comorbid psychological disorders in epilepsy. So far, only one study has estimated the gender differences in the incidence of comorbid anxiety and depression in PWE and found that female patients with epilepsy are more susceptible to be affected by depression than male patients, but there is no gender difference in the prevalence of anxiety [(10). Therefore, more evidence is needed to increase the understandings of the gender differences in comorbid anxiety and depression in PWE.

Based on the gender discrepancy in psychiatric disorders, we hypothesized that there should be differences between male and female PWE in comorbid anxiety and depression. So the objective of the present study was to seek out gender differences in sociodemographic, clinical, incidence and risk factors of epilepsy comorbid anxiety/depression symptoms by investigating adult PWE in our hospital located in northwest China. Using statistical analyses of data obtained by self-rating questionnaires, we found differences between genders in the most predictors but not prevalence of epilepsy comorbid negative emotions.

SUBJECTS AND METHODS

Participants and Recruitment

All 158 (male 93, female 65) adult PWE were recruited from the outpatient and inpatient Department of Clinical Neurology, the 940th Hospital of Joint Logistics Support Force of People's Liberation Army (Lanzhou, Gansu Province, China). Patients were consecutively enrolled in this study between March 2017 and November 2017. The included patients should meet following criteria: (1) older than 18 years, (2) no other systemic disease, and (3) epilepsy was diagnosed by experienced epileptologists according to the International League against Epilepsy, (4) cognitively capable of communicating with physicians and understanding the questionnaires. After recruited the patients, resident doctors sent the questionnaires to the patients and helped them to fill in; physicians, attending doctors were responsible for collecting and analyzing data.

This study was approved by the Institutional Review Board prior to initiation, and each participant was consent and signed the patient's medical notes to collect demographic and clinical information, including age, gender, years of education, occupational status, age at onset, duration of seizures, the number of antiepileptic drugs (AEDs) used daily, frequency of AED use, self-reported adverse effects of AEDs and frequency of seizures.

Assessment Scales

Self-Rating Anxiety Scale and Self-Rating Depression Scale

We adopted the Self-rating Anxiety Scale (SAS) and the Self-rating Depression Scale (SDS) to assess anxiety and depression in the enrolled PWE. The SAS and SDS are widely used questionnaires among PWE, and their Chinese versions were administered in a previous study (11, 12). Either the SAS or the SDS composes 20-item symptom inventories, and each item is rated on a scale from 1 to 4. The total score is multiplied by 1.25 and is then converted into a standardized score ranging from 25 to 100, with higher scores reflecting more severe anxiety and depression. The criteria of severities applied in China are as the following: mild anxiety (score 50–60) and mild depression (score 53–62), moderate anxiety (score 61–70) and moderate depression (score 63–72), and severe anxiety (score>70) and severe depression (score>72) (13).

Quality of Life in Epilepsy-31 Inventory

Quality of life was assessed using the Chinese version of the Quality of Life in Epilepsy-31 (QOLIE-31) inventory, which is widely acknowledged epilepsy-specific QOL instruments and shows good validity and reliability in Chinese populations (14–16). The QOLIE-31 contains seven multi-item scales: seizure worry, overall quality of life (QOL), emotional well-being, energy/fatigue, cognitive function, medication effects and social function. The QOLIE-31 overall score is the sum of the weighted average of each subscale score, ranging from 1 to 100, with higher scores meaning a more favorable QOL (17, 18).

Pittsburgh Sleep Quality Inventory

The Pittsburgh Sleep Quality Inventory (PSQI) is a self-report questionnaire for assessing sleep. The global score ranges from 0 to 21, with a higher score indicating poorer sleep quality. The scale consists of seven subscales, comprising subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbance, use of sleep medications and daytime dysfunction. A total PSQI score of 5 or more points indicates poor sleep quality, and more than 10 points is considered severely disturbed sleep (19).

Statistical Analysis

All data were analyzed using the software package SPSS 23.0. Descriptive statistics were calculated for all variables. Quantitative data are expressed as the mean ± standard deviation (SD). Qualitative data are shown and summarized as numbers and proportions.

Gender differences were compared using Student's t-test for continuous variables and Chi-square tests for categorical variables. To explore the relationships between depression/anxiety and sociodemographic, psychosocial and clinical variables, Pearson's r was calculated. The independent variables correlating with anxiety or depression, which were defined as the scores of SAS (≥ 50) and SDS (≥ 53), respectively, were introduced into a logistic regression model with stepwise selection. All tests for statistical significance were two-sided, and $p < 0.05$ was considered significant.

RESULTS

Demographic and Clinical Characteristics

The demographic and clinical characteristics of 158 PWE (93 male and 65 female) are summarized in **Table 1** and **Figures 1** and **2**. Most parameters were similar in male and female patients. The significant difference between genders was only found in terms of education years in which male patients had a longer education time than female patients (**Figure 1C**).

TABLE 1 | Gender differences in demographic and clinical characteristics.

Characteristics	Total sample (N = 158)	Men (N = 93)	Women (N = 65)	*p*
Gender, n (%)				
Male	93 (58.9)	93 (100)	–	
Female	65 (41.1)	–	65(100)	
Age, years (mean ± SD)	26.39 ± 10.10	26.02 ± 9.11	26.92 ± 11.44	0.583
Settlement, n (%)				0.064
Rural area	76 (48.1)	39 (41.9)	37 (56.9)	
Urban area	82 (51.9)	54 (58.1)	28 (43.1)	
Education years (mean ± SD)	10.27 ± 3.86	10.80 ± 3.35	9.52 ± 4.42	0.041
Occupation status, n (%)				0.098
Full-time student	38 (24.1)	20 (21.5)	18 (27.7)	
Employed	67 (42.4)	46 (49.5)	21 (32.3)	
Unemployed	53 (33.5)	27 (29.0)	26 (40.0)	
Age of onset, years (mean ± SD)	20.33 ± 3.86	20.28 ± 11.47	20.41 ± 12.22	0.946
Duration of seizures, years (mean ± SD)	5.98 ± 7.00	6.07 ± 7.59	5.83 ± 6.08	0.833
Number of AEDs used daily, n (%)				0.082
0	66 (41.8)	43 (46.2)	23 (35.4)	
1	57 (36.1)	28 (30.1)	29 (44.6)	
2	23 (14.6)	12 (12.9)	11 (16.9)	
≥3	12 (7.6)	10 (10.8)	2 (3.1)	
Frequency of seizures, n (%)				0.319
Seizrue free > 1 year	50 (31.7)	29 (31.2)	21 (32.3)	
≥1 per 1 year	15 (9.5)	7 (7.5)	8 (12.3)	
≥1 per 6 months	27 (17.1)	13 (14.0)	14 (21.5)	
≥1 per 3 months	23 (14.6)	15 (16.1)	8 (12.3)	
monthly	19 (12.0)	15 (16.1)	4 (6.2)	
weekly	18 (11.4)	11 (11.8)	7 (10.8)	
daily	6 (3.8)	3 (3.2)	3 (4.6)	
Type of epilepsy, n (%)				0.662
Generalized epilepsy	138 (87.3)	82 (88.2)	56 (86.2)	
Partial epilepsy	17 (10.8)	10 (10.8)	7 (10.8)	
Unclassified epilepsy	3 (1.9)	1 (1.1)	2 (3.1)	

AEDs, antiepileptic drugs; SD, standard deviation.

Prevalence of Anxiety and Depressive Symptoms

The SAS and SDS scores for all participants are listed in **Table 2** and **Figures 3A, B**. Of the 158 patients, the mean overall scores of the SAS and SDS were 45.28 (SD = 12.29) and 49.28 (SD = 13.20), respectively. No significant difference was found between genders in the percentage of subjects with high SAS and SDS scores.

Other Inventory Results

The overall and subscale scores of the QOLIE-31 and PSQI are shown in **Table 3** and **Figure 3**. For the overall sample, the mean overall QOLIE-31 score was 62.29 (SD = 16.30). According to the PSQI rating scale, only 28.5% of the total PWE participated declared good quality of sleep, but 51.3% had poor sleep, and 20.3% reported severely disturbed sleep, respectively (**Figure 3C**). The female PWE had higher Sleep Quality score than the male ($p = 0.023$), and no significant gender difference was found in terms of any other total or subscale scores of the PSQI and QOLIE-31 (**Figures 3D–K**).

Correlation Analysis

Gender Differences in the Correlation Between Clinical Variables and Anxiety (SAS Scores)

Table 4 outlines a univariate correlation analysis between various factors and SAS scores in men and women with epilepsy. The correlation analysis revealed that anxiety (SAS scores ≥50) in both men and women with epilepsy was positively correlated with the frequency of seizures (men: r = 0.207, $p < 0.05$; women: r = 0.467, $p < 0.05$), the overall QOLIE-31 scores (men: r = −0.522, $p < 0.01$; women: r = -0.606, $p < 0.01$) and the PSQI scores (men: r = 0.467, $p < 0.01$; women: r = 0.445, $p < 0.01$). In addition, anxiety in women with epilepsy was positively correlated with occupation (r = −0.394, $p < 0.01$), years of education (r = −0.422, $p < 0.01$) and settlement (r = −0.330, $p < 0.01$). There were also significant differences in the correlations between anxiety and each subscale domain of the QOLIE-31 and PSQI, which are listed in **Table 4**.

Gender Differences in the Correlation Between Clinical Variables and Depression (SDS Score)

The correlational analysis showed that depression in men with epilepsy was positively correlated with education years (r = −0.279, $p < 0.01$), frequency of seizures (r = 0.321, $p < 0.01$), QOLIE-31 scores (r = −0.615, $p < 0.01$) and PSQI scores (r = −0.501, $p < 0.01$). In contrast, depression in women with epilepsy was positively correlated with occupation (r = −0.394, $p < 0.01$), years of education (r = −0.422, $p < 0.01$), settlement (r = −0.330, $p < 0.01$), QOLIE-31 scores (r = −0.615, $p < 0.01$) and PSQI scores (r = −0.501, $p < 0.01$).

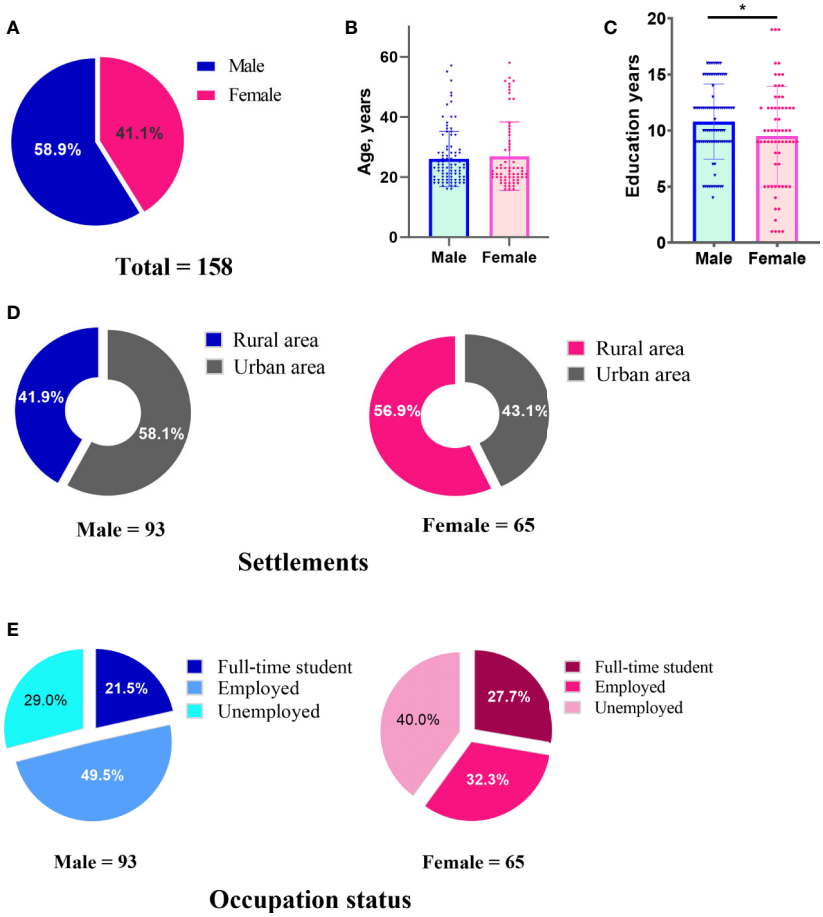

FIGURE 1 | Demographic characteristics of male and female patients with epilepsy included in this study. The number of cases **(A)**, ages **(B)**, education years **(C)**, settlements **(D)** and occupation status **(E)** of male and female patients were indicated, respectively.

Logistic Regression Analysis

Gender Differences in Predictors of Anxiety

Logistic regression models for the male group explained the independent predictors of the SAS score. These predictors included energy/fatigue (OR = 0.432) as the subscale of the QOLIE-31 and the PSQI score (OR = 1.339) (**Table 5**). In the female group, energy/fatigue (OR = 0.151) and frequency of seizures (OR = 3.001) significantly predicted the SAS score (**Table 5**).

Gender Differences in Predictors of Depression

Logistic regression analysis indicated that education years (OR = 0.769), energy/fatigue (OR = 0.482) and social function (OR = 0.698), as the subscales of the QOLIE-31, independently predicted depression for the male group (**Table 6**). In the female group, only energy/fatigue (OR = 0.265) was a significant indicator of depression (**Table 6**).

DISCUSSION

Gender discrepancy usually exists in psychiatric disorders. However, that difference(s) between genders may not always be the same in all kinds of scenarios. The current study investigated gender differences in the demographic variables, incidence and risk factors of comorbid anxiety and depression in PWE and have three key findings in the present study: (1) Expected gender differences in the prevalence of anxiety and depression are not actually seen in PWE. (2) Most of the important predictors for anxiety and depression in PWE vary by gender. (3) QOL energy/fatigue is the common influential factor of anxiety and depression in PWE and influences male and female PWE to different extents.

Large-scale epidemiological studies suggest that anxiety disorders are much more prevalent in women than in men, and this gender disparity is even more pronounced in other neuropsychiatric disorders, such as sleep disturbance (20). However, this study revealed no gender difference in the high prevalence rates of comorbid anxiety in PWE, which was consistent with previous studies (21, 22). Women in general are also documented to be more prone to depression than men (9, 10, 23), and recent evidence (22) also showed the female PWE had a higher ratio of comorbid depressive symptoms than the male. Paradoxically, the present study showed that men with epilepsy were just as likely as female patients to exhibit markedly

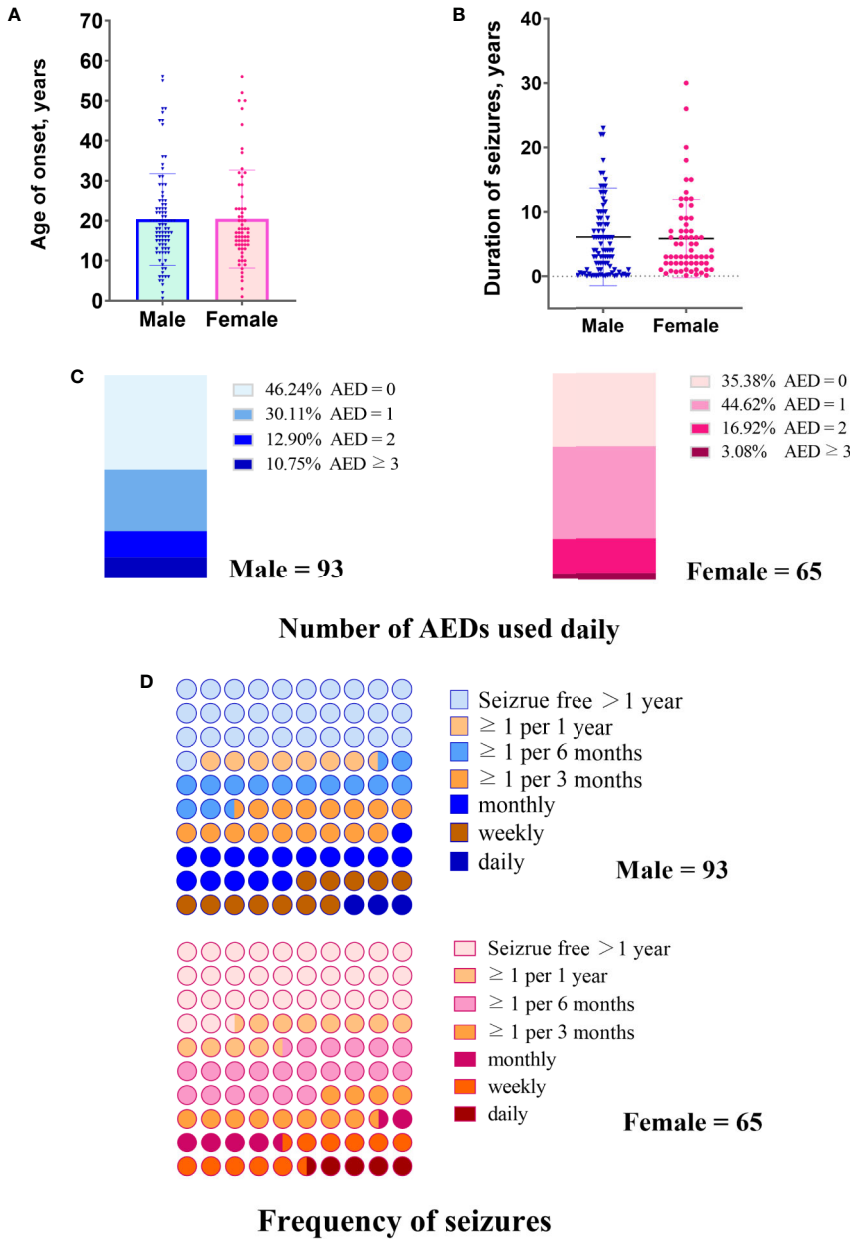

FIGURE 2 | Clinical characteristics of male and female patients included in this study. **(A)** Age of onset years. **(B)** Duration of seizures. **(C)** Number of antiepileptic drugs (AEDs) used daily. **(D)** Frequency of seizures.

higher levels of depression in comparison to the general population. Such discrepancies may be attributed to the different features of the selected samples among these studies. Significantly, the patients in this study were mainly from Gansu, a province of northwest China where economy and healthcare conditions are relatively under-developed. The unemployed and poorly educated situations may greatly impact the emotions of those participants. Nevertheless, our findings indicate that psychiatric comorbidities in male PWE must be concerned as important as in female patients, and the psychological state of PWE should not be equated with that of the general population.

Although the expected gender difference in the prevalence of anxiety and depression in PWE was not observed in this study, the important predictors of comorbid anxiety were found in this study quite complex and differed between male and female PWE. In men with epilepsy, poor sleep is the most dangerous factor in comorbid anxiety disorders. Sleep problems occur more frequently in PWE than in healthy controls (24). Sleep disturbance is a well-documented risk factor for developing or worsening anxiety disorders (25, 26), and women in the general population are more likely than men to report insomnia (25–27). Surprisingly, this study showed that men and women with

TABLE 2 | Mood self-evaluation inventory scores.

Items	Total sample(N = 158)	Men(N = 93)	Women(N = 65)	*p*
Anxiety and depression				
SAS (mean ± SD)	45.28 ± 12.29	45.56 ± 12.59	44.88 ± 11.92	0.732
Normal, n (%)	108 (68.4)	61 (65.6)	47 (72.3)	0.671
Mild	28 (17.7)	19 (20.4)	9 (13.8)	
Moderate	13 (8.2)	7 (7.5)	6 (9.2)	
Severe	9 (5.7)	6 (6.5)	3 (4.6)	
SDS (mean ± SD)	49.28 ± 13.20	48.75 ± 13.62	50.03 ± 12.65	0.551
Normal, n (%)	100 (63.3)	57 (51.6)	43 (66.2)	0.773
Mild	31 (19.6)	20 (21.5)	11 (16.9)	
Moderate	17 (10.8)	11 (20.4)	6 (9.2)	
Severe	10 (6.3)	5 (6.5)	5 (7.7)	

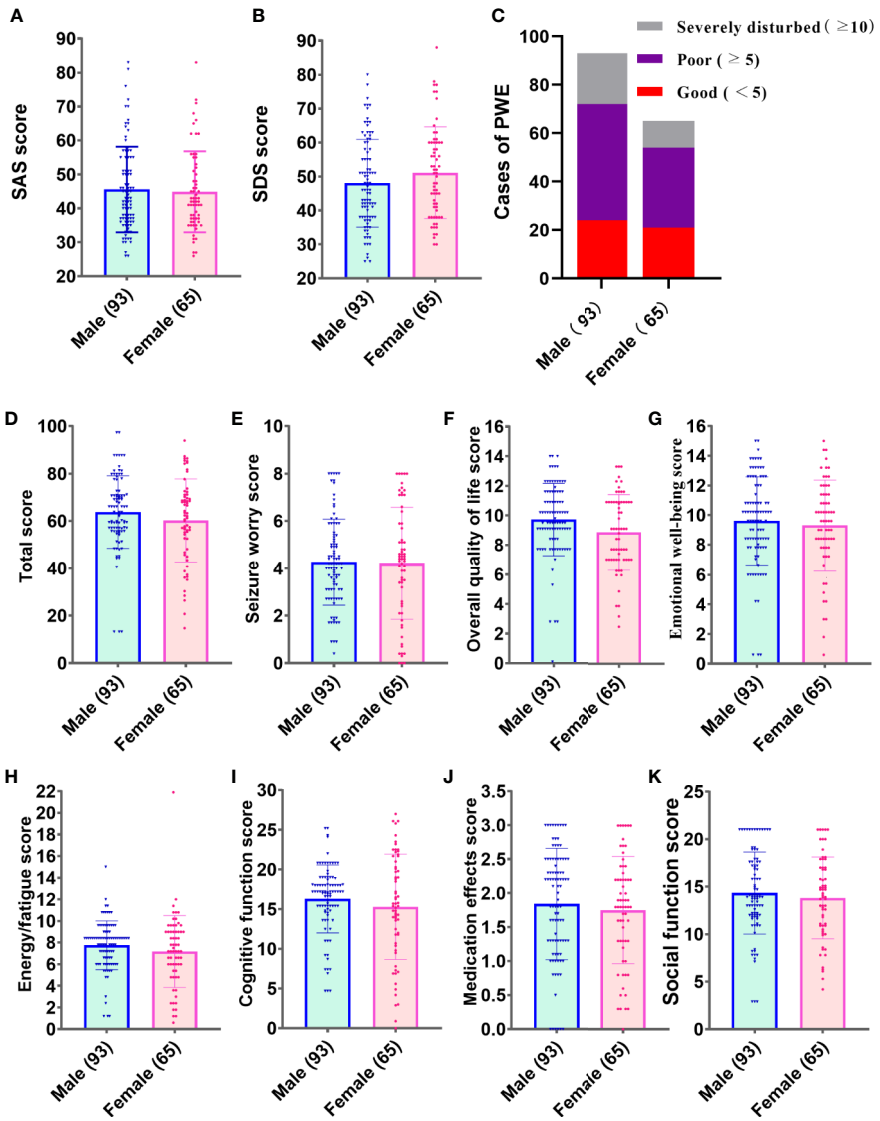

FIGURE 3 | Inventory scores of male and female patients included in this study. **(A)** Self-rating Anxiety Scale (SAS) score. **(B)** SAS score. **(C)** Cases of people with epilepsy (PWE) in different grades of sleep quality. **(D–K)** Total and subscale scores of the Quality of Life in Epilepsy-31 (QOLIE-31) of seizures.

TABLE 3 | Other inventory scores.

Items	Total sample (N = 158)	Men (N = 93)	Women (N = 65)	*p*
QOLIE-31				
Total score (mean ± SD)	62.29 ± 16.30	61.48 ± 17.03	63.45 ± 15.25	0.467
Seizure worry	4.24 ± 2.05	4.42 ± 2.13	4.28 ± 1.95	0.848
Overall quality of life	9.37 ± 2.52	9.11 ± 2.75	9.74 ± 2.11	0.124
Emotional well-being	9.48 ± 3.00	9.52 ± 3.12	9.42 ± 2.86	0.841
Energy/fatigue	7.51 ± 2.76	7.36 ± 2.65	7.72 ± 2.91	0.421
Cognitive function	15.88 ± 5.38	15.84 ± 5.69	15.95 ± 4.93	0.896
Medication effects	1.81 ± 0.81	1.84 ± 0.80	1.75 ± 0.81	0.493
Social function	14.12 ± 4.30	13.76 ± 4.52	14.62 ± 3.96	0.217
PSQI, n (%)				
Good (<5)	45(28.5)	24 (25.8)	21 (32.3)	0.555
Poor (≥5)	81(51.3)	48 (51.6)	33 (50.8)	
Severely disturbed (≥10)	32(20.3)	21 (22.6)	11 (16.9)	
Total score (mean ± SD)	6.75 ± 3.97	7.12 ± 4.10	6.23 ± 3.75	0.168
Sleep quality	1.08 ± 0.78	1.19 ± 0.81	0.91 ± 0.70	0.023
Sleep latency	1.32 ± 1.01	1.37 ± 1.00	1.25 ± 1.02	0.481
Sleep duration	0.66 ± 0.83	0.70 ± 0.87	0.62 ± 0.76	0.533
Sleep efficiency	0.76 ± 0.96	0.81 ± 0.98	0.70 ± 0.94	0.510
Sleep disturbances	1.27 ± 0.85	1.32 ± 0.92	1.20 ± 0.74	0.421
Sleep medication	0.11 ± 0.54	0.10 ± 0.53	0.12 ± 0.55	0.763
Daytime dysfunction	1.58 ± 1.17	1.66 ± 1.26	1.48 ± 1.03	0.347

TABLE 4 | Correlation analysis of factors influencing mood of patients with epilepsy.

Variables	Anxiety (SAS)		Depression (SDS)	
	Men (N=93)	Women (N=65)	Men (N=93)	Women (N=65)
Demographic characteristics				
Age	–0.007	0.156	–0.073	0.152
Occupation	0.102	–0.394**	0.125	–0.400**
Education years	–0.153	–0.422**	–0.292**	–0.416**
Settlement	–0.027	–0.330**	0.004	–0.360**
Clinical characteristics				
Onset age	–0.088	0.010	–0.081	–0.033
Duration	0.175	0.081	0.026	0.111
Numberof AEDs	0.151	–0.075	0.090	0.064
Frequency of seizures	0.207*	0.467**	0.230*	0.377**
Type of epilepsy	0.072	–0.248*	0.096	–0.205
QOLIE-31				
Total score	–0.522**	–0.606**	–0.564**	–0.557**
Seizure worry	–0.248*	–0.579**	–0.303**	–0.544**
Overall quality of life	–0.371**	–0.251*	–0.429**	–0.262*
Emotional well-being	–0.521**	–0.576**	–0.511**	–0.642**
Energy/fatigue	–0.654**	–0.697**	–0.675**	–0.676**
Cognitive function	–0.432**	–0.392**	–0.423**	–0.363**
Medication effects	–0.225*	–0.338**	–0.181	–0.260*
Social function	–0.306**	–0.416**	–0.441**	–0.348**
PSQI				
Total score	0.467**	0.445**	0.388**	0.330**
Sleep quality	0.318**	0.277*	0.263*	0.287*
Sleep latency	0.362**	0.370**	0.280**	0.324**
Sleep duration	0.145	0.109	0.113	0.015
Sleep efficiency	0.117	0.182	0.079	0.162
Sleep disturbances	0.468**	0.342**	0.348**	0.291*
Sleep medication	0.252*	0.275*	0.230*	0.227
Daytime dysfunction	0.402**	0.483**	0.344**	0.306*

*$p < 0.05$.
**$p < 0.01$.

epilepsy suffered equally from sleep problems. In contrast, the frequency of seizures was the most important indicator that independently related to anxiety in women with epilepsy in this study. Anxiety and depression are always correlated with seizure frequency, both before and after treatment, and depressive symptoms and seizure frequency influence mutually, indicated by both cross-sectional and longitudinal studies (28, 29). Our data are consistent with this finding in terms of anxiety but not depression. The analysis showed that anxiety symptoms associated seizure frequency stronger in women than in men in this study. This phenomenon may result from women's higher level of psychological burden when facing a higher frequency of seizures, compared with male patients. Therefore, seizure-related factors impact the anxious status of women much more in comparison with men. For female PWE, good control of epilepsy and maintaining a positive and optimistic attitude toward seizures may help to reduce anxiety.

Predictors of depressive symptoms in PWE also varied by gender. It was notable in this study that depression was independently associated with education years and QOL social function in men with epilepsy but not in women. As reported, epilepsy often leads to poorer educational and vocational outcomes including unemployment and limited career prospects, especially for male patients (30). Data in the present study indicated that longer education years and better social function meant a lower risk of depression; on the contrary, poorly educated male PWE may have more psychological barriers and less-flexible coping strategies. In general, social support means differently to men and women. Women commonly relied on searching for social and religious support, while most men showed alexithymia and more difficulties on coping negative emotions (31), which may explain the gender differences in the predictors of depression in PWE in our study.

Our findings also suggest the importance of the relationship between fatigue and mental comorbidity in people with epilepsy. QOL medication effects, as the most important indicators of depression in both genders (32), were significant in our regression model of men with epilepsy. In women with epilepsy, rather than sociodemographic factors, QOL energy/fatigue almost exclusively account for the variance in depression. The degree of fatigue was found higher in adult PWE than in the general population (4, 33). In this study, the bidirectional relationship between negative emotions and epilepsy was well established: QOL energy/fatigue was confirmed as the common predictor of comorbid anxiety and depression in both genders with epilepsy. Moreover, the male patients were influenced by QOL energy/fatigue to a greater extent than the female patients. Although the mechanism of fatigue remains unclear, previous studies have found that fatigue was prominent in people with major depressive disorder even when depression was atypical (34). Thus, the association between depression (or anxiety) and fatigue should be fully understood, and fatigue should

TABLE 5 | Logistics analysis for anxiety in men or women with epilepsy.

	B	S.E.	Wald	OR	*p*
Men					
Duration	0.101	0.058	3.095	1.106	0.079
Energy/fatigue (QOLIE-31)	–0.839	0.217	14.991	0.432	<0.001
PSQI	0.292	0.107	7.491	1.339	0.006
Women					
Frequency of seizures	1.099	0.517	4.517	3.001	0.034
Seizure worry (QOLIE-31)	–0.873	0.586	2.217	0.418	0.137
Energy/fatigue (QOLIE-31)	–1.891	0.728	6.751	0.151	0.009

TABLE 6 | Logistics analysis for depression in men or women with epilepsy.

	B	S.E.	Wald	OR	*p*
Men					
Education years	–0.263	0.119	4.873	0.769	0.027
Energy/fatigue (QOLIE-31)	–0.729	0.194	14.163	0.482	0.000
Medication effects (QOLIE-31)	0.907	0.498	3.314	2.477	0.069
Social function (QOLIE-31)	–0.360	0.124	8.426	0.698	0.004
PSQI	0.163	0.097	2.863	1.177	0.091
Women					
Seizure worry (QOLIE-31)	–0.621	0.348	3.186	0.537	0.074
Energy/fatigue (QOLIE-31)	–1.328	0.398	11.108	0.265	0.001
Medication effects (QOLIE-31)	1.762	0.757	5.417	5.826	0.202
MoCA	–0.201	0.104	3.736	0.818	0.053

particularly be ameliorated in epilepsy patients. Seizure frequency and sleep-related problems have been found crucial risk factors for fatigue in epilepsy patients (33, 35), which may help to explain why PWE with anxiety disorders share fatigue as a common risk factor without gender differences.

In clinical practice, the main reason for psychiatric disorders in PWE usually is orchestration of large number of factors. However, our findings uncovered some interesting correlations between those factors, most of which serving as predictors for depression seem regulatable. Our study suggests that male and female PWE should be treated differently, particularly regard to negative emotional comorbidities. Further social support for male PWE and effective health education on epilepsy for female PWE may increase their confidence in treatment and relieve their anxiety about seizures.

Limitations

The following limitations should be considered when our data are being interpreted. (1) The cases were acquired from a single epilepsy center, and the sample size was relatively small. Thus the conclusion from these data cannot be applied to other patient groups directly. (2) A definitive diagnosis of depression or anxiety disorders should meet the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria by a comprehensive clinical examination. The SAS and SDS are brief screening instruments to assess the severity of anxiety or depressive symptoms and more appropriate for epidemiological research. (3) The data in our study were collected by participants' self-reports with no confirmation by professional psychiatric staff. Therefore, the patients may have over-evaluated or underreported their actual situation of epilepsy. (4) The main type of epilepsy in most PWE of our sample is generalized epilepsy, while focal epilepsy has a higher prevalence in the adults.

CONCLUSION

The data of this study suggest that there may be no gender difference in the prevalence of epilepsy comorbidity of anxiety and depression, but gender discrepancy plays a significant role in the incidence of psychiatric comorbidities in epilepsy. Most of the important predictors for anxiety and depression in PWE vary between genders: male patients are more likely to be affected by psychosocial factors, while female patients are more influenced by seizure-related events. In addition, common indicators (e.g., energy/fatigue) may influence the psychiatric comorbidities of epilepsy differently in male and female PWE. To improve the psychiatric condition and QOL of PWE, psychological interventions and related medical care should be tailored according to gender-specific predictors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the 940th Hospital of Joint Logistics Support force of committee: Chinese People's Liberation Army (Department of Neurology, The 940th Hospital of Joint Logistics Support Force of People's Liberation Army, Lanzhou, Gansu, China.). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SW and FK conceived of and designed the study. ZL, RY, ZF, HF, HW, and BS were involved in data acquisition. ZL and FK analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Probable Autoimmune Depression in a Patient With Multiple Sclerosis and Antineuronal Antibodies

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Background: In a subgroup of patients with mood disorders, clear-cut organic disorders are responsible for depressive symptoms (e.g., autoimmune diseases such as multiple sclerosis or systemic lupus erythematosus). In these cases, an organic affective disorder can be diagnosed.

Case Presentation: The authors present the case of a 59-year-old male patient who developed a severe depressive episode over approximately 6 months and was, therefore, admitted to the hospital. In retrospect, he reported that, at age 39, he suffered from self-limiting sensory disturbances and muscle weakness in both legs. The current magnetic resonance imaging of his brain showed several conspicuous FLAIR-hyperintense supratentorial white matter lesions compatible with chronic inflammatory brain disease. Imaging of the spinal axis revealed no clear spinal lesions. Cerebrospinal fluid (CSF) analyses showed CSF-specific oligoclonal bands. Therefore, multiple sclerosis was diagnosed. Further CSF analyses, using tissue-based assays with indirect immunofluorescence on unfixed murine brain tissue, revealed a (peri-)nuclear signal and a strong neuritic signal of many neurons, especially on granule cells in the cerebellum, hippocampus, and olfactory bulb, as well as in the corpus callosum. Additionally, antinuclear antibody (ANA) titers of 1:12,800 and a lymphopenia were detected in blood tests. Further system clarification showed no suspicion of rheumatic or oncological disease. Anti-inflammatory treatment led to rapid and sustained improvement.

Conclusion: The present patient suffered from a probable "autoimmune depression" in the context of newly diagnosed multiple sclerosis with typical MRI and CSF pathologies, alongside mild concomitant latent systemic autoimmune process (with high-titer ANAs and lymphopenia) and unknown antineuronal antibodies. The case report illustrates that a

depressive syndrome suggestive of primary idiopathic depressive disorder may be associated with an autoimmune brain involvement. The detection of such organic affective disorders is of high clinical relevance for affected patients, as it enables alternative and more causal treatment approaches.

Keywords: depression, multiple sclerosis, connective tissue disease, autoimmune encephalitis, autoantibody

BACKGROUND

Mood disorders are one of the most common mental illnesses, and they are the most limiting factors regarding quality of life. In a small subgroup of patients with depressive episodes, organic disorders are responsible for depressive symptoms (e.g., multiple sclerosis, systemic lupus erythematosus, stroke, and hypothyroidism). In these cases, an organic affective disorder can be diagnosed (1). In particular, a number of autoimmune diseases with central nervous system (CNS) involvement can lead to affective symptoms (2–4). Depressive symptoms occur in about 50% of patients with multiple sclerosis (5). However, they can also occur in the context of different connective tissue diseases, especially in systematic lupus erythematosus (SLE) (6, 7). Predominant brain involvement of SLE refers to neuropsychiatric SLE (8, 9). However, affective symptoms can also occur in the context of Hashimoto encephalopathy (4, 10) or other autoimmune encephalitides, such as anti-NMDA receptor encephalitis (11). Most of these autoimmune syndromes are associated with neuropsychiatric symptoms (e.g., focal neurological deficits in multiple sclerosis or epileptic seizures in autoimmune encephalitis) or other organ involvement (e.g., joint involvement in SLE) (2, 4, 12). The extent to which isolated depressive syndromes are caused by clear autoimmune pathophysiology is still largely unknown. Various blood tests, including the measurement of antineuronal autoantibodies, electroencephalography (EEG), magnetic resonance imaging (MRI), [^{18}F]-fluorodeoxyglucose positron emission tomography (FDG-PET), and cerebrospinal fluid (CSF) diagnostics may contribute to the detection of an autoimmune disorder of the CNS (13). The rationale of this article is to present a patient with probable "autoimmune depression".

CASE PRESENTATION

Here, the authors present the case of a 59-year-old male patient who, over approximately 6 months, developed a severe depressive episode with depressed mood, loss of interest, reduced energy, reduced concentration and attention, pessimistic views of the future, disturbed sleep, and distressing inner restlessness. The psychopharmacological treatment with sertraline, trimipramine, trazodone, and cognitive behavioral therapy did not lead to an improvement, which is why the patient was admitted to our psychiatric day-care hospital. Focal neurological symptoms or other general medical symptoms or signs (e.g., skin changes) were not present. The patient had already experienced one mild depressive episode when he was 55 years old. The possibility of multiple sclerosis had already been discussed at the age of 39. At that time, he had suffered from sensory disturbances and muscle weakness of both legs (emphasized on the right side). Already at that time, CSF-specific oligoclonal bands (OCBs) and MRI white matter (WM) lesions had been noticed. However, with clinical symptoms fading away (without treatment) those MRI images had got lost over the years. When the patient was 44, autoimmune hepatopathy had been discussed due to slightly elevated transaminases, evidence of fatty liver in abdominal ultrasound, and elevated antinuclear antibodies (ANAs; titer: 1:3,200; reference, <1:50) without specification for extractable nuclear antigens (ENAs).

Diagnostic Findings

An MRI of the neurocranium showed several conspicuous FLAIR-hyperintense supratentorial WM lesions (among others, ovoid periventricular WM lesions on both sides, in the corpus callosum, and in the right side of the crus cerebri) without contrast enhancement which was assessed to be compatible with chronic inflammatory brain disease (**Figure 1**). Imaging of the spinal axis revealed no clear spinal lesions. Serum ANA titers of 1:12,800 (reference: <1:50) were found using indirect immunofluorescence technique on HEp-2000® cells without specification for ENAs or double-stranded DNA (ds-DNA). The CSF diagnostics revealed CSF-specific OCBs and local immunoglobulin M (IgM) synthesis. The MRZ reaction was negative. A large panel of established antineuronal antibodies against cell surface (NMDA-R, LGI1, CASPR2, AMPA1/2-R, GABA-B-R, and DPPX), and intracellular (Yo, Hu, CV2/CRMP5, Ri, Ma1/2, Tr, Zic4, SOX1, GAD65, and amphiphysin) antigens were negative. The tissue-based assay using CSF with indirect immunofluorescence on unfixed murine brain tissue revealed a (peri-)nuclear signal, likely reflecting the ANAs. However, CSF-testing also displayed an arborizing neuronal signal of many neurons, especially on granule cells in the cerebellum, hippocampus and olfactorius bulb (most likely axonal, not present in control CSF). The neuronal signal was also detectable in the corpus callosum (**Figure 2**). This signal was less detectable in the serum. The EEG and FDG PET of the brain and the whole body showed no relevant abnormalities. The peripheral electrophysiological examinations were essentially normal. Comprehensive blood analyses revealed persistent lymphopenia (minimum 0.59 10^9/L; reference 1.1–3.2 10^9/L). The lymphocyte panel showed a deficiency of CD8+ T cells, and the CD4/CD8 quotient was elevated (6.92), however the sarcoidosis markers were unremarkable. Further medical investigations including gastroscopy, coloscopy, ultrasound of the abdomen, and an x-ray of the thorax did not produce any evidence for rheumatic or oncological disease. Neuropsychologically, a clear psychomotor slowing and deficits in

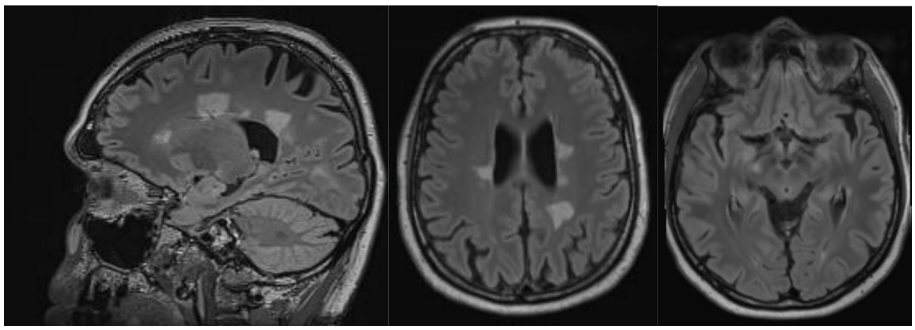

FIGURE 1 | Magnetic resonance imaging showed conspicuous supratentorial white matter lesions without contrast enhancement. This is compatible with chronic inflammatory brain disease.

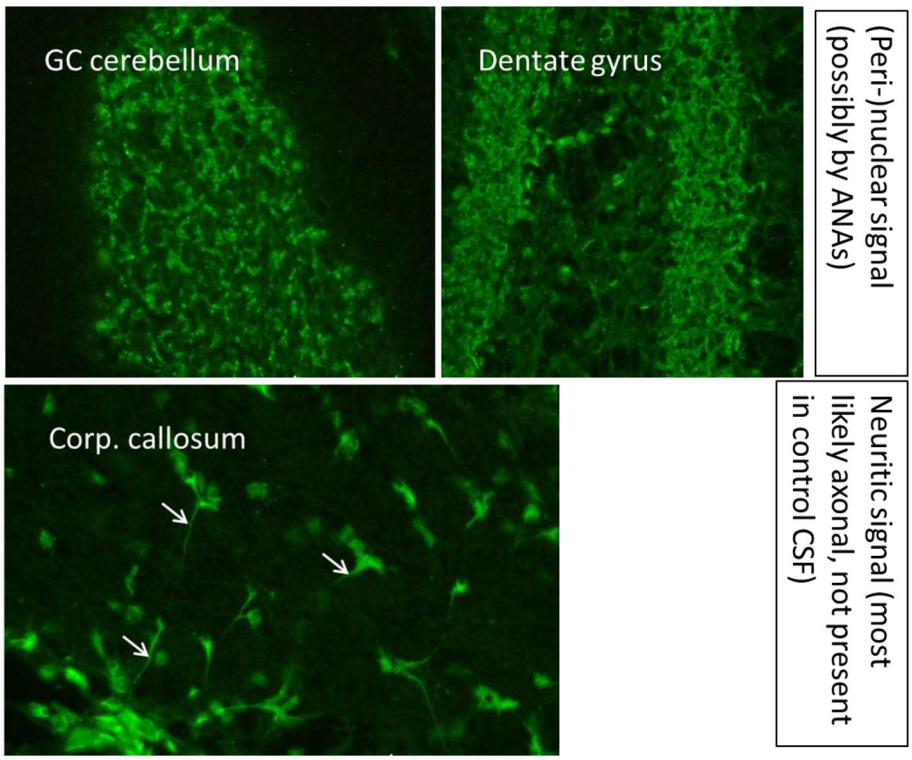

FIGURE 2 | A tissue-based assay using indirect immunofluorescence screening on brain sections of rodents showed binding of especially cerebrospinal fluid antibodies to granule cells in the granule cell layer of the cerebellum and the hippocampus and in the corpus callosum. Methodological aspects of the antibody test are described in Kreye et al. (14) and Edwin Thanarajah et al. (15). ANA, antinuclear antibodies; CSF, cerebrospinal fluid; GC, granule cell; Corp., corpus.

verbal memory were observed. All diagnostic findings are summarized in **Table 1.**

Developmental, Somatic, and Family Histories

The patient's history was negative for in-utero/birth complications, febrile convulsions/epileptic seizures, severe infectious diseases, or craniocerebral traumata. There were no indications of developmental disorders. An obsessive-compulsive personality structure was perceivable without ever fulfilling the criteria of a personality disorder. The family history (including his parents, grandparents, and siblings) was clear of any diagnosed psychiatric, neurological, cancer, or autoimmune disorders except for his mother suffering from beginning late-onset dementia.

Treatment and Outcome

Following diagnostic clarification and in the absence of a response to antidepressants, immunosuppressive treatment with a steroid pulse (5 × 1000 mg methylprednisolone over 5 days) was performed. Directly after steroid pulse treatment, the inner restlessness receded, and the mood improved. Within the next two to three weeks, the depressive syndrome fully remitted. Neuropsychological follow-up testing showed an improvement in working memory and mental flexibility, and the ANA titers decreased to 3200. Maintenance therapy with azathioprine led to pancreatitis, therefore maintenance therapy with methotrexate (15 mg per week) was established. The individual decision for methotrexate treatment was made under the initial assumption of a possible "overlap syndrome" with multiple sclerosis and a systemic autoimmune disease. At the time of publication, the patient was recommended to start a long-term, specific treatment of multiple sclerosis. Vitamin B12/D and folic acid were substituted. After improvement of the depression, the antidepressant treatment was tapered off. Three months later, the MRI was unchanged. Eight months later, one small, newly detectable lesion in the right cerebellar peduncle was observed (in the previous MRI examination, however, the area was artifact overlaid). Over 8 months, all depressive symptoms had vanished, except a slight sensory overload in vibrant situations.

DISCUSSION

The present case study describes a patient most likely presenting with "autoimmune depression" in the context of multiple sclerosis with typical MRI and CSF pathologies, with mild concomitant latent systemic autoimmune response, unknown antineuronal antibodies in the CSF, and a good response to immunosuppressive treatment.

Diagnostic Considerations

MRI images with several supratentorial WM lesions and inflammatory CSF syndrome with OCBs and transient local IgM synthesis are compatible with multiple sclerosis. When evaluating the clinical episode of ~20 years ago, the patient had experienced one clinical attack compatible with an episode of multiple sclerosis. The detection of OCBs replaces dissemination in time, according to the 2017 revised McDonald criteria (16); therefore, multiple sclerosis was diagnosed. Thus, immunosuppressive treatment of the underlying multiple sclerosis could also indirectly improve depression. In order to diagnose multiple sclerosis, more likely differential diagnoses may need to be excluded. The high ANA titers in combination with lymphopenia and brain involvement could indicate connective tissue disease. However, ENA differentiation and anti-ds-DNA antibodies remained unremarkable. Therefore, the criteria of the American College of Rheumatology (ACR) and Systemic Lupus Collaborating Clinics (SLICC) for SLE were not fulfilled [only three (brain involvement, lymphopenia, ANAs) out of four required ACR criteria, and three (brain involvement, lymphopenia, ANAs) of four required SLICC criteria were fulfilled) (17, 18). In addition, an axo-dendritic anti-neuronal autoantibody signal (which is not to be expected in healthy controls and cannot be typically observed in patients with multiple sclerosis, according to experiences in the Department of Neurology and Experimental Neurology, Charité Berlin from HP) was detected in indirect immunofluorescence on unfixed murine brain tissue on many neurons, especially not only on granule cells in the cerebellum, hippocampus, and olfactory bulb, but also in the corpus callosum. It raises the possibility of additive effects of antineuronal antibodies (which can be found in patients with autoimmune encephalitis) causing depressive symptoms. However, the criteria of possible autoimmune encephalitis were not fulfilled due to the lack of subacute onset (19).

Clinical Significance

Irrespective of the discussed differential diagnostic considerations, the presented case shows that an autoimmune pathophysiology could hide behind a classical manifestation of a depressive syndrome. It is well known that fatigue symptoms can occur in patients with MS. However, the symptoms in the presented patient clearly go beyond a fatigue symptomatology. Thus, the case demonstrates the importance of extended diagnostics also for patients with classic depressive syndromes. Clinical signs pointing to autoimmune depression in our case were the poor response to a classical treatment and the MRI and CSF abnormalities. The testing of CSF on unfixed mouse brain slices could finally shed light on associated autoantibodies. The subsequent immunosuppressive therapy has to be regarded as a more causal treatment of the psychiatric disorder and rapid clinical improvement validated the whole concept. The individual maintenance treatment with methotrexate might also have helped to prevent the progression of the disease. The frequency of such cases in clinical samples with depressive disorder is largely unclear. However, CSF studies reveal that 6.5% of depressive patients receiving CSF analysis display OCBs (20). This observation suggests that similar cases might occur more frequently than previously thought.

Limitations

The functional relevance of the antineuronal autoantibodies and the exact epitope target remain unclear, reflecting the still early development of this approach in psychiatry. In future similar cases, a scientific analysis of the detected autoantibodies would be useful if the patients agree to such studies. This could increase our understanding of the additional effect of the autoantibodies. Even a purely comorbid presence of a depressive disease in an independently existing multiple sclerosis seems possible; following these considerations, an antidepressant effect of corticosteroids without any anti-inflammatory effects would be conceivable. However, in the authors' view—in consideration of the findings of inflammatory CSF and MRI changes, the antineuronal antibodies, poor response to classic antidepressant medication, and rapid and sustained improvement under anti-inflammatory

TABLE 1 | Diagnostic findings performed during the patient's day clinic stay, 6 months after the onset of symptoms.

Blood analyses	•Normal electrolytes, C reactive protein not increased, normal lipase, **slightly increased GPT** (63 U/l; referenc, 10–50 U/L). Blood cellcount showed **lymphopenia** throughout (minimum 0.59 Tsd/µl; reference, 1.1–3.2 10^9/L). •**Vitamin B12** (168 pg/ml; reference, 197–771 pg/ml), **folid acid** (2.6 ng/ml; reference, 5.6–45.8 ng/ml), and **selenium** (68 µg/l; reference, 75–140 µg/l) were decreased. **Vitamin D** was not optimal (21.9 ng/ml; optimal: >30 ng/ml). •Thyroid-stimulating hormone, triiodothyronine, and thyroxine levels were in normal ranges. Autoantibodies against thyroglobulin, TSH receptor, and thyroid peroxidase were not increased. •Serologies for Lyme borreliosis, syphilis, and HIV were negative. •No antibodies against the intracellular onconeural antigens Yo, Hu, CV2/CRMP5, Ri, Ma1/2, SOX1, or the intracellular synaptic antigens GAD65/amphiphysin were found (using Ravo line assay®). •Antibodies against different neuronal cell surface antigens (NMDA-R, AMPA-R, GABA-B-R, DPPX, VGKC-complex [LGI1, Caspr2]) were negative (using Euroimmun biochip-assay®). •**Tissue-based assay with indirect immunofluorescence (IF) on unfixed murine brain tissue showed a slight (peri-) nuclear signal, but also a neuronal arborized signal of many neurons, especially granule cells in the cerebellum and hippocampus as well as in the olfactorius bulb (most likely axonal). The neuronal signal was also detectable in the corpus callosum.** •Aquaporin 4 and MOG antibodies were negative (using Euroimmun assay®). •**Screening for antinuclear antibodies (ANA) in IIF showed increased titers (1:12,800; reference, <1:50)** without specification for ENAs (*anti-SnRNP/Sm*, *anti-Sm*, *anti-SS-A/Ro*, *anti-Ro-52*, *anti-SS-A/Ro*, *anti-Ro-52*, *anti-SS-B/La*, *anti-Scl-70*, *anti-PM-Scl*, *anti-Jo-1*, *anti-centromere*, *anti-PCNA*, *anti-nucleosome*, *anti-histone*, *anti-ribos. P-protein*, *anti-AMA-M2*, *anti-DFS70*, *anti-Mi-2 alpha/beta*, *anti-Ku*, *anti-PM-Scl100*, *anti-Pm-Scl75*, *anti-Jo-1*, *anti-SRP*, *anti-PL-7/12*, *anti-EJ*, *anti-OJ*, *anti-Ro-52*, *anti-Tif1g*, *anti-MDA5*,*anti-NXP2*, *anti-SAE1*) or ds-DNA. Anti-neutrophil cytoplasmic antibodies, antiphospholipid antibodies were not clearly positive (+) without increased anti-MPO and PR3 antibodies. Rheumatoid factor and anti-mitochondrial antibodies were negative. No changes in the complement system (C3, C4, CH50, C3d) were observed. •IgG levels were normal, **IgA was increased** (4.37 g/L; reference, 0.70–4 g/L) and **IgM was decreased** (0.2 g/L, reference 0.4–2.3 g/L); immunofixation showed no monoclonal antibody production. •**T-cell panel showed a deficiency of CD8+ T cells. The CD4/CD8 quotient was elevated (6.92).** •"Sarcoidosis parameters" (interleukin-2-receptor, neopterin, and ACE) were not increased.
Cerebrospinal fluid analyses (CSF)	•No evidence of a malignant process. •Normal white blood cell count (1/µL; reference, <5/µL). •Normal protein concentration (310 mg/L; reference, <450 mg/L), and normal age-corrected albumin quotient: 3.6; age-dependent reference, $<8 \times 10^{-3}$). •**CSF specific oligoclonal bands**; IgG index not increased (0.63; reference, ≤0.7). •**Local IgM synthesis** (no longer detectable in the control examination).•CSF lactate not increased (1.87 mmol/L; reference, 1.7–2.6 mmol/L). •Antibodies against neuronal cell surface antigens (*NMDAR*, *AMPA-R*, *GABA-B-R*, *DPPX*, *VGKC-complex [LGI1*, *Caspr2]*) were negative (Euroimmun Biochip assay®). •**"Tissue-based assay with indirect immunofluorescence on unfixed murine brain tissue showed a strong (peri-) nuclear signal, but also a neuritic signal of many neurons, especially granule cells in the cerebellum and hippocampus as well as in the olfactorius bulb (most likely axonal). The neuritic signal was also detectable in the corpus callosum.**
Cerebral magnetic resonance imaging	•**Several conspicuous FLAIR-hyperintense supratentorial white matter lesions (among others ovally configured on both sides periventricular as well as in the corpus callosum and in the crus cerebri on the right side) without contrast agent uptake, compatible with chronic inflammatory brain disease.**
Magnetic resonance imaging of the spinal axis	•No clear spinal lesions (with partial artifact superimposition of the thoracic spine).
Electroencephalography	•Normal alpha rhythm, no epileptic pattern or pathological slowing.
[^{18}F]fluorodeoxyglucose positron emission tomography	•Unsuspicious brain metabolism. •No metabolic changes or structural lesion suspicious of malignancy on whole-body PET/CT. **Low-grade increase in metabolism axillary and inguinal lymph node left pronounced, most likely unspecific.**
Peripheral electrophysiological investigations	•Inconspicuous tibialis and medianus SEPs on both sides with inconspicuous suralis neurography on the right. •In the MEPs no indication of impaired efference to the arms and legs.
Gastroscopy, coloscopy	•No evidence of malignancy in the upper gastrointestinal tract, **mild, chronic, inactive antral and corpus gastritis. Coloscopy showed a rectal polyp (tubular adenoma with low grade intraepithelial neoplasia).**
Sonography of the abdomen	•**Steatosis hepatis grade II, uncomplicated liver cyst in segment VI, separated gallbladder.**
X-ray thorax	•No indication of fibrotic or granulomatous changes. No tumor suspicious round heart.
Cardiological examinations	•Normal electrocardiography and transthoracic echocardiography, especially no indication of right heart strain.

treatment—it seems more likely that the response to corticosteroids is caused by an anti-inflammatory effect from the corticosteroids. Due to stable multiple sclerosis signs (for over 20 years) and the possible overlap with a systemic autoimmune disease, individual maintenance therapy with methotrexate was started first. At the time of publication, the beginning of a classical relapse prophylaxis for multiple sclerosis (e.g., with teriflunomide) to avoid the development of progressive multiple sclerosis was recommended to the patient. He also had vitamin B12 and folic acid deficiencies. A depressive disorder that was either caused or aggravated by this cannot be excluded. Notably, substitution initially did not lead to any improvement. Only anti-inflammatory treatment led to a

strong and sustained improvement of the depressive symptoms; therefore, we do not assume that the symptoms were caused by the vitamin deficiency alone.

CONCLUSIONS

This case report describes a patient presenting with probable autoimmune depression in the context of multiple sclerosis with additional antineuronal autoantibodies with a yet unspecified target epitope, which was detected by a tissue test producing an axo-dendritic signal in several brain regions. The importance of autoantibodies in such constellations requires further investigation. The detection of organic causes of psychiatric disorders is of high clinical relevance because it enables alternative and more causal treatment approaches, as demonstrated in the current case report.

DATA AVAILABILITY STATEMENT

All necessary information is mentioned in the article.

ETHICS STATEMENT

The patient has given his written informed consent for this case report, including the presented images, to be published.

AUTHOR CONTRIBUTIONS

DE, PS, SR, and LT treated the patient. DE performed the data research and wrote the paper. MM supported the data collection. SR, RD and HP performed the neurological interpretation. SR and RD performed the CSF basic analyses, HP performed the tissue testing. NV performed the rheumatological tests and immunological interpretation. KE performed and interpreted the MRIs. KR, BLF, KN, SM, and KD supported the clinical and laboratory interpretation. All authors were critically involved in the theoretical discussion and composition of the manuscript. All authors contributed to the article and approved the submitted version.

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DNA Methyltransferases in Depression: An Update

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Depression is one of the most common psychiatric disorders affecting public health. Studies over the past years suggest that the methylations of some specific genes such as *BDNF*, *SLC6A4*, and *NR3C1* play an important role in the development of depression. Recently, epigenetic evidences suggest that the expression levels of DNA methyltransferases differ in several brain areas including the prefrontal cortex, hippocampus, amygdala, and nucleus accumbens in depression patients and animal models, but the potential link between the expression levels of DNA methylatransferases and the methylations of specific genes needs further investigation to clarify the pathogenesis of depression.

Keywords: depression, DNA methylation, DNA methyltransferase, neurodevelopment, DNMT3L, DNMT3A, DNMT3B, DNMT1

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HIGHLIGHTS

1. We did a thorough analysis of progress of this field on the basis of literature search in Pubmed for the last 5 years.
2. We proposed a new model of gene-environment interaction through DNA methylation, connecting the missing links between stressors and depression signs, which could be used as potential targets for the depression therapy.
3. We suggested some important questions for the further exploration in this field, especially on *DNMT3L*, a little studied modulator of *de novo* DNA methylation during brain development, its expression relatively rich in hippocampus and amygdala, which are important brain areas for depression.

INTRODUCTION

Depression, also known as major depressive disorder (MDD), is a common chronic and recurrent mental disorder characterized by at least two weeks of presenting with low mood and aversion to daily activities, which affect normal social life (1). In 2017, the prevalence counts of MDD were about 163 million people, together with other depressive disorders, making them the third largest group of disease burden in the world (2). There are many hypotheses regarding the pathogenesis of depression, including those with neurotransmitters (e.g., biogenic amines), genetic, endocrine, and inflammatory mechanistic bases, but no single one can explain all aspects of the depression (3).

Recent studies support a gene-environment interaction model, where epigenetic modifications are the key connectors, whose changes may cause the gene expression alteration in different pathways involved in this disorder, as a way of adaptation (4). These epigenetic modifications include DNA methylation and demethylation, histone acetylation and deacetylation, non-coding RNA, and chromatin remodeling, among which DNA methylation is the most stable modification that could be passed to the next generation, and DNA methyltransferases (DNMTs) are critical enzymes whose activities underlie these processes. Growing evidences suggest that DNA methylation and DNMTs are involved in the development of depression (5). DNMTs expression changes have been found in different brain areas in depression patients (6, 7) and animal models (8, 9). Among these studies, frontal cortex and amygdala are the common affected areas in both human and animal, suggesting DNMTs may contribute to the cognitive and emotional domains of depression endophenotypes, which are also cortisol-related (10, 11). The other affected areas include hypothalamus in depression patients and hippocampus and nucleus accumbens in animal models. However, we still do not know, how are these DNMTs regulated in the neural circuits of depression, and what are the target genes of these DNMTs in this scenario.

In the current review, we summarized the recent progress on DNMTs studies in depression, discussed the controversies in this field, and provided potential directions for further exploring the mechanism of DNMTs in depression. We hope it can help us to understand the pathogenesis of depression, as well as provide a new perspective for the targeted therapy of depression.

DNA METHYLATION

DNA methylation, one of the most important epigenetic modifications, was first discovered in 1940s (12). DNA methylation mainly occurs at cytosine nucleotides in DNA sequence where a cytosine nucleotide is followed by a guanine nucleotide (CG sites), though non-CG sites methylation also exists (13, 14). The methylation in promoter region is usually associated with the repression of gene expression, whereas the methylation in the gene body region may be associated with increased gene expression (12). The process involves the addition of a methyl group from the cofactor SAM (S-adenosyl-methionine) to the 5th carbon of the cytosine residue to form 5mC and it is catalyzed by DNMTs. The 5mC can be actively demethylated by ten–eleven translocations (TETs) or activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC) enzymes, together with thymine DNA glycosylase (TDG) (12). This methylation modification can physically impede the binding of transcription factors to the gene itself, or by recruiting protein complexes, contribute to many physiological processes such as genomic imprinting, X-chromosome inactivation, and the regulation of the chromatin structure and gene expression (12). However, an increasing number of studies suggest the mechanisms of how DNA methylation regulates gene expression are more complicated than we have expected.

DNA METHYLTRANSFERASES

The catalysis and maintenance of DNA methylation depend on DNMTs. In mammals, DNMTs include DNMT1, DNMT2, and DNMT3. DNMT3 includes DNMT3A, DNMT3B, and DNMT3L (15). DNMT2 only catalyzes RNA methylation, which uses tRNA as its substrate, and has no DNA methylation activity (16). So the DNMTs that participate in DNA methylation consist of DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT1 maintains DNA methylation while DNMT3A and DNMT3B help *de novo* DNA methylation (12). DNMT3L can't catalyze DNA methylation directly, but it can modulate the DNA methylation through activating DNMT3A and DNMT3B (17, 18).

The function of DNMTs in the brain has been investigated in *Dnmt1/Dnmt3a* knockout mice, where *Dnmt1* knockout in embryonic brain led to demethylation of the *Gfap* promoter in neural precursor cells, promoting astrogliosis (19, 20), and *Dnmt1/Dnmt3a* double knockouts in forebrain postmitotic neurons caused the deficits in synaptic plasticity and learning and memory (21). The expressions of DNMTs in the developing brain are different among mice (21), rats (22), primates (23), and human (24), but for most DNMTs, they share a common pattern that the expression peaks in early development and declines in adult, high in proliferating cells and low in differentiated cells.

DNA METHYLATION IN DEPRESSION

Many of the studies on DNA methylation in depression used animal models. There are several approaches to induce the depression-like behaviors in rodents, such as early life stressors, corticosterone supplementation, learned helplessness, social defeat stress, and genetic modification (25). These approaches can usually generate behavioral phenotypes analogous to depression signs required for depression diagnosis, especially anhedonia (25, 26). Although it is difficult to mimic the depression signs in animals due to its heterogeneous nature, there are some consensual depression-like behavioral tests being used by researchers in this field to model the signs of depression, such as the coat state assessment (CSA) and sucrose consumption test (SCT) for anhedonia-like behavior, the restraint stress test (RST), the tail suspension test (TST) and the forced swim test (FST) for helplessness and despair-like behavior, the novel object recognition (NOR), spatial memory test (SMT) and the Morris water maze (MWM) for cognitive changes, the open field test (OFT) for locomotor and anxiety-like behavior, and the affective bias test (ABT) for reward learning and processing (25, 27–29). However, given the heterogeneity of this disease and the limitations of animal models, results from these studies should be interpreted with caution (26).

Recent studies demonstrated that the methylations of some specific genes such as *P11* (30–33), *BDNF* (34–38),

SLC6A4 (39–42), and *NR3C1* (43–45), were closely correlated to depression. For example, Svenningsson et al. found that the *P11* knockout mice showed anhedonia-like and despair-like behaviors assessed with above mentioned SCT, TST, and FST (33). When the *P11* gene was re-introduced into the nucleus accumbens of the *P11* knockout mice, the anhedonia-like and despair-like behaviors could be effectively eliminated (33). Seo et al. found that P11 knockdown in the lateral habenula alleviated the stress-induced anhedonia-like and despair-like behaviors in rats, while overexpressing P11 in dopamine D2 receptor-containing lateral habenula neurons of control mice induced anhedonia-like and despair-like behaviors assessed with SCT, RST, TST, and FST (32).

With the progress of methylation measurement, there are increasing numbers of clinical studies showing the correlation between the gene methylation changes and depression symptoms. For example, one study found the hypermethylation of *BDNF* promoter in the peripheral blood cells was correlated with the severity of the depression (34). Another clinical study found similar changes of *BDNF* hypermethylation in the buccal tissue of depression patients (36). Serotonin dysregulation is one of the most studied pathways in depression. In a monozygotic twin study, Zhao et al. found the DNA methylation levels of the *SLC6A4* promoter were positively correlated with the Beck Depressive Inventory scores for depressive symptoms (42). In another study, Lam et al. found *SLC6A4* DNA methylation was associated with depression status in the presence of specific genotype (40). Glucocorticoid receptor (GR) is another well-studied molecule in depression based on stress model. Melas et al. found that the hypermethylation changes of the promoter of GR gene *NR3C1* were correlated with childhood adversities in depression patients (43). Na et al. found decreased *NR3C1* methylation changes in non-psychotic depression patients (45). Additionally, several methylome-wide association studies have shown global DNA methylation changes in depression patients, with enriched genes involved in neurodevelopment (5).

In summary, DNA methylation might be the essential link between the environment and gene expression in depression, but its detailed mechanism needs thorough research. Though some results are contradictory to each other, most studies support the hypothesis that the hypermethylation changes in specific genes induce decreased expressions in depression patients or animal models and all these changes are involved in the pathways related to depression (5, 46). However, we do not know exactly what causes the methylation changes and how the genes are specifically targeted for the methylation changes. We can speculate that environmental changes (stressors) during brain development may activate the hypothalamic–pituitary–adrenal (HPA) axis, releasing glucocorticoids, which can activate the immediate early genes (IEGs) through GRs; the IEGs then stimulate the expressions of DNMTs. The DNMTs target specific genes assisted by transcription factors (TFs) and non-coding RNAs (ncRNAs), whose activation may represent a stress response or plasticity in cells, imitating a stem cell state when DNA methylation is active. These specific gene expression changes can affect the balance of neurotransmitters and therefore cause depression symptoms. This is supported by the observation that the DNA methylation was dynamically regulated, and environmental enrichment in adolescent rats that were exposed to adversity stressors rescued the DNA methylation profiles and recovered their typically observed behavior in non-treated animals (47), and DNA methylation inhibitor zebularine normalized the aberrant behavior in FST observed in rats exposed to maltreatment during infancy (48).

DNA METHYLTRANSFERASES IN DEPRESSION

DNMT1

DNMT1 was first discovered in 1988, which is involved in the maintenance of DNA methylation (49, 50). It was found that the mRNA level of DNMT1 was decreased in the peripheral blood of patients with depression in attacking stage, but there was no difference between depression patients in remission stage and healthy subjects (51). The results in postmortem patients with depression also showed that the expression levels of DNMT1 mRNA in the prefrontal lobe and amygdala were decreased, which was the same as that in the peripheral blood (6).

However, contrary to above findings, the experiments in animal models showed opposite results. Morris et al. found that mice with *Dnmt1* knockout in forebrain showed anxiolytic and antidepressant-like effects assessed with elevated plus maze (EPM), FST, and novelty suppressed feeding test (NSF) (52). Melas et al. discovered that treating depressive rats with escitalopram could down-regulate the expression of Dnmt1 in prefrontal cortex (31). And in hippocampus, genipin reduced despair-like traits (assessed *via* TST and FST) by inhibiting the expression of Dnmt1 and normalizing the expression of BDNF (53). Additionally, Wright et al. found that the expression of Dnmt1 was increased in the central nucleus of amygdala in the depression mouse model established by chronic social failure stress (assessed *via* a social interaction test (SIT)), but only in the female mice (54). Park et al. used male rats to establish the depression model through maternal deprivation. It was found that the expression levels of Dnmt1 were significantly increased in the hippocampus of depression-like rats, and decreased after antidepressant treatment with escitalopram (55).

In the depression animal model established by maternal deprivation and social isolation, Dnmt1 expression and *Nr3c1* promoter methylation level of female rats were higher than those in male rats. It is believed that early life stress destroyed the protective effect of estrogen. This is consistent with the current high prevalence of depression among women, but it is not clear whether it can be seen as the principle cause of the difference in the incidence of depression between men and women (55). It is still not known what causes this discrepancy; the DNMT1 expression might be tissue specific and age and sex dependent, which need deliberate investigation.

DNMT3A and DNMT3B

DNMT3A and DNMT3B have direct catalytic activity for DNA methylation, as functional DNA methyltransferases. DNMT3B

plays an important role in the process of early brain development and neurogenesis whereas DNMT3A is more essential to mature neuron than DNMT3B (21, 56). According to a postmortem study from suicide patients with MDD, there was no significant change of DNMT3A expression in the prefrontal cortex, amygdala, and paraventricular nucleus of hypothalamus, but the DNMT3B expressions were increased in the prefrontal cortex and paraventricular nucleus and decreased in amygdala of patients compared to healthy controls (6). In another study, the DNMT3B expressions in the dorsolateral prefrontal cortex of depression patients were increased, and the DNMT3A expressions showed no change compared to those in healthy controls (7). This is consistent with a study done with peripheral white blood cells (51).

Although no correlation was found between DNMT3A and depression in clinical studies, it is different in the animal models. Several studies have shown a causal relationship between Dnmt3a and depression, but the results are not consistent. In one of the studies, Elliott et al. found that the depressive mice established by chronic social defeat expressed lower Dnmt3a in the medial prefrontal cortex than wild type mice did (57). The *Dnmt3a*-knockout mice also had anxiety-like behavior [assessed *via* OFT, dark-light transfer (DLT), EPM, and home cage locomotion], whereas Dnmt3a overexpression in the medial prefrontal cortex induced an anxiolytic effect. However, in another study, Sales et al. found adult rats underwent learned helplessness displayed despair-like behavior (assessed *via* numbers of escape failures) accompanied by an increment of Dnmt3a and Dnmt3b expressions in medial prefrontal cortex and that treatment with antidepressant imipramine down-regulated their expression levels in the prefrontal cortex (9). Melas et al. also demonstrated that Dnmt3a expression level was decreased in prefrontal cortex after the depressive rats (Flinders Sensitive Line) (58) were treated with antidepressant escitalopram (31). This discrepancy may reflect the different depression models and behavioral tests used under the same depression umbrella, but it also indicates the significance of DNMT3A in all the models.

Since different brain areas may play different roles in the pathogenesis of depression, it is not surprising that Dnmt3a alterations are different in these areas. Besides prefrontal cortex, Dnmt3a changes were also found in nucleus accumbens and hippocampus in animal experiments. One study showed that Dnmt3a expressions in the nucleus accumbens were increased after the rats underwent social chronic defeats and Dnmt3a overexpressions in the nucleus accumbens promoted the occurrence of depression-like behaviors (social avoidance and despair-like behavior assessed *via* SIT and FST) (59). In another study, exposure to subchronic variable stress induced depression-associated behaviors (assessed *via* splash test, SCT, FST, EPM, and locomotor activity) and increased the expressions of Dnmt3a in the nucleus accumbens of mice, but the expression levels were higher in female mice than those in male mice (60). The different sensitivities of mice to depression were determined by the expression of Dnmt3a in the nucleus accumbens. After Dnmt3a coding genes in male mice were enhanced in the nucleus accumbens, the sensitivities to subchronic variable stress were increased, which were similar to those observed in female mice. Oppositely, after Dnmt3a genes were specifically knocked out in female mice, their adaptabilities to subchronic variable stress were significantly enhanced, which were similar to those of male mice (60).

Critical for learning and memory and mood regulation, hippocampus is the brain region associated with cognitive and emotional domains of depression (61). The expressions of Dnmt3a in this region were also investigated in depression models. In one study, the Dnmt3a expressions in the hippocampus of depressed rats (maternal deprivation) were increased, which could be alleviated by antidepressant treatment with escitalopram (55). Specifically, this alteration was found in the dorsal hippocampus but not ventral hippocampus, accompanied by increased DNA methylation. Additionally, in another study, injection of DNA methylation inhibitors into the hippocampus of the depressed mice could decrease the levels of DNA methylation in the hippocampus, and it had antidepressant-like effects (confirmed *via* FST and TST) (62). Whether this antidepressant effect is related to the inhibition of Dnmt3a activity in the hippocampus needs further study.

Compared to DNMT3A, DNMT3B is little studied due to its early expression during brain development and may play a less important role than DNMT3A. However, one study suggests DNMT3B may also change in depression, paralleling DNMT3A. In this study, the Dnmt3b expressions in the prefrontal cortex of depressive mice were higher than those in normal controls. After treatment with the antidepressant imipramine, the Dnmt3b expression in prefrontal cortex was decreased compared with that in the group without treatment (9).

In summary, given the contradictory observations among the studies of DNMT3A and DNMT3B in depression patients and depression-like animal models, it is still early to draw a conclusion based on the available data. However, the above-mentioned data do support the correlation and some causal relationship between DNA methylation and depression signs in patients or endophenotypes in animal models. The discrepancy may be caused by different experimental methods, depression models, sex and age or environmental changes. Also, we believe that DNA methylation is a dynamic process, and there are individual differences on its interaction with genes.

DNMT3L

DNMT3L has no direct catalytic effect, but it can be directly combined with DNMT3A and DNMT3B, and their enzyme activities can be increased by about 1.5–3 times (18). Some studies showed that DNMT3L increased the enzyme activity of DNMT3A more than DNMT3B (63). The only report on DNMT3L expression in depression patients showed the expression of DNMT3L in blood samples of patients with depression was not significantly different from that of psychiatrically healthy controls (51).

However, DNMT3L may play an important role during neurodevelopment. *DNMT3L* is located on chromosome 21, and its overexpression may cause the hypermethylation and alter the gene expression that controls the neurodevelopment (64). Ethanol exposure could decrease the Dnmt3l mRNA in the

amygdala of adolescent rats (65). There are few data about the expression of DNMT3L in the developing brain. The RNA expressing data from the Allen Institute showed that the expression of DNMT3L in the human brain increased from gestational age of 16 weeks until adult. Though at low levels, it distributed broadly, including cerebral cortex, cerebellar cortex, hippocampus, striatum, thalamus, and amygdala (24). In primate brain, the Dnmt3l distributed mainly in the ventricular and subventricular zone, and limited to hippocampus and amygdala after birth (23). Opposite to DNMT3L, DNMT3A, and DNMT3B expressions peaked around gestational age of 16 weeks and decreased after until adult (24). In primate brain, they mainly distributed in the ventricular zone, subventricular zone, hippocampus and amygdala, where the neural stem cells locate (23). Consistent with these observations in human and primates, a recent study in rats showed that the mRNA expression of Dnmt3l in the hypothalamus and hippocampus followed a pattern opposite to the other Dnmts, that was low expression in newborns and increasing expression with age (66). This indicates DNMT3L may function differently compared with other DNMTs, important in neurogenesis and neural plasticity, which are essential processes during brain maturation, a stage when the prevalence of early-onset depression is high. A summary of the DNMTs changes in depression patients and animal models is listed in **Table 1**.

A MODEL OF GENE-ENVIRONMENT INTERACTION THROUGH DNA METHYLATION

Many studies have suggested that early life adversity (ELA) such as maternal separation and child maltreatment is a major risk factor of MDD (68). ELA as a stressor causes abnormal nervous system arousal during early neurodevelopment. It continually activates the amygdala, which then sends signals to the hypothalamus to activate the HPA axis for the release of glucocorticoids, which functions through GRs and mineralocorticoid receptors (MRs) to regulate the body's response to stress (69). These ELA events may cause HPA hyperactivity and GR resistance, which are correlated with later life psychiatric disorders including MDD. ELA is also associated with structural changes in different brain areas, including amygdala, hippocampus, and prefrontal cortex (70, 71). The correlation of DNA methylation in GR gene *NR3C1* with ELA and depression have been reported in several studies in

TABLE 1 | DNMTs Expression in Depression Patients and Animal Models.

Sample	Tissue	Phenotype	DNMT	Reference
Suicide patients with MDD	Frontal cortex, Amygdala, Paraventricular nucleus	MDD	Frontal cortex: DNMT1 mRNA decreased; DNMT3A mRNA no change; DNMT3B mRNA increased. Amygdala: DNMT1 mRNA decreased; DNMT3A mRNA no change; DNMT3B mRNA decreased. Paraventricular nucleus: DNMT1 mRNA decreased; DNMT3A mRNA no change; DNMT3B mRNA increased	(6)
Patients with MDD and BPD	Peripheral white blood cells	MDD, BPD	DNMT1 mRNA decreased: DNMT3B mRNA increased; DNMT3A mRNA no change; DNMT3L mRNA no change	(51)
Patients with MDD	Dorsolateral prefrontal cortex, Cingulate cortex, Leucocyte	MDD	Dorsolateral prefrontal cortex: DNMT3B mRNA increased; DNMT3A mRNA no change; DNMT1 mRNA no change	(7)
Chronic defeat stress in mice	Nucleus accumbens	Depression-like behaviors	Dnmt3a mRNA increased	(59)
Rat depression model	Hippocampus	Depression-like behaviors	DNA methylation inhibitors induced antidepressant-like effects	(62)
Flinders Sensitive Line genetic rat model of depression	Prefrontal cortex	Reversing depressive-like behaviors	Increased Dnmt1 and Dnmt3a mRNA expression were decreased after SSRI treatment	(31)
Subchronic variable stress in mice	Nucleus accumbens	Depression-like behaviors	Dnmt3a mRNA increased	(60)
Dnmt1 and Dnmt3a knockout mice	Forebrain	Anxiolytic and antidepressant-like properties	Dnmt1 knockout	(52)
Adult mice with chronic social defeat stress	Medial Prefrontal Cortex	Anxiety-like behaviors	Dnmt3a mRNA decreased	(57)
Prenatally stressed dams in rats	Hippocampus	Reversing depression-like behaviors	Increased Dnmt1 mRNA and protein expression were decreased after Genipin treatment	(53)
Rat pups with maternal separation	Hippocampus	Reversing depression-like behaviors	Increased Dnmt1 and Dnmt3a mRNA expression were decreased after citalopram treatment	(67)
Stress model of depression in rats	Dorsal and ventral hippocampus, Prefrontal cortex	Depression-like behaviors	Dnmt3a and Dnmt3b protein expression increased in the prefrontal cortex, reversed by imipramine treatment	(9)
Two-hit stress model in mice	Hippocampus	Depression-like behaviors	Dnmt1 mRNA increased	(55)

patients (43, 72–78), with most cases showing increased methylation in the promoter region of *NR3C1*.

Consistent with the observations in patients, multiple studies have found the increased DNA methylation in *Nr3c1* and decreased GR expression in hippocampus of ELA mice and rats (79–85). Correspondingly, the DNA methylation in *Crh* promoter was decreased and its expression increased in the hypothalamus of ELA models. The other genes in the HPA axis such as AVP and ACTH were also reported to have similar DNA methylation changes in ELA models (44, 86–88). Supporting this machinery, the DNMTs were also found to be increased in these ELA models (89, 90). However, the global methylation changes affect many other genes resulting both hypermethylation and hypomethylation. Taken together, the DNA methylation changes of genes in HPA axis connect the ELA with neural activity, which may increase the susceptibility of the later life psychiatric disorders though HPA axis hyperactivity and GC resistance, associated with increased inflammation and imbalanced neurotransmitters (69, 91–96). It thus makes a model of gene-environment interaction for the pathogenesis of MDD (**Figure 1**), where the DNA methylation changes of genes in HPA axis are adaptive to the stress stimulation, which are reversible once the stress is removed; it induces depression though if the stressor stays for a long period. However, this could be oversimplified since there are individual differences on susceptibility and resilience reported due to different ELA types, sex, age, developmental time, and genetic background (69). Also, the DNA methylation mechanism in the regulation of gene expression is complicated. How does ELA change the expression of DNMTs? How does DNMTs specifically regulate genes in HPA axis? And how does HPA axis changes lead to increasing incidence of MDD? All these questions need thorough investigations.

DISCUSSION

The depression studies have a long history, but the study of DNA methylation and DNA methyltransferase is still in its infancy. At present, many studies have shown that changing the DNA methylation state in brain can alleviate the depression-like behaviors in animal experiments. For example, Sales et al. found that systematic injection of DNA methylation inhibitors (5-AzaD and RG108) into depressed mice could rescue the despair-like behavior and effectively relieve hypermethylation in hippocampus and prefrontal cortex caused by stress (101).

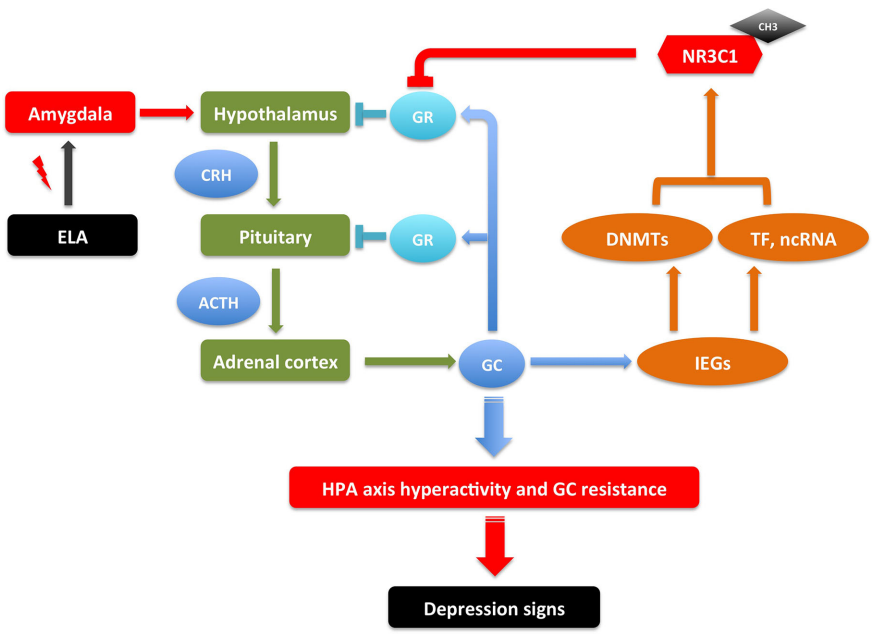

FIGURE 1 | An illustrative model of gene-environment interactions underlying depressive behaviors through *NR3C1* methylation. ELA events send signals to neurons in amygdala, which then send signals to hypothalamus to activate the HPA axis to release GC. In a feedback loop, GC activates GR in hypothalamus and pituitary to inhibit the HPA axis activation; it also activates IEGs that increase the transcription of DNMTs, TFs, and ncRNAs, which together up-regulate the DNA methylation in the promoter of *NR3C1*, inhibiting the expression of GR. These serial signals transduction results in HPA axis hyperactivity and GR resistance, which may cause increased inflammation and imbalanced neurotransmitters, therefore the susceptibility of depression. The depression signs in patients include depressed mood, anhedonia, lack of motivation, psychomotor changes (agitation/retardation), psychotic changes (delusion, hallucination), and cognitive changes (verbal memory, working memory, executive function). The depression-like signs in animal models include anhedonia-like behavior, despair-like behavior, anxiety-like behavior, locomotor changes, and cognitive changes (97–100). This is a reversible process. The depression signs can be treated by regaining the GR activity with drugs that interfere with DNA methylation or GR expression (69, 91–96). ACTH, Adrenocorticotropic hormone; CH3, DNA methylation; CRH, Corticotropin-releasing hormone; DNMTs, DNA methyltransferases; ELA, early life adversity; GC, glucocorticoids; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; IEGs, immediate early genes; ncRNA, non-coding RNA; TF, transcription factor.

Xing et al. also found that inhibition of DNMT activity in prefrontal cortex could play an antidepressant role (102). It may be related to the changes of DNMTs expressions and the increases of DNA methylation in these regions. The methylation levels of BNDF coding gene in hippocampus and prefrontal cortex were increased in patients with depression, and the results of autopsies showed atrophy in hippocampus and prefrontal cortex (103, 104). These changes are consistent with the changes of DNMTs in the corresponding brain regions, but the causal relationship between them is still not clear.

At present, we can confirm that DNMTs are involved in the pathogenesis of depression, but we do not know what causes the DNMTs changes, and how the change of DNMT expression in brain tissue affects the expression of specific genes. Pacaud et al. believe that transcription factors bind to DNMTs, thus exerting biological effects (105). Combining the changes of gene expression and DNMTs mentioned in this review, we can speculate as follows: 1) there are some tissue specific or cell specific transcription factors that can bind to the promoter region of related genes such as *P11*, *BDNF*, and *SLC6A4*, and DNMTs can bind these transcription factors and regulate the expression of the targeting genes; 2) DNMTs may function through catalyzing DNA methylation to regulate the gene expression, assisted by the transcription factors, miRNA or lncRNA to get to the specific targeting genes; 3) environmental changes may activate some IEGs, which in turn activate or inhibit the DNMTs expression; 4) the current depression model is based on the loop formed between several brain regions of the limbic system, and the completion of the loop function depends on the normal secretion, release and transport of excitatory and inhibitory neurotransmitters. Therefore, whether the changes of DNMTs in these related brain regions affect the expression of these neurotransmitters is also a question that needs to be further investigated.

Other studies have shown that gender differences exist for the expression of MeCP2, SLC6A4, and other genes in blood samples of depression patients (106), but it is not clear whether it is related to the sex difference in the expression of DNMTs mentioned in this review. Kolodkin et al. found that Dnmt3a expression in the amygdala of rats at birth had a sex difference, and the expression of Dnmt3a in the female rats was higher than that in the male rats, but the gender difference vanished at the 10th day of birth (107). The prefrontal cortex, nucleus accumbens, amygdala, and hippocampus form reward loops closely related to addiction and mood regulation. According to the sex difference of Dnmt3a expression in nucleus accumbens and amygdala and the characteristics of decreased estrogen and progesterone levels in women during menopausal period (108), we can infer: 1) estrogen can affect the expression of DNMT3A in amygdala; 2) the high incidence of depression in women may be due to the inhibitory effect of estrogen on DNMT3A expression in amygdala, which is higher than that of androgen; 3) the sex difference in DNMT3A expression in the nucleus accumbens and DNMT1 in the amygdala may be due to the different depression patterns in men and women; 4) the higher incidence of menopausal depression in women may be due to the decrease of estrogen, so the inhibitory effect on DNMT3A is reduced.

To test the above conjectures, we need following studies: 1) to explore the mechanism of DNMT3L regulating the activities of DNMT3A and DNMT3B under different conditions; 2) to observe the expression of DNMT1, DNMT3A, DNMT3B, and DNMT3L in different brain regions and the effect of estrogen on them under physiological conditions; 3) under physiological conditions, to observe the process of DNA methylation dynamically and capture the changes of DNMT expression in different brain regions at different time points, then record the time sequence of DNMT expression changes; 4) to study the expression and activity of DNMT at cellular level; 5) to study the methylation process of DNMTs to specific gene promoter sequences at molecular level; 6) to start with the genes with altered expression in depression, use DNMTs and DNA methylation as the intermediary to find the signal pathway that regulates the expression of specific genes.

It is difficult to obtain the brain tissue of patients with depression, which brings some difficulties to the exploration of the pathogenesis of depression. However, with the invention of methods to noninvasively visualize epigenetic processes *in vivo*, such as PET radiotracers for histone deacetylases (HDACs) (109, 110), we can expect the development of PET radiotracers for DNMTs too and dynamic DNA methylation changes could then be traced in depression patients. Recent technical advancement on induced pluripotent stem cells may help to solve this problem by making cellular model by using the fibroblasts from depression patients. Besides this, by making use of other molecular techniques such as CRISPR, methylation editing, optogenetics, and single cell technologies, we could work on these questions and will better understand the mechanisms of DNMTs in the pathogenesis of depression.

AUTHOR CONTRIBUTIONS

JL: conceived the topic of manuscript. JL and ZD: collected the data and carried out the main analysis. All authors contributed to the article and approved the submitted version.

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Anterior Cingulate Cortex Glutamate Levels Are Related to Response to Initial Antipsychotic Treatment in Drug-Naive First-Episode Schizophrenia Patients

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The glutamatergic system has previously been shown to be involved in the pathophysiology of schizophrenia and the mechanisms of action of antipsychotic treatment. The present study aimed to investigate the relationship between the levels of glutamate (Glu) or Glu/total creatine (Glu/Cr+PCr) in the anterior cingulate cortex (ACC) and psychiatric symptoms as well as the response to antipsychotic treatment. We performed proton magnetic resonance spectroscopy (^{1}H–MRS) to measure Glu and Glu/Cr+PCr in the ACC of 35 drug-naïve first-episode schizophrenia (FES) patients and 40 well-matched healthy controls (HCs). After scanning, we treated the patients with risperidone for eight weeks. Remission status was based on the Positive and Negative Syndrome Scale (PANSS) scores at week 8. At baseline, there were no significant differences in the levels of Glu or Glu/Cr+PCr in the ACC between drug-naïve FES patients and HCs. Lower baseline levels of Glu/Cr+PCr but not Glu in the ACC were associated with more severe negative symptoms of schizophrenia. Compared to the remission group (RM), the non-remission group (NRM) had lower baseline ACC Glu levels ($P < 0.05$). Our results suggest that ACC Glu levels may be related to the severity of symptoms in the early stages of schizophrenia and therefore may be a marker with which to evaluate the treatment effect of antipsychotics in schizophrenia patients.

Keywords: glutamate, magnetic resonance spectroscopy, ACC, anterior cingulate cortex, Cr, creatine, FES, first-episode schizophrenia

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INTRODUCTION

Schizophrenia is a complex and severe mental disorder with a lifetime prevalence of 1% worldwide (1). Although it has been over 60 years since the first antipsychotic, chlorpromazine, was initially developed, a third of patients with schizophrenia have a suboptimal response to first-line antipsychotic treatment, most of which mainly target dopamine D2 receptors (2, 3). The dopaminergic hypothesis can partially explain the psychopathology of positive symptoms in schizophrenia. However, dopaminergic antipsychotics have only a limited effect on negative symptoms and cognitive deficit, which are the best predictive factors of persistent disability and a poor response to antipsychotics (4, 5).

Converging lines of evidence from pharmacological and neuropharmacological studies suggest that glutamatergic dysfunction also contributes to deficits in schizophrenia (6–8). Studies have shown that administration of phencyclidine (PCP), dizocilpine (MK801), and ketamine, antagonists of the N-methyl-d-aspartate glutamate receptor (NMDAR), to healthy volunteers or rodents can induce schizophrenia-like symptoms (9–11). In pharmacological animal models of schizophrenia, compared with other drugs, such as amphetamine, NMDAR antagonists can cause positive and negative symptoms that more closely mimic those of schizophrenia (12). Genetic animal models also provide a wealth of data showing that decreased NMDAR activity can lead to changes in the brain and behavior, similar to those observed in schizophrenia (13). First-episode schizophrenia studies have shown that approximately one-quarter of first-episode schizophrenia (FES) patients have persistent psychosis symptoms despite adequate initial treatment (14), and this finding, together with the glutamatergic hypothesis, may suggest that dopaminergic drugs are ineffective throughout illness in the subgroup of schizophrenia patients, who have an inadequate response to initial treatment with dopaminergic drugs.

Proton magnetic resonance spectroscopy (^{1}H-MRS) is an efficient and non-invasive method for detecting the concentration of Glu in the human brain. Many studies have found differences in glutamate levels in target areas of the brain between schizophrenia patients and healthy controls (15–18), and glutamate levels have been associated with the severity of symptoms and cognitive function (19–21). These studies have aroused great interest in whether the levels of glutamate in the brain can predict the response to antipsychotics and the outcome of schizophrenia. The current hypothesis is that inadequate response to conventional dopaminergic antipsychotics is more likely to be associated with the glutamatergic system (22). In previous ^{1}H-MRS studies, the results of measurements of glutamate levels in the brains of schizophrenia patients were mixed (23), and the findings of the relationship between glutamate levels and the severity of symptoms of schizophrenia were also inconsistent (24). The discrepancy of the above results may be due to the effect of age, stage of the disease, and treatment with antipsychotics (25, 26).

In this study, we implemented a longitudinal design to explore the relationship between glutamate levels and symptom severity as well as the response to antipsychotics. To reduce the effect of the confounding factors mentioned above, we selected drug naïve first-episode psychiatric patients as the experimental cohort. In previous studies, the ACC has been shown to play a critical role in cognitive impairment and psychotic symptoms in schizophrenia patients (27, 28). Therefore, we selected the ACC as the target region. At baseline, ^{1}H-MRS scans were performed on patients and healthy controls who met the study criteria. We assessed the symptom severity of the patients using the PANSS scale at both baseline and week 8 after risperidone treatment. We chose risperidone as the administered antipsychotic because it is widely prescribed and very inexpensive in China. We expected to determine whether baseline levels of Glu or Glu/Cr+PCr in the ACC are associated with the severity of psychiatric symptoms and with the response to initial antipsychotic administration in drug naïve FES.

RESULTS

Comparison of Demographic Characteristics and Metabolite Levels Between HC and FES Patients

The demographic characteristics and metabolite levels are summarized in **Table 1**. Thirty-five FES patients met the inclusion criteria, were first diagnosed with schizophrenia, and had never been treated with antipsychotics. There was no significant difference in age, gender, education, or tobacco or alcohol use between drug-naïve FES patients and HCs. As shown in **Table 1**, no significant differences were observed in Glu and

TABLE 1 | Demographic, clinical characteristics and MRS data of controls and schizophrenia patients.

	Controls (n = 40)	Patients (n = 35)	P-value
Age, mean ± SD, y	22.75 ± 4.19	22.286 ± 4.46	0.643
Gender (male/female)	25/15	22/13	0.975
Education, mean ± SD, y	11.50 ± 1.68	11.06 ± 2.33	0.343
Handedness (right/left)	40/0	35/0	-
Tobacco use (yes/no)	6/29	10/30	0.407
Alcohol use (yes/no)	0/40	0/35	-
ACC-Glu, mean ± SD	8.79 ± 0.91	8.92 ± 1.14	0.572
ACC-Glu/Cr+PCr, mean ± SD	1.61 ± 0.18	1.65 ± 0.27	0.413
ACC-FWHM, mean ± SD	0.065 ± 0.021	0.069 ± 0.017	0.339
ACC-S/N, mean ± SD	24.73 ± 2.91	23.97 ± 2.70	0.251

ACC, anterior cingulate cortex; Glu, Glutamate; Cr, creatine; PCr, phosphocreatine; FWHM, full width at half maximum; S/N, signal-noise-noise ratio.

Abbreviations: ^{1}H–MRS, proton magnetic resonance spectroscopy; ACC, anterior cingulate cortex; Glu, glutamate; Cr, creatine; PCr, phosphocreatine; CRLBs, Cramér Rao lower Bounds; FWHM, full width at half maximum; SNR, signal-noise ratio; HC, healthy control; FES, first-episode schizophrenia; PANSS, Positive and Negative Syndrome Scale; NMDAR, N-methyl-D-aspartate receptor; RM, remission; NRM, non-remission.

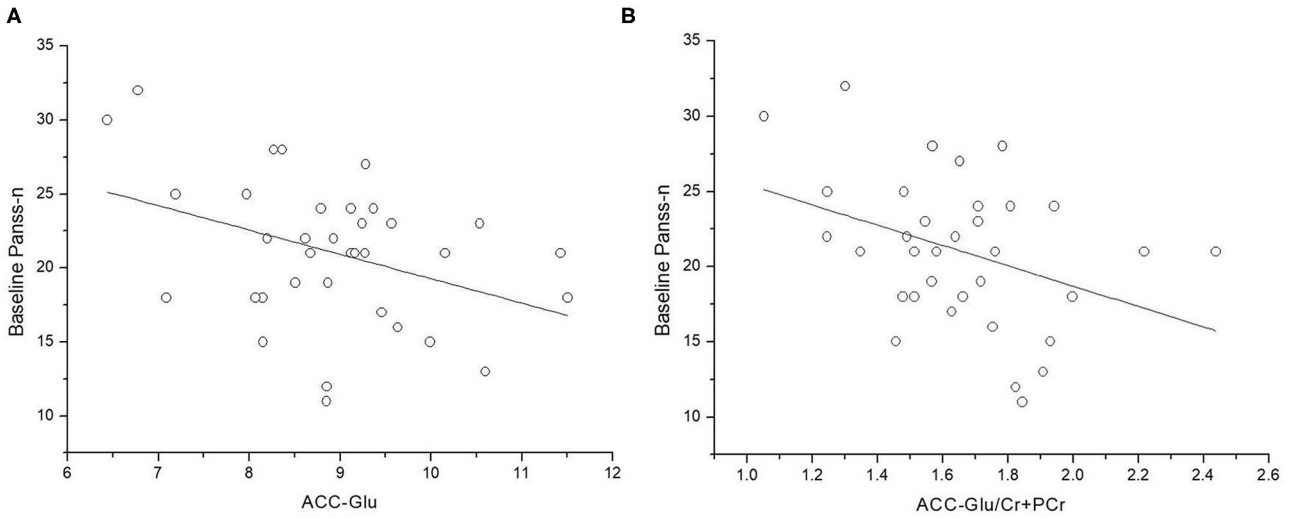

FIGURE 1 | Relationship between levels of glutamate (Glu and Glu/Cr+PCr) in anterior cingulate cortex (ACC) and PANSS negative symptom scores. **(A)** ACC-Glu and PANSS negative symptom scores at baseline ($n = 35$, $r = -0.360$, $p = 0.034$). **(B)** ACC-Glu/Cr+PCr and PANSS negative symptom scores at baseline ($n = 35$, $r = -0.432$, $p = 0.010$).

Glu/Cr+PCr levels in the ACC between HCs and FES patients at baseline. There were no significant differences in the Glu and Glu/Cr+PCr levels of the males and females between the patient group and the control group. Both *p*-values were higher than 0.05 (**Supplementary Table 1**).

Quality of ^{1}H-MRS Spectra

The mean ± SD of the FWHM from the LC Model in the ACC was 0.069 ± 0.017 ppm for the FES patients and 0.065 ± 0.021 ppm for the HCs. The mean ± SD of the S/N in the ACC was 23.97 ± 2.70 for FES patients and 24.73 ± 2.91 for HCs. There were no significant differences in the FWHM and S/N in the target voxels between FES patients and HCs (FWHM, T73 = 0.849 $p = 0.399$; S/N, T73 = −1.156, $p = 0.251$). The CRLB% values of Glu, Cr, and PCr in the ACC for both the FES patients and HCs were <20%. Data on the quality of the ^{1}H-MRS spectra are summarized in **Table 1**.

Correlations of Metabolite Levels With Clinical Symptoms

At baseline, lower levels of Glu and Glu/Cr+PCr in ACC were associated with more severe negative symptoms (**Figure 1**) (Glu and PANSS-n: $r = 0.360$, $p = 0.034$; Glu/Cr+PCr and PANSS-n: $r = -0.432$, $p = 0.010$). After controlling for age, the relationship between the Glu level in the ACC and negative symptoms was not significant ($r = -0.323$, $p = 0.062$), but lower Glu/Cr+PCr levels remained associated with a higher PANSS-n score ($r = -0.446$ $p = 0.008$).

Remission vs. Non-remission Patients

According to the standards proposed by the Remission in Schizophrenia Working Group (29), 25 patients (71%) met the remission criteria, and 10 patients (29%) were in non-remission status. Non-remission patients were younger, had more severe symptoms, and were treated with a higher dose of risperidone during the study (**Table 2**).

TABLE 2 | Demographic, clinical characteristics, and MRS data of remission and non-remission.

	Remission (25)	Non-remission (10)	*P*-value
Age, mean ± SD, y	23.48 ± 4.52	19.30 ± 2.58	0.010
Gender (male/female)	14/11	8/2	0.259
Risperidone dose, mean ± SD	3.60 ± 0.50	4.30 ± 0.48	0.001
Education, mean ± SD, y	11.24±2.49	10.60 ± 1.90	0.470
(Baseline)Panss-T	80.68 ± 12.11	94.50 ± 13.55	0.006
(Baseline)Panss-P	21.88 ± 4.71	24.60 ± 6.20	0.168
(Baseline)Panss-N	16.68 ± 3.52	22.60 ± 4.17	<0.001
(Baseline)Panss-G	42.12 ± 8.91	47.30 ± 7.71	0.117
(Week8) Panss-T	53.72 ± 8.27	81.40 ± 9.88	<0.001
(Week8) Panss-P	11.36 ± 2.40	20.40 ± 5.21	<0.001
(Week8) Panss-N	11.88 ± 2.35	19.60 ± 4.25	<0.001
(Week8) Panss-G	30.48 ± 1.16	41.40 ± 5.68	<0.001
ACC-Glu	9.22 ± 1.05	8.17 ± 1.08	0.013
ACC-Glu/Cr+PCr	1.67 ± 0.26	1.53 ± 0.28	0.117

PANSS, Positive and Negative Symptoms Scale; PANSS-T, PANSS total scores; PANSS-P, PANSS positive symptom scores; PANSS-N, PANSS negative symptom scores; PANSS-G, PANSS general psychopathological symptom scores.

Compared with the remission group, non-remission patients had a significantly lower Glu level in the ACC (T33 = −2.634 $P = 0.013$). However, the difference in Glu/Cr+PCr levels in the ACC between the remission and non-remission groups was not significant (1.67 ± 0.26 vs. 1.53 ± 0.28, T33 = −1.612, $p = 0.117$) (**Figure 2**, **Table 2**).

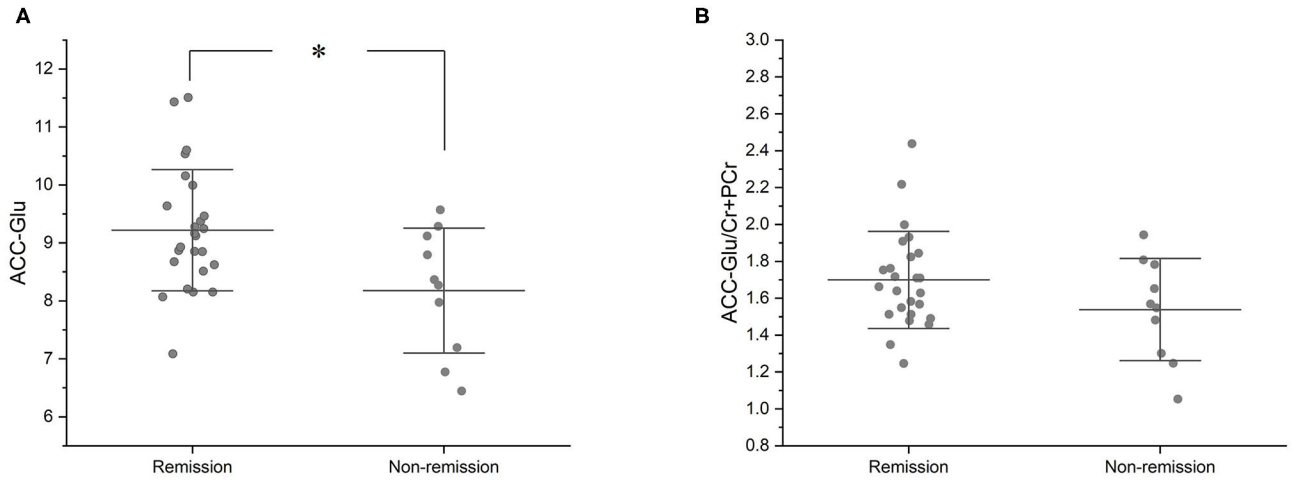

FIGURE 2 | Baseline Glu and Glu/Cr+PCr in ACC of remission and non-remission groups. **(A)** At baseline, the remission group had a significantly higher Glu level than the non-remission group (mean values, 9.22 ± 1.05 vs. 8.17 ± 1.08; T33 = −2.634, $P = 0.013$). **(B)** At baseline, the remission group had a higher Glu/Cr+PCr than the non-remission group, but there was no statistically significant difference between the two groups (mean values, 1.67 ± 0.26 vs. 1.53 ± 0.28; T33 = −1.612, $p = 0.117$). *$p < 0.05$.

Prediction of Non-remission

To distinguish between remission and non-remission patients, we implemented a binary logistic regression model. Baseline Glu or Glu/Cr+PCr in the ACC was associated with the likelihood of remission in FES patients after treatment with risperidone. In the Glu model (Nagelkerke $R^2 = 0.256$, B = −1.048, Wald = 4.945, $p = 0.026$, EXP[B] = 0.351), 77.1% of FES patients (96% remission patients and 30% non-remission patients) were correctly segregated. In the Glu/Cr+PCr model (Nagelkerke $R^2 = 0.110$, B = −2.602, Wald = 2.367, $p = 0.124$, EXP[B] = 0.074), 74.3% of FES patients (96% remission patients and 20% non-remission patients) were correctly segregated. When age and PANSS-total score were included in the two models, the accuracy increased to 85.7% (92% remission patients and 70% non-remission patients, Nagelkerke $R^2 = 0.674$) in the model with baseline Glu and 82.9% (88% remission patients and 70% non-remission patients, Nagelkerke $R^2 = 0.655$) in the model with baseline Glu/Cr+PCr (**Table 3**).

TABLE 3 | Models of binary logistic regression.

Models	Nagelkerke R^2	HL-test	Accuracy of Re (%)	Accuracy of non-Re (%)	Overall Accuracy (%)
Model 1	0.256	0.895	96	30	77.1
Model 2	0.110	0.472	96	20	74.3
Model 3	0.670	0.987	92	70	85.7
Model 4	0.561	0.477	88	70	82.9

HL-test, Hosmer–Lemeshow test; Re, remission; non-Re, non-Remission.
Model 1: Glu; Model 2: Glu/Cr+PCr; Model 3: Glu; Age; Panss-total score; Model 4: Glu/Cr+PCr; Age; Panss-total score.

DISCUSSION

Glutamate is the most important excitatory neurotransmitter in the nervous system (30), and NMDAR is widely distributed throughout the human brain. The glutamate measurement provided by ^{1}H-MRS reflects the amount of glutamate in the target region in the brain and is also considered an indicator of motor cortical excitability (31, 32). Previous studies have observed that phencyclidine (PCP) and ketamine, which are antagonists of NMDAR, can transiently induce positive psychotic symptoms, such as delusions, hallucinations, agitation, and catatonic behavior, and negative psychotic symptoms including blunted affect, anhedonia, avolition, and inattention (6, 33). Evidence from MRS and microdialysis studies has shown that PCP or ketamine and other antagonists of NMDAR can increase glutamate release (34–37). To verify that glutamatergic system dysfunction is involved in the psychopathology of schizophrenia, a large number of studies on glutamate levels in the brain were conducted through MRS, and according to the ketamine or PCP model, glutamate concentrations in the brain should increase in first-episode schizophrenia patients. Supporting the ketamine model, recent studies based on samples of first-episode schizophrenia have observed glutamate concentrations in the frontal, prefrontal, and anterior cingulate cortexes (38), and the striatum (26, 39). Contrary to this hypothesis, some studies found reductions in glutamate concentrations in the ACC (16) and the medial frontal cortex of first-episode schizophrenia patients (40). In our study, there were no significant differences in glutamate levels in the ACC between drug-naïve first-episode schizophrenia patients and healthy controls. The inconsistency in these studies may suggest that the ketamine model does not accurately reflect the mechanisms underlying schizophrenia.

Accumulating evidence has shown that glutamatergic dysfunction may be involved in the pathogenesis of

schizophrenia, and glutamate levels may be associated with the outcome of schizophrenia and the severity of psychiatric symptoms (41, 42). In our study, we found lower Glu/Cr+PCr was correlated with more severe negative symptoms as assessed by the PANSS at baseline. Some studies with samples of chronic schizophrenia patients report that the severity of negative symptoms was associated with lower levels of Glu levels in the brain (41, 43, 44). However, in contrast to our results, several studies found that more severe negative symptoms were associated with higher levels of Glu in first-episode psychosis (16, 45, 46). The inconsistencies were most likely due to the effects of age, antipsychotic administration, and disease stage on glutamate concentration. Natsubori et al. (47) found that the Glx level of chronic and medicated schizophrenia patients in the mPFC and ACC was lower than that in the healthy control group, patients at ultrahigh risk for psychosis, and patients with first-episode schizophrenia. One study found that Glu levels in the ACC were significantly lower than those at baseline in patients with first-episode schizophrenia after 4 weeks of amisulpride administration (46). Therefore, demographic characteristics of different samples can influence the Glu level, which affects the determination of the correlation between Glu levels and symptoms. In the present study, our subjects were younger than those in many studies and had never taken antipsychotic drugs. Recently, a study (48) with samples that had a similar mean age with ours (22.3 $\pm$ 4.4 years) found that the Glu level positively correlated with the cognitive function of the patients. According to our results, a lower level of Glu/Cr+PCr indicates more severe negative symptoms in patients with schizophrenia in the early stage before treatment. ACC glutamate levels were associated only with negative symptoms, suggesting that lower glutamate levels are linked to poor clinical outcomes.

Longitudinal studies on the relationship between antipsychotic response and Glu levels before treatment are rare. Recently, a multi-center ^{1}H-MRS study found that first-episode schizophrenia patients with a worse response to amisulpride had higher ACC glutamate levels at baseline before treatment (46). The advantage of this study was that amisulpride has high selectivity to dopamine D2 and D3 receptors (49, 50); thus, in schizophrenia patients, Glu levels could be linked to the dopamine-blocking effect, rather than to the effect of blocking other receptors. However, this study did not require that the patients taking part in the study had never taken the antipsychotics, and they were also examined without a withdrawal period before the experiment. Additionally, a large proportion of the patients in this study were users of other substances. These factors make it impossible to eliminate the possible effect of pre-study drug therapy and substance use on the patients' Glu levels and the outcome of amisulpride administration (51). In our study, we selected individuals with schizophrenia who had never been treated by antipsychotics and excluded substance users. We found that patients with a worse response to risperidone had lower Glu levels than patients with a better response. Binary logistic regression models showed that the overall discrimination accuracy of remission by ACC Glu and Glu/Cr+PCr was 77.1 and 74.3%, respectively. When the age and PANSS-total scores were introduced into the regression models, the discrimination accuracy of remission reached 85.7 and 82.9%, respectively, which also showed clinical significance.

Ketamine has been shown to induce schizophrenia-like symptoms (6, 33, 52). The advantage of the ketamine model is that the relative proportions of positive and negative symptoms induced by the drug are more similar to schizophrenia than the respective proportions of symptoms induced by amphetamine or LSD (33, 53). According to the hypothesis of the ketamine model, schizophrenia patients should have higher levels of glutamate, which is associated with more severe psychiatric symptoms. However, this popular model is not without its limitations. First, some studies have found that ketamine also has a high affinity for D2 receptors (54, 55), while a recent study found that ketamine has no affinity for D2 receptors (56). Other studies have found that ketamine can change the level of glutamate in the brain of humans or animals (57), so the psychotomimetic effects of ketamine may be related to the dopaminergic system. Second, schizophrenia is considered a neurodevelopmental disorder in which the nervous system undergoes a long process to reach the disease state (58). Therefore, it is not reasonable to study the pathological process of schizophrenia using the more acute ketamine model. Third, many results based on MRS, including ours, are inconsistent with or even wholly contrary to the hypothesis of the ketamine model (16, 44, 48). Therefore, future studies on glutamate levels based on MRS should not only rely on the hypothesis of the ketamine model but should also be considered from multiple perspectives.

Our study also had some limitations. First, we did not perform ^{1}H-MRS scans on patients at week 8, so we could not evaluate the changes in glutamate concentration caused by risperidone, which was not considered in our initial design. Second, we assessed only the psychiatric symptoms by the PANSS but did not evaluate the cognitive and social functions, so we could not fully judge the overall functional status and outcome of the drug-naïve FES patients. Third, because ^{1}H-MRS scans require individuals to be relatively calm, we excluded patients with underlying impulsivity, agitation, and excitement symptoms, so our sample was not representative of all first-episode schizophrenia patients. Fourth, our sample size is small, and the lower limit of the age of the inclusion criteria was relaxed to 16 years old, so the influence of age on Glu concentration cannot be excluded entirely. In addition, non-remission patients were younger, had more severe symptoms, and were consequently treated with higher doses of risperidone. The difference in risperidone dosage will affect the final outcome of the patient. Fifth, we asked only the patient himself/herself and his/her guardians about the patient's use of tobacco, alcohol, and substances. We did not perform urine or blood tests on each subject to determine whether they used tobacco, alcohol, or other substances. Thus, we cannot guarantee the authenticity of the above reported data.

In conclusion, we observed that there were no significant differences in baseline Glu or Glu/Cr+PCr in the ACC between drug-naïve FES patients and HCs. However, we found that lower Glu/Cr+PCr levels were highly associated with serve negative symptoms and that the patients with worse outcomes had lower baseline Glu levels. Our results suggested that baseline glutamate levels in the ACC may be used as a

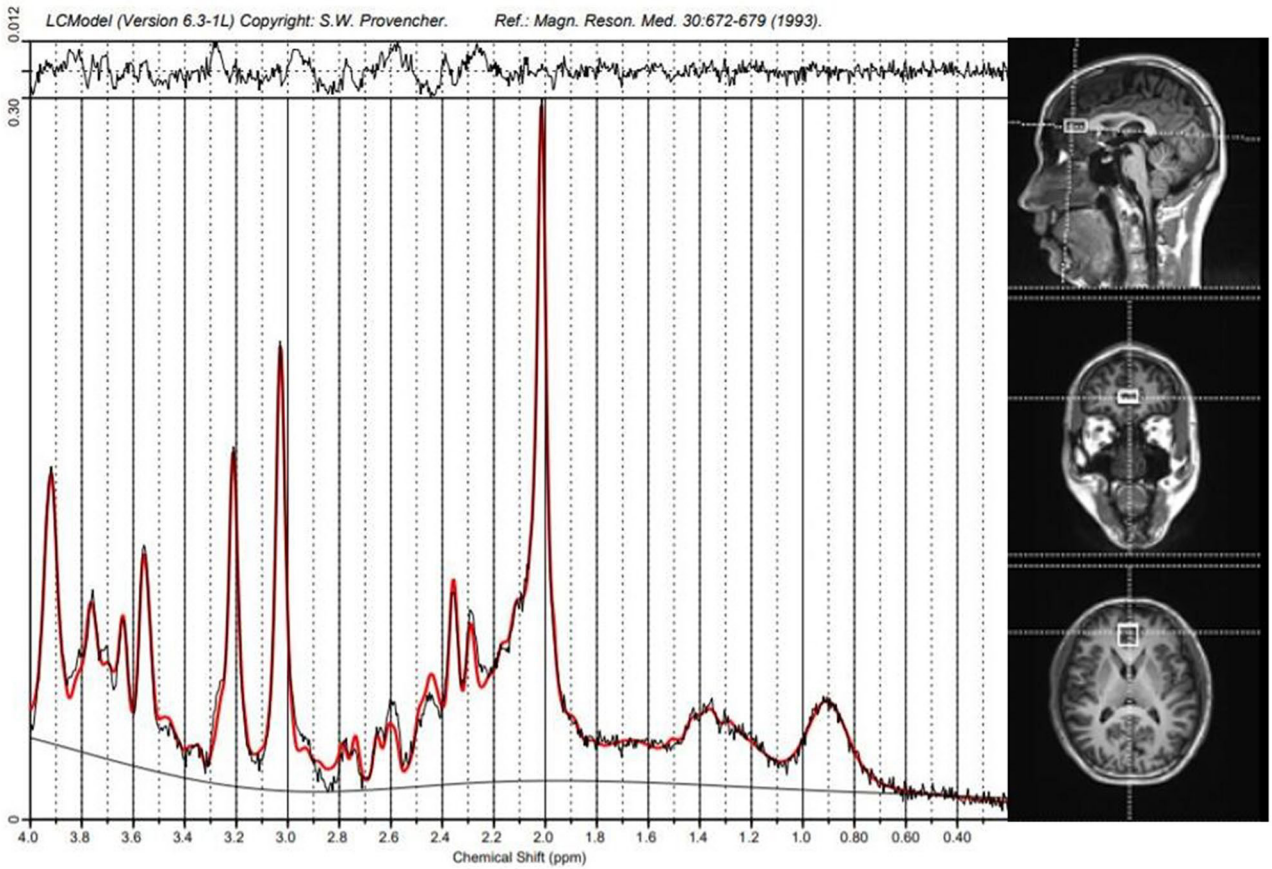

FIGURE 3 | Spectroscopic voxel placement and proton magnetic resonance (^{1}H-MRS) spectra. Voxel placement in the anterior cingulate cortex (ACC) and Sample ^{1}H-MRS spectra acquired from ACC by LCModel.

marker to evaluate the treatment effect of antipsychotics in schizophrenia patients.

MATERIALS AND METHODS

Participants

We recruited 39 drug-naïve FES subjects from outpatients in the Second Xiangya Hospital, Central South University, China, and 42 HC subjects through advertisements posted at the same time. All FES patients were first diagnosed with schizophrenia using the *DSM-IV* criteria with a Mini-International Neuropsychiatric Interview (59) by two experienced senior psychiatrists. The inclusion criteria of all subjects were as follows: (1) right-handed Han Chinese individuals aged 16–30 years; (2) education≧6 years; (3) no prior exposure to antipsychotics; (4) no history of substance use; and (5) no major medical or neurological illness. None of the HC subjects met the diagnostic criteria for any mental illness in the *DSM-IV* or had a history of mental illness or a family history of mental disorders. Data from four patients (two lost to follow-up and two with MR contraindications) and two healthy controls (one with MR contraindications and one with an anatomical abnormality on structural scan) were excluded. Data from 35 FES patients and 40 HCs were included in the final analyses. The study was approved by the Second Xiangya Hospital Ethics Committee (No. S008,2012). All participants were aware of the detailed procedures of our study and signed informed consent forms.

^{1}H-MRS

All subjects were scanned in a 3T scanner (Siemens, Verio, Germany) with a 16-channel head coil at the Magnetic Imaging Centre of Hunan Children's Hospital, China. Scans of patients were performed before treatment with antipsychotics, while healthy controls had no time limit on undergoing the scan. Patients with acute psychotic symptoms underwent an ^{1}H-MRS scan within a few hours and took risperidone immediately after the scan was completed. Patients with predominantly negative symptoms had an ^{1}H-MRS scan later in the day, taking risperidone in the evening or morning the next day. Participants wore specially made foam pads to reduce head motion and scanning noise. T1-weighted anatomical MRI images were acquired with 3-dimensional magnetization-prepared fast gradient echo sequences (TR/TE = 2530 ms/2.33 ms; FOV = 256 × 256 mm; slice thickness = 1.0 mm; gap = 0 mm; NEX = 1; and 192 sagittal slices). Target voxels were placed in the ACC (10 ×

20 × 20 mm), and the ^{1}H-MRS data were acquired using a point-resolved spectroscopy sequence (PRESS) (svs_se; TR = 3000 ms; TE = 30 ms; NEX 80) (**Figure 3**).

^{1}H-MRS spectra were analyzed with a linear combination model (LCModel version 6.3–1B) (**Figure 3**) at the Second Affiliated Hospital, Shantou, China. The cerebrospinal fluid in the target voxels was used as the internal reference to calculate the absolute concentration of Glu and Cr+PCr. The FWHM and SNR were checked to guarantee the quality of the MRS data, and only those spectra with an FWHM <0.1 ppm and an SNR> 10 were retained. Furthermore, data with a Cramer–Rao minimum variance ≥ 20% were discarded.

Medication

All patients were taking antipsychotics for the first time and were treated with risperidone monotherapy for eight weeks following the scanning. It is contraindicated for co-use with mood stabilizers and antidepressants. None of the patients dropped out of the study because of severe side effects.

Clinical Assessments

We assessed the severity of illness at both baseline and week 8 using the 30-item Positive and Negative Syndrome Scale (PANSS) (60). The assessment was carried out by two psychiatrists (interrater reliability score > 0.8). Remission status was defined by the criteria proposed by the Remission in Schizophrenia Working Group (29), but it did not include the six-month observation period. This standard requires that the score of the following items of the PANSS be less than 3: PANSS-Positive (P1, P2, P3), PANSS-Negative (N1, N4), PANSS-General (G5, G9).

Statistical Analysis

We performed all statistical analyses in SPSS (version 20 IBM Inc., New York, USA), with statistical significance indicated by two-tailed p-values < 0.05. Group differences in demographical characteristics, levels of Glu and Glu/Cr+PCr, and PANSS scores were assessed using independent-samples t-tests or chi-square tests. Relationships between metabolite levels and baseline symptom scores of the PANSS were described with Pearson's correlation analysis. The association between baseline metabolite levels of Glu and Glu/Cr+PCr and patient outcome (remission and non-remission) at week 8 was analyzed with binary logistic regression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Second Xiangya Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL, JT, HH, and XC designed the study. JL, ZL, HR, DW, XM, and LY collected the samples and clinical information. CL carried out the brain scanning. JL, HR, ZL, HH, JZ, and JT analyzed and discussed the experimental result. JL and HR wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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State-Independent and -Dependent Structural Connectivity Alterations in Depression

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Some brain abnormalities persist at the remission phase, that is, the state-independent abnormalities, which may be one of the reasons for the high recurrence of major depressive disorder (MDD). Hence, it is of great significance to identify state-independent abnormalities of MDD through longitudinal investigation. Ninety-nine MDD patients and 118 healthy controls (HCs) received diffusion tensor imaging scanning at baseline. After 6-month antidepressant treatment, 68 patients received a second scan, among which 59 patients achieved full clinical remission. Differences in whole-brain structural connectivity (SC) between patients with MDD at baseline and HCs were estimated by two-sample *t*-tests. Masked with significantly changed SCs in MDD, two-sample *t*-tests were conducted between the remitted MDD subgroup at follow-up and HCs, and paired *t*-tests were implemented to compare the differences of SC in the remitted MDD subgroup before and after treatment. Significantly decreased SC between the right insula and the anterior temporal cortex (ATC), between the right ATC and the posterior temporal cortex (PTC), between the left ATC and the auditory cortex as well as increased connectivity between the right posterior cingulate cortex (PCC) and the left medial parietal cortex (MPC) were observed in the MDD group compared with the HC group at baseline ($p < 0.05$, FDR corrected). The decreased connectivity between the right insula and the ATC and increased connectivity between the right PCC and the left MPC persisted in the remitted MDD subgroup at follow-up ($p < 0.05$, FDR corrected). The decreased SC between the right insula and the ATC and increased SC between the right PCC and left MPC showed state-independent characters, which may be implicated in the sustained negative attention bias and motor retardation in MDD. In contrast, the decreased SC between the right ATC and the PTC and between the left ATC and the auditory cortex seemed to be state-dependent.

Keywords: major depressive disorder, structural connectivity, state-independent, state-dependent, DTI

INTRODUCTION

Major depressive disorder (MDD) is characterized by a high rate of recurrence and a high rate of lifetime prevalence, which brings an enormous burden to the patients, families, health system and society (1). Many patients with MDD have residual depressive symptomatology at remission, which seems to be a significant predictor of relapse (2). MDD residues include persistent subclinical illness symptoms and potential persistent brain abnormalities. Therefore, identification of state-independent biomarkers may provide insights into MDD pathophysiology and pathogenesis.

Whole-brain white-matter structural connectivity (SC), derived from diffusion tensor imaging (DTI) tractography, are the substrate of distributed functional interactions among brain regions. Tymofiyeva et al. compared the DTI-based structural networks in a cohort of 57 depressed adolescents and 41 matched healthy controls (HCs) and found that MDD patients showed reduced SC between the insula and the right caudate (3). Korgaonkar et al. used the inter-regional SC of the entire cortex to characterized MDD. They found that the most discriminant features included the SC of the right insula to the right inferior parietal (4). Furthermore, Qin et al. explored the topological properties of structural brain networks of MDD and found that both current and remitted patients exhibited a decrease in node strength of the right insula compared with HCs (5). Another investigation in patients with remitted geriatric MDD revealed an altered component including 18 regions and 19 SCs in the right hemisphere. Compared with the HCs, all the connectivity in the component was decreased in the patients. The regions were mainly paralimbic (insula, parahippocampal gyrus, and superior/middle temporal gyrus), subcortical (hippocampus, caudate nucleus, putamen, pallidum, and thalamus) regions (6). Taken these findings together, abnormal SC with insula in MDD patients seems to show state-independent characters. In addition, many previous studies had found decreased inter-regional SC in the temporal-limbic, frontolimbic, and parietal-limbic circuits in MDD patients relative to HCs (4, 7–9). Recently, reduced connections related to the superior temporal gyrus, both for functional and structural connectivity, were also found in MDD patients relative to HCs (10). After electroconvulsive therapy, the decreased SCs among the temporal, frontal lobe, and limbic structures in MDD patients were reversed (11). Additionally, increased communication between the left superior temporal gyrus and the right precuneus was reported in remitted MDD relative to current MDD (12). In this way, abnormal SC with the temporal lobes seems to show a state-dependent character. These previous studies revealed the state-independent and -dependent SC in MDD to some extent. However, these conclusions are inconvergent, and most of them are based on cross-sectional studies. Only a few prospective follow-up studies examined the course of SC changes in MDD with a 2-month antidepressant treatment or a short-time electroconvulsive therapy (5, 11). Besides, these few studies had a small sample size and showed a lack of HCs or a lack of controlling medication. Therefore, large-scale follow-up studies with a more extended period are needed to overcome these shortcomings.

To pinpoint the state-independent and -dependent SC abnormalities in MDD patients clearly, a group of MDD patients without medication for at least 2 weeks at baseline were recruited in this study. Patients were treated with paroxetine during the 6-month follow-up, and they received a DTI scan respectively at baseline and the sixth-month follow-up. Drawing on the findings of former studies, we hypothesized that the insula-related SC abnormalities may be state-independent, and the temporal-related SC abnormalities may be state-dependent.

METHODS AND MATERIALS

Subjects and Design

A total of 107 depressed patients were recruited at the outpatient or inpatient departments of Zhumadian Psychiatric Hospital, Henan province, China. Both MDD patients and 123 demographically matched HCs underwent the MRI scan at baseline. The frequent inclusion criteria for the MDD group and the HC group were: (1) right-handed; (2) 18–55 years old; (3) education $\geq$6 years; Additional inclusion criteria for the MDD group were: (1) diagnosis of a current major depressive episode by an attending psychiatrist using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders-IV (SCID); (2) scored more than 20 on the Hamilton Depression Rating Scale (HAM-D_{24}); (3) no psychotropic drugs for at least 2 weeks (fluoxetine for 6 weeks) before inclusion. Inclusion criteria for HC group: (1) scored less than eight on the HAM-D_{24}; (2) no history of mental disorders. Exclusion criteria for both groups included a history of severe somatic diseases, pregnant or breast breeding women, having used medications for thyroid diseases, glucocorticoids, or anticoagulants (heparin, warfarin, etc.) in the past 3 months, abnormal urine toxicology or thyroid screening results, and current or past alcohol or substance abuse or dependence. Subjects with psychiatric diagnoses other than depression were excluded.

After baseline assessment, patients received a 6-month paroxetine treatment. In the first week, patients received paroxetine 10 mg/day (d), the minimum dose for the study. In the second week, patients received paroxetine 20 mg/d or higher doses depending on clinical symptoms, side effects, and clinical antidepressant effects. The maximum dose of paroxetine was 60 mg/d. Depressive symptoms were assessed with the HAM-D_{24} per month for the next 6 months. Patients received other clinical assessments and a second scan at the endpoint of the sixth month. Of the initial 107 patients, one was not scanned, and seven experienced manic onsets in the follow-up period. In consequence, 99 patients were included for baseline analysis. During follow-up, seven patients received other antidepressant medications or electroconvulsive therapy, 24 patients did not continue to participate as some uncontrollable factors, and 68 patients finished 6-month treatment and underwent a second scan. A flow chart was shown in **Figure 1** to introduce the patients' information in detail after inclusion, exclusion, medications, and MRI scanning. Patients who scored less than eight on HAM-D_{24} for at least two consecutive months and persisted till the end of the 6 months of follow-up were examined clinically remitted. After treatment, 59 patients were considered

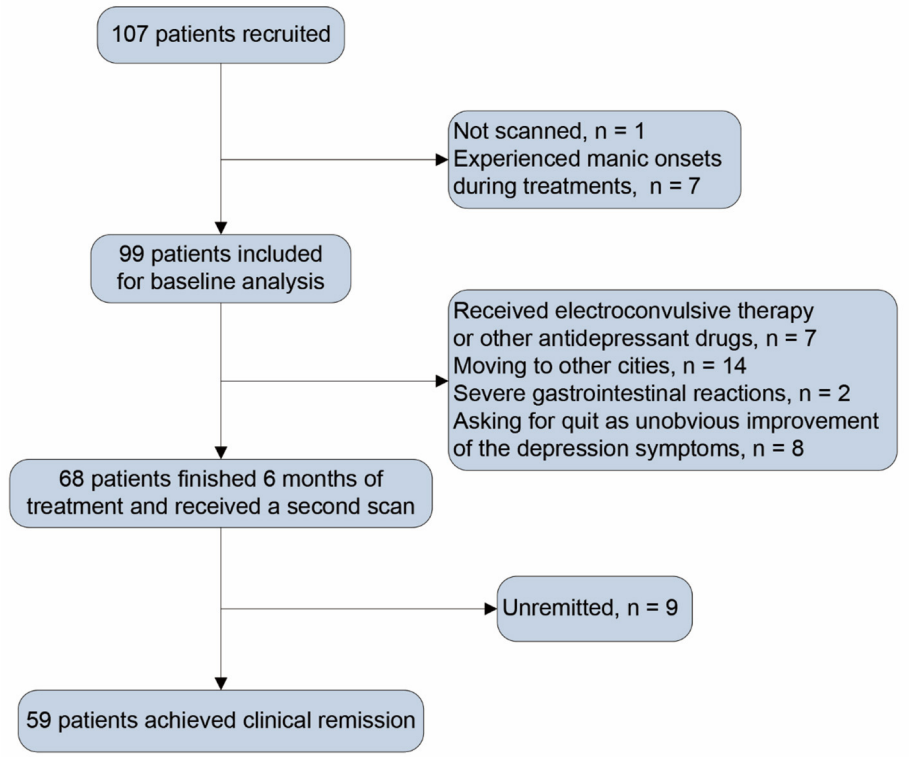

FIGURE 1 | Flow chart.

clinically remitted (remitted MDD subgroup), and nine failed to achieve clinical remission. Due to the small sample size of the non-remitters, there was a lack of sufficient powers while comparing the non-remitters with other groups. We thus focused on analyzing the SC changes in remitted MDD subgroup.

This longitudinal investigation was approved by the Ethics Committee of Zhumadian Psychiatric Hospital and the Ethics Committee the Second Xiangya Hospital of Central South University, and written informed consent was obtained from all subjects.

Image Acquisition and Preprocessing

DTI data were collected using a 3T Signa HDxt scanner. Diffusion tensor images were acquired adopting a single-shot echo-planar imaging sequence (EPI), 32 non-collinear directions ($b = 1{,}000$ s/mm^2), one non-diffusion weighted volume ($b = 0$ s/mm^2), TR = 13,000 ms; slice thickness = 3 mm, FOV = 256 × 256 mm, 128 × 128 matrix, NEX = 1, and 50 gap-free transverse slices covering the whole brain. We also obtained high-resolution 3D structural images using a T1-weighted BRAVO sequence, TR = 6.8 ms, slice thickness = 1 mm, slice gap = 0 mm, flip angle = 9°, TE = 2.5 ms, turnover time (TI) = 1,100 ms, FOV = 256 × 256 mm, 256 × 256 matrix, NEX = 1. The 3D brain image of each subject contains 192 consecutive sagittal slices.

All DTI data were processed with the PANDA package based on FSL (FMRIB's Software Library, http://www.fmrib.ox.ac.uk/fsl) (13).

The DTI data were preprocessed by the following four steps: (1) eddy current and motion artifact correction; (2) diffusion tensor estimation, (3) fractional anisotropy (FA) calculation, and (4) diffusion tensor tractography. The eddy current distortions and motion artifacts were corrected by applying an affine alignment of each diffusion-weighted image to the non-diffusion weighted image. After this step was completed, the DTI elements were evaluated by solving the Stejskal and Tanner equation. Next, three eigenvalues and eigenvectors were acquired by diagonalizing the reconstructed tensor matrix. And then according to the three eigenvalues, the FA value of each voxel was calculated. The tractography was performed using the "fiber assignment by continuous tracking (FACT)" method (14) in the last step. All the tracts in the dataset were calculated by seeding per voxel with a FA $\geq$ 0.2. The tractography was terminated if it turned an angle $\leq 45^\circ$ or reached a voxel with a FA $\leq$ 0.2 (14). The tractography was implemented in each participator to generate three-dimensional curves representing the connectivity of fiber bundle (15, 16). Tens of thousands of streamlines were drawn, etching out the main white matter fiber tracts.

SC Network Constructions

Network Nodes Definition

The procedure for defining nodes has been formerly described (17). Here, we adopted a functional parcellation of the human cerebral cortex and striatum (18, 19), which had been used in previous studies (20–22). The parcellation divided the cerebral

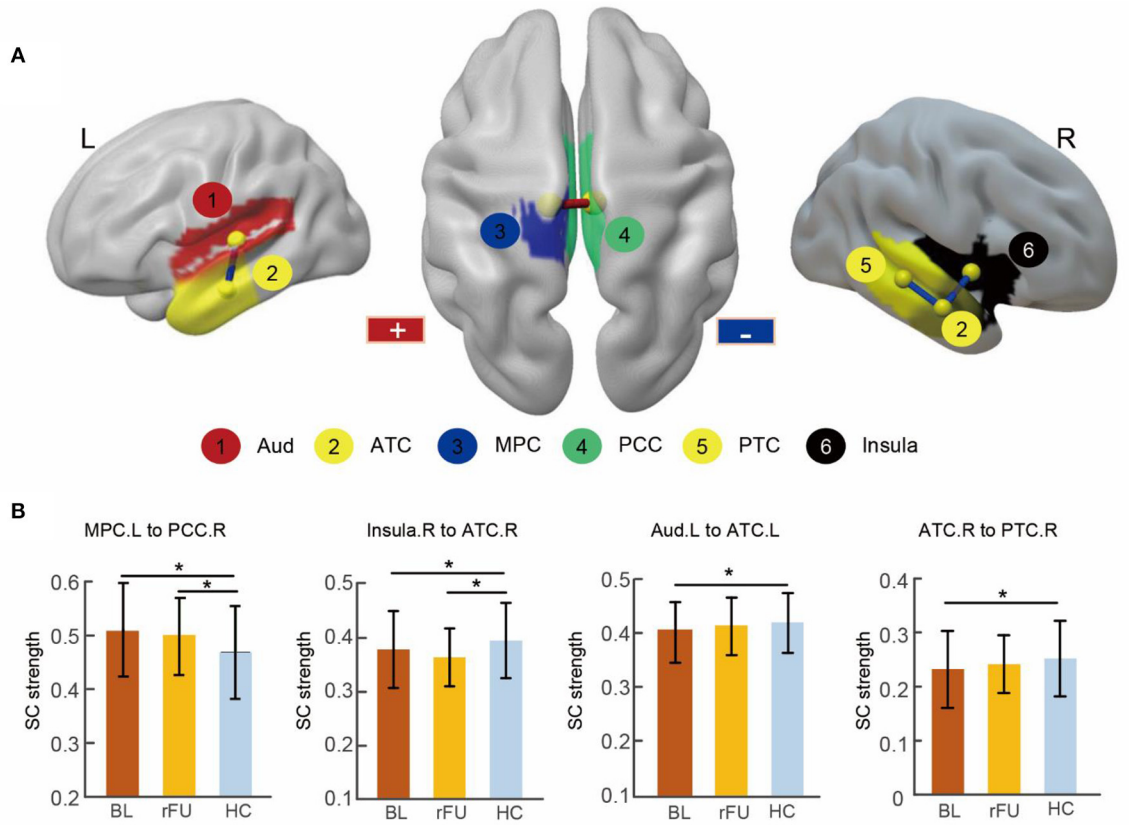

FIGURE 2 | Abnormal SC in MDD patients at baseline and remitted patients at follow-up. **(A)** Three-dimensional representations of the abnormal connectivity in patients at baseline. The regions were mapped onto the cortical surface at a lateral and superior view. The red line represents increased SC in patients at baseline, blue lines represent decreased SC in patients at baseline. **(B)** The bar figures showed the connectivity strengths across the three groups. * Two-sample *t*-tests ($p < 0.05$, FDR corrected). BL, MDD patients at baseline; rFU, remitted MDD subgroup at follow-up; HC, healthy control group; L, left; R, right; Aud, auditory cortex; ATC, anterior temporal cortex; MPC, medial parietal cortex; PCC, posterior cingulate cortex; PTC, posterior temporal cortex.

cortex and divided striatum into 132 regions totally according to the 17-functional network parcellation of the human brain (not including the cerebellar regions). These regions were applied to represent nodes in the SC networks. The list of these brain regions of the SC network was shown in **Supplementary Table 1**. The original parcellation was in the Montreal Neurological Institute (MNI) space. To establish the nodes of the SC network in each participant, regions should be defined in the native diffusion space (23). In short, the T1-weighted images of a subject were coregistered to the b0 image in the native diffusion space. And the converted T1 images were converted into the ICBM152 T1 image in the MNI space nonlinearly. And then the inverse transformations were applied to the above parcellation. In this way, we obtained subject-specific parcellation of the brain, each region representing a node in the network in the native diffusion space.

SC Definition

To define the network edge across each pair of the 132 regions, the average FA value of all voxels connected by tracts between each pair of areas was calculated. We set the average FA value of the connected fibers between two areas as the weight of the network edge. To reduce the potential impact of data acquisition and pre-/post-processing on noise or other factors during diffusion tractography, we took two regions as structurally connected if the number of streamlines between two regions was >3 (17, 24). The threshold selection decreased the risk of false-positive connectivity owing to the limitations or noise in the tractography. Consequently, a FA-weighted SC network for each subject was constructed, a sparse and symmetric 132 × 132 matrix.

Statistical Analyses

One sample *t*-test was performed to extract SCs that met a significant level in HCs and MDD patients at baseline. The identified connectivity within either MDD patients or HCs were selected as connectivity of interest (COI) in the following two-sample *t*-tests.

For each connectivity in the COI, a two-sample *t*-test was conducted to explore significantly different connectivity between MDD patients at baseline and HCs. With the abnormal SCs in MDD as masks, two-sample *t*-tests were accomplished between the remitted MDD subgroup at follow-up and HCs. And

TABLE 1 | Demographic and clinical characteristics by groups.

Characteristics	BL (Mean ± SD)	HC (Mean ± SD)	rFU (Mean ± SD)	BL vs. HC	rFU vs. HC
[a]Age (years)	34.17 ± 8.79	35.01 ± 8.86	35.42 ± 9.15	$p = 0.49$	$p = 0.77$
[b]Gender (F/M)	57/42	65/58	35/24	$p = 0.71$	$p = 0.59$
[a]Education (years)	10.54 ± 3.40	10.65 ± 3.25	10.78 ± 3.48	$p = 0.80$	$p = 0.81$
Onset age	32 ± 9.08	–	33 ± 8.59	–	–
Total illness length (months)	42 ± 52.30	–	51 ± 60.95		
[a]HAM-D_{24}	31 ± 7.55	1.66 ± 1.94	2.42 ± 2.39	$p < 0.001$	$p = 0.061$
[a]HAMA	19.03 ± 6.67	–	2 ± 2		

BL, MDD patients at baseline; rFU; remitted MDD subgroup at follow-up; HC, healthy control group. vs., versus; SD, standard deviation. HAMA, Hamilton anxiety scale.
[a]p-values were acquired by two-sample t-tests.
[b]p-values were acquired by chi-square tests.

paired *t*-tests were implemented to compare the differences in the remitted and unremitted MDD subgroup before and after treatment. Two-sample *t*-tests were accomplished between the patients who finished the 6-month follow-up procedure and the drop-outs at baseline. The abnormal connectivity was shown with the Surf Ice tool (https://www.nitrc.org/projects/surfice/).

Pearson correlation analyses were conducted to explore the relationship between HAM-D_{24} scores and the abnormal SCs in the MDD group at baseline.

Validation Analyses

To validate the results with different parcellation schemes, we re-analyzed the data using nodes defined in the Automated Anatomical Labeling (AAL) parcellation. The AAL parcellation (25) has been widely applied to assess SC alteration in MDD (26). The FA-weighted values between each pair of regions were defined as the edges.

To validate the results with different definitions of the network edge, we re-analyzed the data by defining the normalized number of fibers as the weight of the network edge. As the number of fibers tracts between regions of interest (ROI) i and ROI j of each individual ($N_{i,j}$) was obtained from the native diffusion space, the number of fibers were normalized by the sum of surfaces of ROI i and ROI j, that is $2N_{i,j}/(S_i + S_j)$ as the weight of each edge (27). S_i and S_j are two-dimension intersect of the individual's white matter with the parcellation ROI_i and ROI_j, respectively.

RESULTS

Demographic and Clinical Details of the MDD Group and HC Group

The demographic and clinical details of the MDD group at baseline, HC group, and remitted MDD subgroup at follow-up were shown in **Table 1**. Demographic, clinical measures were presented as mean ± SD. The MDD group at baseline and the HC group did not significantly differ on age, gender, and years of education. The remitted MDD subgroup and HC group also did not significantly differ on age, gender, and years of education.

SC Abnormalities in MDD Patients at Baseline

Three hundred and fifty-four connectivity in HCs and 378 connectivity in patients at baseline survived in one-sample *t*-tests ($p < 0.05$, FDR corrected). The average number of fibers tracts between different nodes of each survived connectivity in HCs and MDD patients at baseline was shown in **Supplementary Figure 1**. COI was then defined as the union of these connectivities, which consist of 378 connectivity.

Two-sample *t*-tests ($p < 0.05$, FDR corrected) revealed that four connections were significantly different in MDD patients at baseline when compared with HCs, including decreased connectivity between the right insula and the right anterior temporal cortex (ATC), between the right ATC and posterior temporal cortex (PTC), between the left ATC and the auditory cortex as well as increased connectivity between the right posterior cingulate cortex (PCC) and the left medial parietal cortex (MPC). These altered connections were shown in **Figure 2** and **Table 2**.

SC Abnormalities in the Remitted MDD Subgroup at Follow-Up

There was no significant difference between patients who finished the 6-month follow-up procedure and the drop-outs at baseline ($p < 0.05$, FDR corrected).

After a 6-month treatment, two-sample *t*-tests conducted between remitted MDD subgroup at follow-up and HC group revealed decreased connectivity between the right insula and the right ATC and increased connectivity between the right PCC and the left MPC in remitted patients ($p < 0.05$, FDR corrected; **Table 2**).

Changes of SC Abnormalities in the Remitted and Unremitted MDD Subgroups Before and After Treatment

Paired *t*-tests revealed that there was no significant difference before and after treatment in remitter or non-remitters ($p < 0.05$, FDR corrected; **Table 2**).

TABLE 2 | Structural connectivity abnormalities in patients at baseline and in remitted patients at follow-up.

Connectivity		Connectivity strength (Mean ± SD)			*p*-value		
Region 1	**Region 2**	**BL**	**rFU**	**HC**	***BL vs. HC**	***rFU vs. HC**	**$^{\Delta}$rBL vs. rFU**
Ins.R	ATC.R	0.37 ± 0.07	0.36 ± 0.06	0.39 ± 0.07	0.044	0.004	0.105
PCC.R	MPC. L	0.51 ± 0.08	0.50 ± 0.07	0.47 ± 0.09	<0.001	0.023	0.171
ATC.R	PTC.R	0.23 ± 0.06	0.24 ± 0.06	0.25 ± 0.07	0.022	0.336	0.348
ATC.L	Aud.L	0.40 ± 0.06	0.42 ± 0.05	0.42 ± 0.06	0.014	0.489	0.112

**Two-sample t-tests, $^{\Delta}$Paired t-tests ($p < 0.05$, FDR-correction).*

BL, the MDD patients at baseline; rFU, remitted MDD subgroup at follow-up; rBL, remitted MDD subgroup at baseline; HC, healthy control group; vs., versus; SD, standard deviation; L, Left; R, right; ATC, Anterior temporal cortex; Aud, Auditory cortex; MPC, medial parietal cortex; PTC, posterior temporal cortex; Ins, Insula; PCC, posterior Cingulate cortex.

TABLE 3 | Association between the HAM-D_{24} scores and four abnormal SCs in patients with MDD at baseline.

Connectivity		*r*	*p*-value
Region 1	**Region 2**		
Ins.R	ATC.R	0.03	0.77
PCC.R	MPC. L	0.08	0.32
ATC.R	PTC.R	−0.09	0.3
ATC.L	Aud.L	−0.12	0.17

L, Left; R, right; ATC, Anterior temporal cortex; Aud, Auditory cortex; MPC, medial parietal cortex; PTC, posterior temporal cortex; Ins, Insula; PCC, posterior Cingulate cortex.

Correlations

There was no significant correlation between HAM-D_{24} scores and four abnormal SCs in the MDD group at baseline (**Table 3**).

Validation Results

The results using AAL parcellation revealed that there was no significant SC alteration in MDD patients at baseline compared with HCs at the whole-brain level. Based on the results using 17-functional network parcellation, we further examined the SC alteration between right insula and the right temporal lobe and between the right PCC and the left PCC and the bilateral precuneus (**Supplementary Table 2**). Detailed analyses and results were described in the **Supplementary Materials**. The results were in line with the results obtained from 17-functional network parcellation.

The results using the normalized number of fibers as the weight of the network edge revealed that there was no significant SC alteration in the MDD patients after treatment, while significant SC alterations were found both in MDD patients at baseline and in the remitted MDD subgroup at follow-up ($p < 0.05$, FDR-corrected, **Supplementary Table 3**). The results were comparable with those of the FA-weighted network analyses (Details of the results were described in the **Supplementary Materials**).

DISCUSSION

This longitudinal work examined SC changes during 6 months of antidepressant treatment in a large sample of MDD patients. We found four abnormal structural connections in depressed individuals at baseline as compared to HCs, which mainly distributed in the bilateral temporal lobe, the right insula, the left MPC, the PCC. After 6-month antidepressant treatment, decreased connectivity between the right insula and the right ATC, and increased connectivity between the right PCC and the left MPC persisted in remitted patients, showing a state-independent character. In contrast, the decreased connectivity between the right ATC and the PTC and between the left temporal cortex and the auditory cortex were reversed at remission, showing a state-dependent character.

The right insula usually co-activates with limbic cortices (temporal pole, amygdala), which are associated with social cognition, attention (28). A previous task-related study reported the activation of the anterior insula during processing emotional stimuli, particularly negative stimuli (29). Similarly, another task-related study reported abnormal activation of the temporal lobe during emotional processing tasks in patients with MDD compared to HCs (30). Furthermore, the presence of attentional biases to negative stimuli in MDD was reported (31). Besides, both current and remitted MDD patients selectively attended to and remembered sad faces with happy faces filtered out, when presented happy or sad faces paired with emotionally neutral faces in a dot-probe task (32), suggesting a state-independent character of attentional bias in MDD. SC reduction of the insula in acute MDD, even in remitted MDD, had been reported in previous studies (3–6). Also, FA reduction in insula has been reported in our earlier study in patients with MDD from the acute episode to remission and was regarded as a state-independent character (33). In the present study, we found that decreased SC of the insula retained from the episode phrase to the remission in MDD patients after 6-month treatment, which provides ampler evidence for the state-independent character of insula. Moreover, no correlation was found between the SC changes of the right insula to the right ATC and the HAM-D_{24} scores in the MDD group at baseline. The result indicates that alterations of the SC values are free of clinical status, further implying decreased SC between the right insula and the right ATC is a state-independent character for MDD. Taking this imaging evidence and state-independent character of attentional bias together, we speculate that the sustained decreased structural connection between the right insula and the anterior temporal lobe might underlie the neural basis of negative attentional biases in MDD patients.

However, more task-related MRI studies of emotional attention bias are needed in the future to elucidate this speculation. Additionally, clinically remitted MDD patients with decreased insula-related SC by MRI scans are likely to be recurrent and require longer antidepressant treatment (34). Besides, the previous study had suggested that the activation of insula can be increased by transcutaneous vagus nerve stimulation (tVNS), so it may be a future direction to develop physical therapy (e.g., tVNS) for insula or its related brain regions (35).

The PCC connected to intrinsic control networks extensively. Increased activity in PCC had been observed in many cases of internally directed attention, such as episodic memory retrieval, daydreaming, and planning (36). Liberg et al. found that the PCC and the posterior MPC were involved in pre-executive motor production during a motor imagery task (37). Pre-executive motor production is a part of the production of movement, including selection, planning, and preparation (38). Motor imagery, rather than performing it openly, is the psychological rehearsal of a specific action. Prior investigations have reported that motor imagery is functionally equivalent to the pre-executive stages of explicit motion (39–41). Liberg et al. further found that the activities in the PCC and the posterior MPC were altered during the pre-executive stages of motor generation in bipolar depression showing psychomotor retardation. Psychomotor retardation is an essential dimension of symptoms in depression. It is manifested as a general slowdown in movement, sagging posture, reduced facial expressions, slower speed, and lowered speech tone. Persisting psychomotor retardation in the remission phase of MDD has been reported, which is independent of the clinical status (42, 43). Moreover, no significant correlation was found between the SC changes of the right PCC to the left MPC and the HAM-D_{24} scores in the MDD group at baseline. The result indicates that alterations of the SC values are also free of clinical status, further implying increased SC between the right PCC and the left MPC is a state-independent character for MDD. Thus, it is reasonable to deduce that the abnormal SC between the right PCC and the left MPC in remitted MDD may contribute to the residual psychomotor retardation symptoms in the remission phase through mediating the pre-executive motor production.

A previous DTI study found that patients with psychosis showed FA reduction affecting fronto-limbic white matter and associative, projective, and commissural fasciculi in the acute phase and showed FA increase over time after symptom remission (44). Another DTI study found that patients with bipolar I disorder (BD-I) showed reduced FA in the right superior, and inferior longitudinal fasciculi and inferior fronto-occipital relative to HCs and remitted BD-I patients, while remitted BD-I patients are not different from HCs in FA (45). The alterations of microstructural white matter from the acute phase to remission were regarded as state-dependent. In the present study, we found that the reduced connections between the right ATC and the PTC and between the left ATC and the auditory cortex were reversed at remission during a 6-month treatment, showing a state-dependent character. Both structural and functional abnormalities in the temporal lobe show high discriminative power in distinguishing MDD patients from HCs (12). Besides, decreased nodal efficiency and thinner cortical gray matter in the temporal lobe have been found in MDD compared with HCs in cross-sectional studies (10, 46). Whereas, thicker cortical thickness in the temporal cortex in remitters than in non-remitters (47), and normalization of the temporal cortex activity with treatment (48) were found. Also, higher FA was detected in the temporal (superior, middle, and fusiform) regions in remitters relative to the non-remitters (49). We found that the reduced SCs of temporal regions are reversed over time after clinical remission in the study, which provides direct evidence for the state-dependent character of temporal regions. Moreover, the auditory cortex is part of the temporal lobe, processing auditory information in human beings. The temporal lobe, together with other regions like the amygdala, played a significant role in emotional processing as well as social cognition (50), which is a well-established region that underpins the pathophysiology of depression (51). Thus, the abnormality of SC between the left ATC and the left auditory cortex, between the right ATC and the PTC may underlie the emotional symptoms in MDD, which seem to be state-dependent. Notably, there was no significant correlation between temporal-related SC alterations and $HAMD_{24}$ scores. Combined with the results that no significant SC alterations were found in the MDD patients after treatment, we are not difficult to infer that the reversion of abnormal SCs is slower than that of depressive symptoms.

This study has a relatively large sample size. However, several limitations should be noted. First, due to some unavoidable reasons (such as migrant workers, severe gastrointestinal reactions, etc.), we lost contact with some of the MDD patients in the follow-up. But patients who finished the 6-month follow-up procedure represent the patients in the entire MDD group at baseline. Thus, the drop-outs at follow-up would not significantly influence our main results. Second, we just collected the MRI scans at two-time points, so we are unable to know more detailed information about the trajectory of SC changes from acute depression to remission. Third, the depressed patients were followed for only 6 months, so it is unable to collect the information of recurrence in the broader period. We are unable to examine whether these state-independent SC abnormalities are associated with a higher risk of recurrence. Fourth, only nine patients did not remit after 6-month treatment during follow-up. Due to the insufficient non-remitters, analyses of this subgroup are exploratory. Fifth, we only analyzed the DTI data, and the results were not able to explain more abnormality of brain functioning. And the subjects were interviewed before and 6 months after treatment, and patients experienced much more during 6 months between two interviews, which we did not know may influence the results of the study.

With a relatively large sample to date, this longitudinal study investigated the state-independent and -dependent SC alterations in patients with MDD. The results demonstrated that SC abnormalities distributed in the right insula, the left PCC, and the right MPC showed a state-independent character, which may be implicated in the sustained negative attention bias and motor retardation in MDD, In contrast, SC abnormalities within the bilateral temporal lobes showed a state-dependent character, which may be associated with the fluctuating affective symptoms in MDD. To investigate the state-independent and

state-dependent SCs across the course of depression thoroughly, longitudinal studies with longer follow-up time and more intensive time points will be needed in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Second Xiangya Hospital of Central South University and Zhumadian Psychiatric Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YF analyzed the data, wrote, submitted, and revised the manuscript. QD, XL, JSun, LZ, and MW collected data. JSu, LP, HS, JR, JL, and BL revised the manuscript. L-LZ, DH, and LL conceptualized the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.568717/full#supplementary-material

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REVIEW
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The Psychobiology of Bereavement and Health: A Conceptual Review From the Perspective of Social Signal Transduction Theory of Depression

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Losing a spouse is considered one of the most stressful life events a person can experience. Particularly in the immediate weeks and months after the loss, bereavement is associated with a significantly increased risk of morbidity and mortality. Despite an abundance of research aimed at identifying risk factors for adverse health outcomes following marital death, the mechanisms through which mental and physical health problems emerge following bereavement remain poorly understood. To address this issue, the present review examines several pathways that may link bereavement and health, including inflammation and immune dysregulation, genetic and epigenetic changes, gut microbiota activity, and biological aging. We then describe how these processes may be viewed from the perspective of the Social Signal Transduction Theory of Depression to provide a novel framework for understanding individual differences in long-term trajectories of adjustment to interpersonal loss. Finally, we discuss several avenues for future research on psychobiological mechanisms linking bereavement with mental and physical health outcomes.

Keywords: bereavement, interpersonal loss, life stress, inflammation, immune system, biological aging, health, disease

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INTRODUCTION

Losing a spouse can be a very stressful life event that places individuals at risk for mental and physical health problems (1, 2). Particularly in the immediate weeks and months following spousal loss, bereavement is associated with increased risk of multimorbidity and mortality (2–5), including an elevation in inflammation-related health problems (6–10), cardiovascular disease (CVD) (8, 11–14), and some types of cancer (12). Despite a large body of research in the trauma literature attempting to identify risk factors for adverse health outcomes following spousal bereavement, however, the mechanisms through which mental and physical health problems emerge following interpersonal loss remain poorly understood.

Over the past 30 years, the field of psychoneuroimmunology has helped elucidate how different life stressors affect autonomic nervous system, neuroendocrine, and immune processes that could in turn be relevant for understanding the psychobiology of bereavement (15, 16). In particular, the past decade has produced a substantial body of knowledge shedding light on how specific types of stressors can trigger increases in inflammation (15, 17–21), which has in turn been linked with the development of numerous disease conditions, including autoimmune disorders, CVD, and some

cancers, as well as mortality (12). Consequently, bereavement-related dysregulation in immune function may be one potential process that underlies the increased risk for morbidity and mortality seen in spousal bereaved individuals (22).

One strategy for better understanding the psychobiology of bereavement involves applying what we know about depression, which is also strongly precipitated by interpersonal loss. One model in particular, the Social Signal Transduction Theory of Depression (20), may be helpful for shedding light on how spousal loss and grief affect neural and immune processes that in turn structure risk for health problems following bereavement. In brief, this theory suggests that both early and later-life stress can promote neuro-inflammatory sensitivity to subsequently occurring stressors and thus heighten a person's vulnerability to physical and mental health problems across the lifespan. In the context of bereavement, this would occur if an individual with a history of past life stress exposure lost a terminally-ill spouse in adulthood after a sustained period of caregiving burden, in turn leading to mental and physical health problems that have an inflammatory basis.

Recently, Knowles et al. (23) published an excellent systematic review that focused on the link between bereavement and immune system functioning. The present review also examines links between bereavement and immune functioning but seeks to go beyond prior work by providing an integrated account of psychosocial, neural, immunologic, and genomic processes linking bereavement and health, as well as a description of how cumulative lifetime stress exposure may alter vulnerability to mental and physical health problems following spousal loss. To accomplish this goal, we conducted a PubMed literature search of all relevant studies published through October 2019 using the following key words: bereavement, mental health, physical health, psychobiology, stress, genetic, epigenetic, neuroendocrine, neuroimmune, inflammation, and immunity. To be considered for this review, articles had to be peer reviewed and written in English (see **Supplementary Table 1**). The psychometric instruments used in the eligible studies are summarized in **Table 1**.

TABLE 1 | Assessment instruments used in the studies evaluated.

Abbreviation	Full name
ATQ-P	Automatic Thoughts Questionnaire-Positive version
CSS	Crisis Support Scale
CTQ	Childhood Trauma Questionnaire
ECR-SF	Experiences in Close Relationships Questionnaire-Short Form
GMRI	Grief and Meaning Reconstruction Inventory
GMS	Grief Measurement Scale
HAMD	Hamilton Anxiety and Depression Scale
HRSD	Hamilton Rating Scale for Depression
HTQ	Harvard Trauma Questionnaire-Part IV
ICG	Inventory of Complicated Grief
ICG-R	Inventory of Complicated Grief-Revised
IES	Impact of Event Scale
LOT	Life Orientation Test
MCMI-III	Millon Clinical Multiaxial Inventory-III
PSS	Perceived Stress Scale
PERI-A	Psychiatric Epidemiology Research Interview-Anxiety Scale
PERI-H	Psychiatric Epidemiology Research Interview-Helplessness-Hopelessness Scale
PFQ-2	Personal Feelings Questionnaire-2
PG-13	Prolonged Grief 13 Items
PSOM	Positive States of Mind
PSQI	Pittsburgh Sleep Quality Index
RPT	Relationship Profile Test
SADS-L	Lifetime Version of the Schedule for Affective Disorders and Schizophrenia
SCID-I	Structured Clinical Interview for DSM-IV Axis I Disorders
SCL-90	Symptom Checklist-90
SF-36	36-Item Short Form Health Survey
SSI	Beck Scale for Suicidal Ideation
STRAIN	Stress and Adversity Inventory
TIPI	Ten-Item Personality Inventory
TSC	Trauma Symptom Checklist
TLEQ	Traumatic Life Events Questionnaire
YES	Yale Evaluation of Suicidality scale

SOCIAL SIGNAL TRANSDUCTION THEORY OF DEPRESSION

Stressful life events, especially those involving interpersonal loss, are known to activate several autonomic, neuroendocrine, and neuroimmune pathways that can lead to increased inflammatory activity (16), which, if sustained, can increase risk for inflammation-related physical and mental health problems (20, 24, 25). Indeed, dysregulation of the immune system is a critical processes involved in the pathophysiology of various diseases, including infections, autoimmune diseases, CVD, and some cancers (19, 26, 27). In addition, inflammatory activity can negatively affect mental health, and has been found to play a role in the development of anxiety disorders, post-traumatic stress disorder (PTSD), and depression (26). In this context, the cytokines interleukin (IL)-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) have been shown to be both upregulated by interpersonal life stress and associated with poor health (17, 28–32).

One lingering question in this context has always been how exactly stressful experiences like interpersonal loss affect neurocognitive processes that in turn increase inflammation and inflammation-related disease risk. This important question is addressed by the Social Signal Transduction Theory of Depression (20, 33), which is a multilevel theory that describes neural, physiologic, molecular, and genomic pathways that link interpersonal stress exposure with internal biological processes that increase risk for depression and depression-related health problems. More specifically, this theory hypothesizes that cumulative lifetime stress exposure, which encompasses both acute negative life events (e.g., interpersonal loss) and chronic difficulties, can increase inflammatory activity through the activation of the autonomic nervous system

(ANS), hypothalamic-pituitary-adrenal (HPA), and systemic inflammatory response. Although the temporary engagement of these systems is critical for survival during times of actual social or physical threat, sustained activation can occur that increase a person's vulnerability to physical and mental health problems that have an inflammatory component (20, 24, 25, 34, 35).

Consistent with this theory, research has shown that genetic factors, epigenetic processes, personality traits (e.g., neuroticism), and social environmental conditions during childhood and adolescence (e.g., social/financial stress, uncertainty, abuse, or neglect) play a role in shaping individuals' sensitivity to later-occurring adverse life events (25, 36). At the molecular level, epigenetic regulation of gene transcription can play an important role in helping individuals adapt to challenges posed by the external social environmental (37). However, stress-induced epigenetic changes can also lead to persistent increases in social stress-related physiological reactivity that last for months or years (38). In sum, therefore, social-environmental and genetic processes can independently and interactively affect the likelihood that a particular adverse life event will get converted into changes in gene expression that have the ability to influence health (38, 39).

A growing body of research is showing that the gut microbiota may also play an important role in shaping the activity of the enteric nervous system, ANS, neuroendocrine pathways, and immune system (40). This research has shown that the signaling pathways linking the central nervous system and gut are sensitive to social-environmental factors (41). Moreover, disruption of these systems can result in altered gastrointestinal function, HPA-axis activation, changes in immune responses, and, therefore, increased inflammation-related behavioral changes and disease susceptibility (42, 43).

In sum, the Social Signal Transduction Theory of Depression provides one illustration of how interpersonal loss can lead to specific changes in sympathetic nervous system (SNS) and HPA-axis activity that interact with genetic, personality, and social-environmental factors to promote immune dysregulation and increased inflammatory activity, especially in vulnerable individuals. These biological changes can in turn lead to depression-like behaviors, including anhedonia, helplessness, social withdrawal, and fatigue (26), which are characteristic of some bereaved individuals, thus making the Social Signal Transduction Theory of Depression a potentially useful framework for understanding psychosocial and biological aspects of bereavement (see **Figure 1**).

PSYCHOBIOLOGY OF BEREAVEMENT FROM THE PERSPECTIVE OF SOCIAL SIGNAL TRANSDUCTION THEORY OF DEPRESSION

For the purpose of this review, the term grief and bereavement have been conceptualized with slightly different meanings. Whereas, grief refers to a person's emotional response to a loss, bereavement refers to the time period when an individual experiences sadness, grief and mourning after a significant loss. Typically, bereavement is the period during which time the most intensive grieving occurs (44).

The loss of a significant person in one's life is a unique social stressor that requires an individual to adapt, which differentiates it from other stressors such as caregiving, a conflictual marital relationship, or unemployment (45). To better understand psychological adjustment to significant interpersonal loss, research has employed concepts from the literatures on attachment theory, cognitive processing, and resilience. Early life attachment has been proposed to impact relationships in adulthood (46). In this regard, the loss of a spouse represents an attachment stressor that inherently has physiological effects, as attachment stress evolutionarily served to maintain proximity between bonded pairs (23).

As a highly stressful event, the death of a significant person is known to trigger biological responses via several autonomic, neuroendocrine, and inflammatory pathways (22). These responses, either directly or via interaction with the social environment, can cause alterations in biological functioning that include the onset of chronic low-grade inflammation, which can increase risk for sickness behaviors, infections, mental and physical health problems, and premature mortality in vulnerable individuals (26, 47–49). Consistent with this thinking, several lines of evidence indicate that stressful life events are strongly associated with altered immune function and the development of depression, especially for persons living in a high-risk environment (e.g., trauma exposure) who possess a genetic predisposition to depression (20, 50–52). Moreover, clinical studies have shown that depressive symptoms are prevalent in widows and widowers during the first 2 years of bereavement, with a particular high risk for individuals with a history of depression (53, 54). To the extent that grief and depression are precipitated most strongly by the same types of major life stressors (i.e., sudden interpersonal loss), it may be possible to apply what we know about the psychobiology of depression (e.g., using the Social Signal Transduction Theory of Depression) to better understand the psychobiology of bereavement.

Importantly, the grief response depends on the nature and quality of the lost relationship (55). For example, the unintentional death of a spouse will be experienced differently than the death of a child, parent, or sibling, or the death of a close other by suicide (56). Furthermore, the unexpected death of a loved one may trigger different grief reactions than the loss of a spouse due to a terminal disease (56, 57). Hence, the psychobiology of bereavement may differ depending on the specific types of loss experienced (58). In the present article, therefore, we focus specifically on spousal bereavement as a particular type of life stressor.

In the following sections, we examine the applicability of the Social Signal Transduction Theory of Depression for studying psychobiological pathways that may link bereavement with multimorbidity and mortality. To do this, we review available research describing the impact of spousal loss on (a) mental and physical health, (b) inflammation and immune dysregulation, (c) genetic and epigenetic changes, (d) gut microbiota activity, and (e) biological aging. We also describe the effects of cumulative life stress exposure on health in bereaved individuals and explore

psychobiological factors that have been linked to vulnerability and resilience to mental and physical health problems following significant interpersonal loss. Finally, we discuss how Social Signal Transduction Theory of Depression can be extended to bereavement and highlight several avenues of research that may be fruitful to pursue on this topic.

The Impact of Bereavement on Mental Health

As alluded to previously, the death of spouse is one of the most stressful life events a person can experience (59). Spousal bereavement due to cancer, for example, is usually preceded by a long illness trajectory, which is associated with high distress due to the spouse's progressive health deterioration, anticipatory grief about the spouse's inevitable death, adoption of supportive responsibilities, financial stressors, and disruption of the caregiver's social and personal life (60). Moreover, partners of patients who have been suffering from an advanced illness have been found to experience more emotional distress and adjustment problems, as well as greater pain, fatigue, sleep problems, and depression (61–65).

There is considerable variability in how people respond to the loss of a spouse. For example, although the vast majority of conjugally bereaved individuals are relatively resilient and adjust adequately without professional support (66, 67), 10–20% of bereaved spouses develop intense, prolonged grief (67–69). In addition, substantial evidence suggests that high levels of traumatic grief, depression, and anxiety at 6 months post-loss predict the development of even more serious mental and physical health problems up to 25 months post-loss in spousal bereaved individuals, including suicidal ideation, cancer, and heart attacks (70).

Grief is a typical psychological and emotional reaction to losing a significant person that is characterized by symptoms of intense distress, anxiety, yearning, longing, and sadness, all of which usually subside over time (71). Although grieving symptoms are similar in some ways to symptoms of Major Depressive Disorder (MDD), as described by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), these two conditions are classified as distinct constructs (72, 73). Prolonged or persistent grief, by definition, is a debilitating condition following a significant loss that consists of persistent and pervasive longing for, or preoccupation with, the deceased person that persists for 6 months or longer. Moreover, it is characterized by its clinical features, including emotional pain (e.g., sadness, guilt, bitterness, anger), difficulty accepting the loss, emotional numbness, feeling that a part of one died, and difficulties in engaging in social or other activities (69). The diagnostic criteria for prolonged grief reactions are specified in the DSM-5 as Persistent Complex Bereavement Disorder (PCBD) (74) and in the International Statistical Classification of Diseases 11th Revision (ICD-11) as Prolonged Grief Disorder (PGD) (75, 76). PGD can be diagnosed at 6 months and PCBD at 12 months following the loss (77).

Bereavement is associated with short- and long-term health consequences. In one study that examined the early bereavement period (i.e., 6 months post-loss), prolonged grief was found in 12.3% of 56 bereaved adults between 20 and 50 years old who lost their spouse due to cancer (78). In another study of 132 adults, almost 30% of bereaved adults who lost a close relative to cancer met criteria for post-traumatic stress disorders 1 month post-loss (79). Over the long term, complicated grief was found to be present in 24.6% of 668 cancer caregivers 9 months post-loss (80), and 48% of 88 cancer caregivers who suffered from increased levels of bereavement-related distress 3–5 years after the loss (81).

As briefly noted above, high levels of prolonged grief symptoms have been associated with greater disability and can compromise psychological and physical functioning for years, resulting in comorbid health problems. These health problems include impaired sleep (82, 83), depression (9, 84–86), suicidal ideation and attempts (12, 87), and anxiety and PTSD (88, 89), as well as adverse health behaviors (12), prolonged sick leave, and increased health services and medication use (90–92). Furthermore, grief severity assessed from 3 to 6 months post-loss has been found to predict functional impairment, depressive symptoms, and impaired sleep up to 18 months following the loss (86, 93).

Of note, depression, and bereavement share considerable commonalities in terms of disease risk (e.g., CVD) and changes in immune function (2, 6, 11, 94–97). Some researchers have thus suggested that it is not interpersonal loss or bereavement but rather depression that is responsible for causing the increases in morbidity and mortality seen in bereaved individuals (9, 12, 49, 89, 98). On the other hand, though, research has shown that grief and depression are independently associated with health-damaging inflammation (99). Therefore, it may be the case that bereavement and depression are associated with similar but distinct patterns of inflammatory activity, and that interpersonal loss is a common precipitating stressful life event that may lead to both grief and depression.

The Impact of Bereavement on Physical Health

Bereavement has also been found to increase individuals' risk for physical health problems and, especially, immune-related illnesses such as CVD and cancer, in addition to increased mortality risk, within the first 3 years following a major loss (12, 92). Indeed, CVD is a major cause of morbidity and mortality following bereavement (100). In terms of underlying mechanisms, strong evidence has linked bereavement-related stress with increased risk for CVD via chronic low-grade inflammation, as commonly measured by circulating IL-6 or C-reactive protein (CRP) (27, 101, 102). In addition, elevated levels of CRP have been shown to be a strong prognostic factor for atherosclerosis and cardiovascular events (103, 104).

In terms of mortality risk, a meta-analysis of 15 prospective cohort studies and 2,263,888 participants found that relative to their married peers, recently bereaved spouses had a 41% increased risk of dying within the first 6 months following bereavement, independent of age and gender (overall RR 1.41; 95% CI 1.26, 1.57) (105). Another meta-analysis of more than 500 million people found a 23% increased risk of mortality among

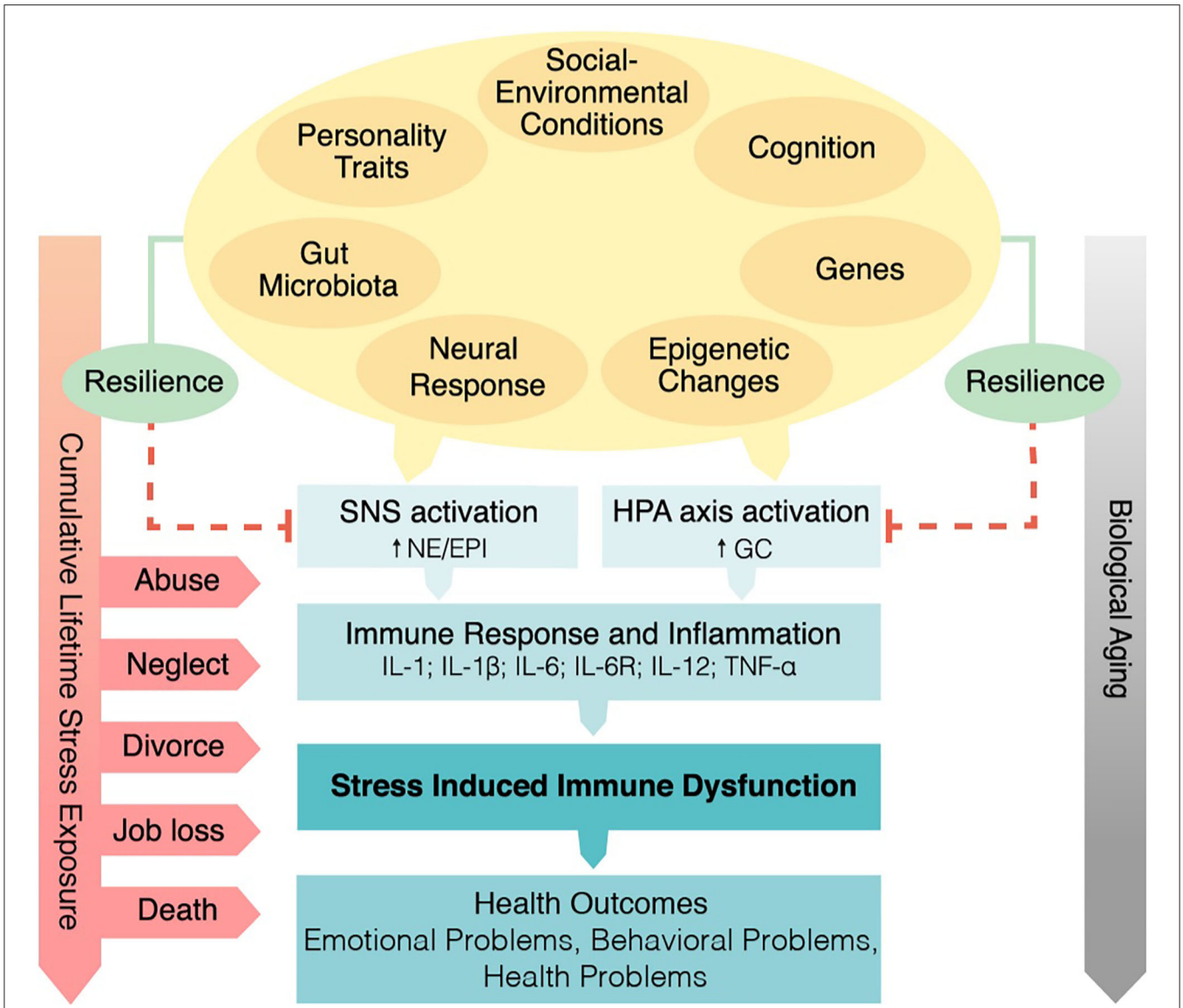

FIGURE 1 | Psychobiological mechanisms linking spousal bereavement and health from the perspective of the Social Signal Transduction Theory of Depression (20). The interplay between genetic factors, personality traits (e.g., neuroticism), and social-environmental conditions during childhood and adolescence (e.g., social/financial stress, uncertainty, abuse, or neglect) are hypothesized to play important roles in shaping individuals' neuro-inflammatory sensitivity to later-occurring life stressors, including interpersonal loss. Exposure to early life stress and subsequent stressors are hypothesized to heighten sympathetic nervous system and hypothalamic-pituitary-adrenal axis dysregulation, and to promote increased inflammatory activity in response to a significant loss. If chronic, elevated inflammatory activity can in turn lead to a variety of adverse emotional, behavioral, and health outcomes. SNS, sympathetic nervous system activation; NE, norepinephrine; EPI, epinephrine; HPA, hypothalamic-pituitary-adrenal; GC, glucocorticoid; IL-1, interleukin-1; IL-1β, interleukin-1beta; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; IL-12, interleukin-12; TNF-α, tumor necrosis factor-α.

widowers as compared to married individuals (HR 1.23; 95% CI 1.19, 1.28), with a relatively higher risk for men (increased risk: 27%) than women (increased risk: 15%) (3). This phenomenon of increased mortality risk for bereaved individuals, specifically within the first 6 months after the interpersonal loss, has been called the "broken-heart phenomenon" (106) or "widowhood effect" (105). Nowadays, these terms have also been used in the context of Takotsubo cardiomyopathy, an acute reversible heart failure syndrome mimicking acute myocardial infarction that is frequently triggered by emotional stress, including loss and grief (107, 108).

Depression and stress are important risk factor for developing CVD, and depression has been associated with poorer coronary outcomes (94). Correspondingly, the death of a significant person is known as a key psychosocial risk factor for CVD (11). Research has shown that as compared to non-bereaved individuals, bereaved individuals exhibit lower heart rate variability and higher heart rate, systolic blood pressure, von Willebrand factor,

factor VIII, and platelet/granulocyte counts (6, 8, 13, 109, 110). These findings have direct clinical relevance, given that prothrombotic changes have been associated with greater risk of CVD and mortality (111, 112). Of note, the adverse effects of stress on cardiovascular and other outcomes are often potentiated by unhealthy behaviors, including poor dietary choices and sleep hygiene, inadequate physical activity, alcohol and/or tobacco consumption, and poor medication adherence (113), all of which are risk factors for CVD in their own right.

Regarding the implications of bereavement for cancer risk, stress hormones, especially glucocorticoids, adrenaline, and noradrenaline, have multiple known effects on human tumor biology (114, 115). Specifically, SNS activation via adrenergic- and glucocorticoid-mediated mechanisms can increase inflammatory activity and alter immune defense mechanisms against tumors with implications for tumor progression (116). Consistent with these pathways, a few studies have suggested that bereavement is associated with increased risk for cancer incidence. For example, a historical study conducted by Prigerson et al. (12) found that bereaved individuals suffering from high levels of grief had a significantly greater risk of developing cancer within 6–25 months post-loss than those with low levels of grief, yet the type of cancer was not described. Additionally, the Pan American Health Organization/World Health Organization examined the effects of parental bereavement on cancer incidence and survival (117). This large cohort study included 6,284 Jewish Israelis who lost an adult son in the Yom Kippur War or in an accident between 1970 and 1977, and who were followed longitudinally for 20 years. There was an increased incidence of lymphatic and hematopoietic malignancies in both war-bereaved parents (OR 1.47) and parents of accident victims (OR 2.01). There also was an increased incidence of melanoma among both war-bereaved parents (OR 1.72) and parents of an accident victim (OR 4.62). Finally, in parents suffering from cancer before the loss, bereavement was associated with a relatively shorter survival time.

These data highlight a possible association between bereavement and cancer. Consistent with this work, data from the psycho-oncology literature suggest that the incidence of cancer recurrence may be higher during bereavement-related distress (114–116, 118–122). On the whole, though, the available evidence linking bereavement and cancer risk is not highly consistent and is certainly limited relative to the relatively large amount of research demonstrating an association between bereavement and CVD risk.

Another condition associated with bereavement is type 2 diabetes, a chronic metabolic disorder characterized by insulin resistance due to insufficient insulin secretion and action (123). A growing body of research indicates that stress plays a role in type 2 diabetes, both as a predictor of new-onset type 2 diabetes and as a prognostic factor for individuals with existing type 2 diabetes. Stress-related biological pathways that are believed to contribute to the pathogenesis of type 2 diabetes include chronic activation of the HPA axis, which can result in neuroendocrine dysfunction and dysregulated cortisol output (123) that leads to glucose intolerance and systemic insulin resistance (124).

Only a limited number of studies have investigated the onset of type 2 diabetes in the context of bereavement. One study reported a 1.4-fold increased risk of incident diabetes in parents who lost their child relative to age- and sex-matched non-bereaved parents (125). Another study found that bereavement-induced prenatal stress in mother increased the risk of insulin resistance by 1.3-fold in their offspring; if grief was due to the death of an older sibling, the risk that the offspring developed type 2 diabetes during childhood or young adulthood was increased by 1.5-fold (126).

Taken together, these data provide relatively strong converging evidence that bereavement is associated with increased risk for poor mental and physical health. In most cases, the mechanisms underlying these bereavement-related health effects remain unclear. To address this issue, in the following sections we review psychoneuroimmunological pathways that may link bereavement with mental and physical health problems. We also discuss how these effects may be understood through the lens of the Social Signal Transduction Theory of Depression.

PSYCHOBIOLOGICAL MECHANISMS LINKING BEREAVEMENT AND HEALTH

Bereavement-Induced Systemic Inflammation and Immune Dysregulation

Neuroendocrine, Sympathetic, and Inflammatory Activation

Several psychological, neural, and immunologic pathways may link bereavement with health. For example, it is well-known that stress activates the HPA axis and sympathetic-adrenal-medullary (SAM) axis, which in turn trigger the release of hormones that modulate immune function. Specific hormones involved in this stress-induced biological cascade include adrenocorticotropic hormone (ACTH), cortisol, growth hormone, prolactin, adrenaline, and noradrenaline (15). Through these mediators, life stress exposure can increase inflammatory activity and reduce anti-viral immune responses (17, 127).

In the context of bereavement, studies have begun to explore bereavement-related alterations in biomarkers of neuroendocrine and immune function. Results from these studies show that within the first 6 months following spousal loss, bereaved individuals exhibit evidence of reduced antibody response to vaccination (128), HPA-axis dysregulation [e.g., as indexed by higher cortisol levels and flatter diurnal cortisol slopes (9, 59, 129–132)], heightened systemic inflammation (6, 10, 49, 99, 133), and impaired immune responses, as indexed by reduced functional activity of natural killer cells (9, 130).

Research has also suggested that these bereavement-related biological alterations, particularly those involving inflammation, may underlie the development of complicated grief. For example, in a recent study of 99 spousally bereaved individuals by Fagundes et al. (99), bereaved individuals with greater grief severity and higher levels of depression showed higher levels of the pro-inflammatory cytokines interferon gamma (INF-γ), IL-6, and TNF-α approximately 3 months after the death of their spouse as compared to those who had more

mild emotional reactions to the loss. Sleep disturbances can also induce inflammation (134). In a study of 54 spousally bereaved and 47 non-bereaved individuals, self-reported sleep disturbance in bereaved individuals was not directly related to elevated inflammatory activity, but bereavement moderated the association between sleep disturbances and inflammation approximately three moths post-loss, after adjusting for depression (133). Therefore, sleep disturbance may be an important pathway linking bereavement with increased inflammation and subsequent health problems.

Mechanistically speaking, the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α are believed to influence the activity of neurotransmitters that can in turn affect mood and induce depressive symptoms in vulnerable individuals (28, 135). Consistent with this understanding, some studies have argued that changes in immune function and related neurochemical processes during bereavement are the result of bereavement-related depression as opposed to experiencing interpersonal loss or bereavement (136). Therefore, additional research is needed to examine the extent to which the increased inflammatory levels sometimes evident during or following bereavement are the result of an intense bereavement period itself vs. increases in depressive symptoms that sometime accompany bereavement.

Immune Dysregulation

Bereavement may also affect health by influencing immunity. Immunity is the natural or acquired resistance of an organism to bacterial or viral invaders, diseases, or infections, while having adequate tolerance to avoid allergy and autoimmune diseases (18). Lymphocytes, including T cells and B cells, as well as natural killer (NK) cells and macrophages, are the main types of cells of the immune system. T cells orchestrate the immune response via the production of cytokines and stimulate B cells to produce antibodies and signal killer cells to destroy antigen-displaying cells (137). Chronic stress, in turn, can induce low-grade inflammation and suppress immuno-protective cell function (18).

Studies investigating immune function in bereaved individuals have demonstrated NK cell activity suppression for up to 6 months following the sudden death of a relative (9, 130), lymphocyte proliferation for up to 2 months following spousal loss (138), and downregulated leukocyte gene expression in individuals who lost their spouse over the prior 2 years as compared to non-bereaved healthy adults (139). Seasonal influenza vaccination provides a useful paradigm to study individual differences in the inflammatory response. In one study that used this immunological challenge model in the context of bereavement, bereaved adults exhibited a reduced antibody titer response to influenza vaccination 1 year post-loss relative to non-bereaved adults (128). Together, these findings illustrate how bereavement can lead to long-lasting impairments in immunity that have implications for health.

Bereavement-Induced Genetic and Epigenetic Changes

Another pathway by which bereavement may influence health is by affecting genetic and epigenetic pathways. Early life stress exposure has been associated with heightened physiological stress sensitivity as indexed by inflammatory activity and reactivity, as well as with greater immune system dysregulation later in life (30, 47, 140–143). In terms of gene expression, stress-induced epigenetic changes can result in altered gene expression (38).

The fields of psychoneuroimmunology, genetics, and genomics have only recently begun to examine genomic mechanisms that may underlie these effects. Although the literature examining bereavement-associated epigenetic changes remains scant, this research has already provided an interesting new window through which we may be able to better understand how bereavement affects health (34, 144). In this context, a few genetic polymorphisms have been investigated that could represent potential protective or risk factors in the face of adversity. For example, one recent cross-sectional study investigated the gene × environment (G × E) interaction effects of different genotypes and inflammation for 36 spousal bereaved older adults who experienced spousal loss over the past 23.75 months vs. 28 married individuals in order to elucidate factors predicting resilience during bereavement. The researchers found that spousally bereaved individuals exhibited higher levels of circulating inflammatory markers relative to non-bereaved individuals. Moreover, the authors identified a SNP in the IL-6-174 region that moderated individuals' vulnerability to increased systemic inflammation following the death of their spouse (49).

Relatedly, a cross-sectional study by O'Connor et al. (139) identified a genotype that helped explain the variability in grief severity experienced by older adults with and without complicated grief following the death of their partner or spouse. Individuals with both complicated grief ($N = 12$) and non-complicated grief ($N = 24$) exhibited the upregulated expression of genes implicated in the activation of the immune response and downregulated expression of genes implicated in B lymphocyte responses. Moreover, individuals exhibiting complicated grief showed a substantial downregulated expression of Type I interferon transcripts.

It is possible that mutations in these genes may lead to the altered regulation of pro- and anti-inflammatory cytokine gene expression, in turn placing individuals experiencing bereavement-related distress at increased risk of experiencing immune-related health problems following bereavement (26). Furthermore, changes in gene expression following bereavement may interact with epigenetic changes that have occurred earlier in life (e.g., from early life stress exposure) to influence how an individual responds to the loss of a spouse in later adulthood. Likewise, bereavement itself may cause epigenetic changes that could increase a person's risk of subsequent health problems through the activation of the previously described inflammatory pathways (34).

Together, these data suggest that increased inflammatory activity may predominantly occur in genetically susceptible individuals who experience the loss of a significant person. This G × E effect may provide a possible mechanism through which bereavement-related stressors increase vulnerability for morbidity and mortality in bereaved individuals. The G × E effect

may also help explain why some bereaved individuals develop mental and physical health problems following the death of a spouse while others do not.

Bereavement-Induced Changes in the Gut Microbiota

In addition, the gut microbiota may play a role in influencing health outcomes following bereavement. Evidence for this possibility comes from research documenting how acute and chronic stressors negatively impact the gut microbiome (41). Animal models provide experimental evidence for associations between early life stress exposure and low-grade inflammation, altered enteric microbiota, exaggerated stress reactivity, and visceral hypersensitivity (42, 145), emphasizing the impact of stress on microbial composition and activity. In humans, it is well-known that stress can cause nausea, vomiting, and abdominal pain (146). In addition, chronic life stressors such as financial problems, unemployment, and loss have been associated with an increased risk of developing functional gastrointestinal disorders, such as irritable bowel syndrome (147, 148). Moreover, accumulating evidence from clinical studies suggests a strong link between stress-induced disturbances along the gut microbiota-immune-brain axis and chronic inflammatory disorders, such as allergies, autoimmunity, and inflammatory gastrointestinal disorders (149, 150), and mental health problems including anxiety disorders and depression (151–155).

In contrast with this general body of research on stress and the gut microbiota, only a few studies to date have investigated how stress may lead to changes in the gut microbiota in the context of bereavement. Moreover, much of the research examining the effects of stress on the gut microbiota is still from animal model studies (43). Therefore, the American Gastroenterology Association and American Psychosomatic Society have encouraged efforts to characterize pathways linking stress and the gut microbiota-immune-brain axis in large, prospective, longitudinal cohort studies to better understand microbiome composition and microbial function in healthy and diseased individuals (156). Ultimately, it is possible that stress may change microbiota and vice versa via neuroendocrine (157), inflammatory (158), and behavioral (e.g., depression) pathways (154), but additional research is needed on this topic in general and especially in the context of bereavement.

Bereavement and Biological Aging

Bereavement may also affect health through stress-induced accelerations in biological aging as indexed, for example, by telomere length. Telomeres are short DNA sequences that are located on the end of chromosomes. Telomeres protect chromosomes from degradation by forming a cap that provides chromosomal stability. Telomeres also regulate cellular replication and cellular lifespan (159). Of clinical importance, telomere length shortens with age (160), and extensive research has also shown that accelerated shortening of telomeres is associated with premature cell death, senescence, apoptosis, and carcinogenesis, in turn increasing morbidity and mortality (161). Furthermore, there is emerging evidence for an association of chronic stress with greater oxidative stress, lower telomerase activity, and shorter telomere length, indicating that stress may contribute to accelerated biological aging, thus providing a possible mechanism linking stress with health (39, 159, 162, 163).

Telomere attrition has emerged as a potentially useful biomarker of cellular aging that has shown associations with increased risk of various diseases and poorer survival (164). Additionally, the long-term effects of bereavement-related stress have been demonstrated in a study that investigated the impact of death or a sudden severe illness of a close family member during prenatal development. The study found that young adults with mothers who had experienced a death during prenatal development had shorter leukocyte telomere length as compared to young adults with mothers who had a pregnancy without experiencing death (165). These findings are of clinical importance for two reasons: first, telomere length in newborns and young adults has been related to maternal stress during pregnancy; and second, cellular aging can be influenced through prenatal stress, thereby potentially increasing an individual's susceptibility to infectious and autoimmune diseases in later life (166, 167).

Moreover, biological aging can affect the immune system by causing a progressive decline in functional immunity, referred to as *immunosenescence*, which diminishes humoral and cellular immune responses. Immunosenescence typically occurs in older individuals ($\geq$65 years old) (168, 169). Declining T-cell function is a well-characterized feature of immunosenescence, contributing to chronic low-grade inflammation (170). However, chronic stress also has been shown to suppress and dysregulate immune function via immunosenescence (162). Consequently, age-associated declines in immune function can contribute to many comorbid conditions and may render older individuals more vulnerable to further assaults on their immune system from things like stress, immune-related illnesses, and infectious diseases (171). For instance, systemic low-grade inflammation, as indexed by circulating levels of CRP and IL-6, has been identified as a significant risk factor for CVD in elderly individuals (102).

The loss of a spouse or partner occurs most frequently in later life and is thus a life event that is often experienced by individuals older than 65 years, mainly due to the loss of a spouse (172). Several studies have found evidence of reduced immunity in older adults who have experienced bereavement. For instance, older bereaved vs. non-bereaved individuals with a mean age of 75 years have been found to show a weaker antibody response to vaccination in the year after the loss of a spouse (128). Furthermore, older bereaved individuals (M_{age} = 72 years old) have been found to exhibit impaired neutrophil function, lower neutrophil reactive oxygen species (ROS) production, and a higher cortisol-to-dehydroepiandrosterone sulfate (DHEAS) ratio relative to younger adults (M_{age} = 32 years old) (173, 174). Of note, cortisol is generally immunosuppressive, whereas DHEAS (secreted by the adrenal gland) is immune-enhancing and counterbalances the effects of cortisol on the innate immune system (175). With aging, though, cortisol and DHEAS respond differently to stress, resulting in a negative regulatory effect on immune function (173).

In addition, from a molecular perspective, older bereaved adults (aged 61–83 years old) have been found to show the

reduced expression of genes involved in the B lymphocyte immune response as compared to a sample of age- and sex-matched non-bereaved adults (139). The salient incidence of immune-related dysregulation in older bereaved individuals suggests a pivotal role of mutually enhancing effects of stress and inflammation on immunosenescence (176). Indeed, bereavement-related distress may promote a natural decline of the immune system and increase older adults' risk of bereavement-related morbidity and mortality (177).

Cumulative Life Stress Exposure and Bereavement

Chronic stress, especially when experienced in early life, can also affect health outcomes in bereaved individuals by setting the stage for long-lasting neurobiological changes that are associated with increased risk of later morbidity and mortality (178). Specifically, stress exposure during childhood can alter behavioral and physiological responses to acute and chronic stress in adulthood that in turn influence later-life risk for mental and physical health problems, including anxiety disorder, depression, CVD, and autoimmune and neurodegenerative disorders (20, 39, 47, 140, 179). Epigenetic changes and immune system dysregulation may be potential pathways that underlie this link (36, 39, 180). Indeed, the presence of multiple childhood adversities has been found to heighten emotional and physical reactivity to subsequent stress, which in turn activates genetic and epigenetic processes that may promote a proinflammatory phenotype (20).

In addition, cumulative life stress exposure is an important moderator of the association between acute life events, such as the loss of a spouse, and vulnerability to mental and physical health problems. As proposed by the Social Signal Transduction Theory of Depression, for example, greater lifetime stress exposure can increase neuro-inflammatory sensitization to adversity that increases a person's risk for immune-related mental and physical health problems in the face of subsequent interpersonal loss (20). Consistent with this formulation, among individuals with a history of childhood maltreatment, those who have experienced spousal loss have been found to be more likely to develop depressive symptoms than those who have not experienced such loss (84). These results suggest that adverse childhood experiences may set the stage for experiencing worse immune-related health outcomes following a significant loss in adulthood.

Psychosocial Factors Affecting Risk and Resilience in Bereavement

Finally, several psychosocial factors can affect risk and resilience for poor health following bereavement. Resilience to stress is characterized by an individual's ability to maintain or restore relatively stable psychological and physical functioning when confronted with stressful life events, such as the death of a spouse (181). It has been hypothesized that resilience to such stressors arises from a combination of genetic factors (e.g., regulatory SNPs), personality traits (e.g., neuroticism, rejection sensitivity), and social-environmental conditions (e.g., life stress exposure, socioeconomic status) (182).

Bonanno et al. have conducted several seminal studies to better understand mechanisms underlying resilience (66, 181). For example, analyzing prospective longitudinal data from individuals enrolled prior to the death of a spouse, these investigators have identified four prototypical grieving trajectories: resilience (66.3%) and chronic depression (14.5%), followed by depressed improved (10.1%), and chronic grief (9.1%) (183, 184). Contrary to expectations, for most individuals (∼60%), grief subsided over the initial weeks and months following the loss, indicating that most people are relatively resilient to interpersonal loss (184). However, even among resilient individuals, a majority showed grief reactions as characterized by transient distress, emotional pain, yearning, intrusive cognition, and rumination (185). In turn, only a small subgroup of grieving individuals exhibited severe grief that persisted for years after the marital loss (183, 184).

Psychosocial factors that appear to help buffer the negative effects associated with interpersonal loss include social support (186–188), secure attachment style (46, 189), positive emotions (129, 188, 190), optimism (191), cognitive flexibility (including positive reappraisal and acceptance) (192), and spirituality, including religiosity (193). In contrast, there are numerous psychosocial factors that have been found to weaken resilience to loss and grief, contributing to grief severity and prolonged grief reactions. These risk factors include the characteristics of spousal death [e.g., context of spousal illness, caregiving strain, lack of preparation for the death, traumatic loss (67, 194)], relationship quality [e.g., close kinship relationship, affection, intimacy, care, understanding, conflicts, ambivalence, and dependency in relationship (67, 195)], intrapersonal factors [e.g., extraversion, neuroticism, insecure, anxious or avoidant attachment styles, poor emotion regulation, negative cognition, pre-loss depression (67, 196, 197)], and interpersonal factors [e.g., low social and emotional support, financial hardships (67)].

In the bereavement literature, most studies that have been conducted so far have investigated group differences or correlates of bereavement, and only a few have examined biomarkers of resilience and vulnerability for bereavement-related morbidity. In this context, Buckley et al. (6) examined predictors of increased risk for thrombotic changes that might contribute to cardiovascular risk in the initial weeks following the death of a spouse and found a potential role for neutrophils, von Willebrand factor antigen, Factor VIII, and platelet/monocyte/granulocytes. Smoking was associated with a higher neutrophil count in acutely bereaved individuals and accounted for 6% of the shared variance ($r^2 = 0.06$, $p = 0.02$). Moreover, duration of relationship between spousal bereaved and deceased spouse ($p = 0.03$) and smoking ($p = 0.001$) were found to be associated with higher platelet/granulocyte aggregate levels, accounting collectively for 14% of shared variance ($r^2 = 0.14$).

In addition to this research, two studies have examined changes in immune function following spousal loss while differentiating between grief severity and depression. The first study by Fagundes et al. (99) found that grief severity and depression were independently associated with increased systemic inflammation (e.g., IL-6, TNF-α, IFN-γ) in bereaved individuals approximately 3 months following the loss of a

spouse. Furthermore, in the same study, MDD was a significant predictor of participants' levels of the proinflammatory cytokines IL-6, TNF-α, INF-γ, and IL-17A following spousal loss. Contrary to expectation, depression did not moderate the association between grief severity and inflammation. Likewise, a second study identified fatigue (as indexed by the SF-36 Energy/Fatigue sub-score) as a predictor of low-grade inflammation (as measured by CRP) in bereaved individuals 1 month following the loss of a spouse (10).

In general, these findings documenting psychosocial factors associated with risk and resilience in bereavement are preliminary and require replication to determine their reliability and relevance for predicting vulnerability to bereavement-related morbidity. Furthermore, most of the studies in this area have used cross-sectional, post-interpersonal loss research designs. Studies that include pre-loss measures of neuroendocrine and immune biomarkers, and that follow participants over time, are therefore needed.

DISCUSSION

Bereavement From the Perspective of the Social Signal Transduction Theory of Depression

As discussed at the beginning of this review, we believe that psychobiological processes linking bereavement and health can be viewed from the perspective of the Social Signal Transduction Theory of Depression in order to provide one possible framework for better understanding processes that may underlie individual difference in risk and resilience to interpersonal loss. More specifically, the Social Signal Transduction Theory of Depression describes neural, physiologic, molecular, and genomic mechanisms linking interpersonal loss and health, as well as several moderators that can influence the effects of life stress on immune function and health, such as age, sex, and early life, adulthood, and cumulative lifetime stress exposure. Another important feature of the theory is that it differentiates between different types of life stressors and also accounts for individual characteristics—including both personality and genetic traits—that may render an individual differentially susceptible to the negative effects of stress and bereavement. Insights from this theory may thus help to address several existing questions in the bereavement literature, including why some individuals are more resilient than others to interpersonal loss, why cumulative life stress exposure is a strong predictor of morbidity and mortality in bereaved individuals, and why bereavement is associated with an increased risk of experiencing physical health problems. In light of this, we believe that identifying how social and biological factors identified by this theory might help explain differences in bereavement severity and persistence is an important topic for future research.

Integrating the consequences of the loss of a significant individual into the Social Signal Transduction Theory of Depression seems to be feasible with some modifications. For example, the Social Signal Transduction Theory of Depression emphasize that stressors experienced over time and exert a cumulative effect on neural and immunologic functioning that sensitizes a person to future stressors, such as interpersonal loss. In this model, the death of a loved one is regarded as a unique, complex stressor that can involve several types of adversity, including housing difficulties, financial strain, physical relocation, retirement, and the loss of daily routines and meaning in life (198, 199).

Relative to other topics in psychoneuroimmunology, the grief, loss, and bereavement literature has generated very few data documenting how lifetime stress exposure affects psychological, neural, and immunologic outcomes in bereaved individuals over time. It is possible that the severity or taboo of discussing grief has rendered the investigation of mental and physical health problems following spousal bereavement difficult. Moreover, obtaining funding for studies designed to examine health outcomes among bereaved individuals (as opposed to patients) can be difficult. As a result of these factors, despite the great importance of this topic, the health consequences of bereavement remain poorly understood (200, 201).

FUTURE DIRECTIONS

To better understand how bereavement affects health, we believe there is a pressing need to study how bereavement leads to changes in psychological, neural, and immunologic functioning. Biobehavioral responses to grief, epigenetic changes, cumulative life stress exposure, and neural sensitivity to stress could each represent potential mechanisms linking bereavement and health. Therefore, it will be important to adopt a multidimensional approach to studying bereavement that involves assessing how these and other processes interact and change over time to structure differences in disease risk and longevity in the context of bereavement. Below, we discuss several direction for future research along these lines.

First, the early identification of individuals who are at the greatest risk of experiencing poor health outcomes following the death of a significant person is an important aim of precision medicine and disease prevention initiatives (202). Encouraging bereaved individuals to provide mental health and biological data will help advance research on how bereavement impacts mental and physical health. This knowledge can in turn help prevent or treat mental and physical health problems in bereaved individuals, especially those deemed to be at high risk as a function of their psychosocial or biological status. Ultimately, identifying biobehavioral mechanisms that can be modified or targeted early on in the bereavement process is an important and clinically relevant step toward the development of treatments that improve health outcomes in bereaved spouses.

Second, additional research is warranted to better understand the clinical significance, timing, duration, and trajectories of immune changes that occur following interpersonal loss (22). Large-scale longitudinal studies that systematically collect data on mental and physical health outcomes prior to and after spousal bereavement are needed. This longitudinal approach will help identify profiles that predict poor responses to the death of a significant person and thus aid in the identification of people who

would benefit from personalized interventions and, moreover, the initial design of such interventions.

Third, studies investigating the biological bases of stress resilience will be important for elucidating processes that may help promote psychosocial resilience to interpersonal loss (203). From the perspective of the Social Signal Transduction Theory of Depression, vulnerability and resilience arises from a combination of social-environmental conditions (e.g., abuse or neglect, interpersonal, or financial difficulties), neurocognitive processes (e.g., perceptions of threat), social factors (e.g., social support), and genetics mechanisms (e.g., regulatory SNPs). According to this perspective, pre-loss neurocognitive, immunologic, and genetic functioning are important factors that help shape how a person is likely to respond to a significant interpersonal loss. Therefore, future studies examining psychobiological factors of resilience in bereavement could benefit from investigating G × E interaction effects by systematically assessing cumulative lifetime stress exposure (204) in addition to other predisposing psychosocial and biological factors that may confer increased vulnerability to the death of a significant person (38).

Fourth, it will be important to study health behaviors, such as smoking, diet, sleep, and exercise, which are not presently accounted for in the Social Signal Transduction Theory of Depression and that are rarely discussed in psychobiological models of bereavement. Some research has shown that bereavement is associated with negative health behaviors, especially diet and sleep (205, 206), but this literature is small for a topic that deserves serious attention.

Finally, investigating the interplay between multiple psychological and biological markers of stress over time involves substantial analytic computational complexity. Therefore, we believe that multilevel statistical approaches such as structural equation modeling and latent growth mixture modeling will be helpful for characterizing inter-individual differences in intra-individual changes over time (207).

CONCLUSION

In conclusion, the death of a spouse is considered one of the most stressful life events a person can experience. In addition to increasing risk for depression, spousal death can lead to increased risk for a variety of somatic and physical diseases, as well as early mortality. Our goal with the present review was to help make sense out of these effects by reviewing social, psychological, immunologic, and genetic processes that have the potential to shape vulnerability to morbidity and mortality following spousal bereavement. We also related these processes to the Social Signal Transduction Theory of Depression, which we believe is one useful, multi-level framework that can be used to understand how social stressors affect psychobiological processes that impact health. In terms of future studies, it will be important to explore the associations described herein to help identify individuals at high risk for poor health outcomes following the death of a significant person. This knowledge could help to elucidate biobehavioral mechanisms that clinicians could in turn target early after a loss to improve health outcomes in bereaved spouses. Given the centrality of interpersonal loss to the human experience, we believe that much more research is needed to understand how exactly spousal bereavement affects health and how we can translate this knowledge to increase psychosocial resilience to such stress.

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All authors developed the concept for this article. The initial draft was written by AS and subsequently edited by RK and GS. All authors read and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

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Tumor Necrosis Factor-α Variations in Patients With Major Depressive Disorder Before and After Antidepressant Treatment

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Tumor necrosis factor-α (TNF-α) had been identified as a key pro-inflammatory cytokine in the pathophysiology of major depressive disorder (MDD) and the mechanism of antidepressant treatment. The primary aim of the present study was to examine the serum TNF-α levels in Chinese inpatients with MDD during the acute phase and to explore the changes in TNF-α levels after effective clinical treatment. Fifty-seven consecutive inpatients with MDD and 30 healthy controls were recruited. The serum TNF-α levels were detected using ELISA. Symptoms of depression were evaluated using the 24-item Hamilton Rating Scale for Depression (HAM-D-24). TNF-α levels and HAM-D-24 scores were assessed at baseline and after 2 and 12 weeks of follow-up. The serum TNF-α levels were higher in the MDD group than in the control group. After 2 and 12 weeks of antidepressant treatment, there were significant improvements in the patients' symptoms and significant decreases in the TNF-α levels. The baseline TNF-α levels significantly correlated with the decreased HAM-D-24 scores, particularly for the depressive symptoms of anxiety/somatization and weight loss. The present findings indicate that depression is accompanied by activation of TNF-α, which also has a predictive value for the antidepressant treatment response in patients with MDD.

Keywords: remission, serum, antidepressant, major depressive disorder, tumor necrosis factor-α

INTRODUCTION

Major depressive disorder (MDD) has emerged as one of the most common psychiatric disorders (1). Extensive research efforts have been made to improve the understanding of its molecular pathophysiology. Although several mechanisms leading to MDD are possible, the inflammatory hypothesis is currently one of the widely accepted theories, also called the "cytokine" theory of depression (2). The inflammatory cytokine system is activated in several somatic diseases that share a number of common symptoms with MDD, such as tiredness. This implies that inflammatory cytokines may play a key role in the development of

depression (3). This theory also well-explained some phenomena of psycho-neuroimmunological dysfunction in patients with MDD, who have an abnormal peripheral immune system (4). It has been established that inflammatory cytokines can induce immune stimulation, which induces depression-like signs and symptoms, and these links have been supported by experimental and clinical evidences (5, 6).

Tumor necrosis factor-α (TNF-α) is one of the essential pro-inflammatory cytokines that has received much attention because of its ability to cause inflammation and apoptotic cell death and to mediate the release of a variety of cytokines (7, 8). Two mechanisms may link TNF-α to the pathophysiology of MDD. First, pro-inflammatory cytokines such as TNF-α may regulate the neuronal serotonin transporter activity and stimulate serotonin uptake (9). Second, pro-inflammatory cytokines activate the tryptophan- and serotonin-degrading enzyme indolamine-2,3-dioxygenase, resulting in a reduced availability of serotonin in depression (10). Although from the mechanism point of view it has been postulated that the increased production of TNF-α might play a causative role in MDD, the levels of TNF-α have been shown to be quite varied among patients with MDD across many clinical investigations. Increased (11, 12), unchanged (13), and decreased (14) levels of TNF-α have been reported in depression. One of the possible explanations for this inconsistency might be that different patterns of TNF-α expression may appear in depressive episodes of different types.

Changes in the TNF-α system are involved not only in the development of MDD but also in mediating the response to antidepressant treatment. Two recent meta-analysis studies have found TNF-α to be decreased in MDD patients after antidepressant treatment, especially for those who are well-responded in the selective serotonin reuptake inhibitor (SSRI) treatment. However, due to the limited number of MDD cohort studies available, and the fact that the MDD sample was highly heterogeneous with respect to symptom severity and treatment response, the association between changes in TNF-α levels and response to antidepressant treatment in MDD was not clear.

Changes in the TNF-α system were involved not only in the development of MDD but also in mediating the response to antidepressant treatment. Two recent meta-analysis studies (15, 16) have found TNF-α to be decreased in MDD patients after antidepressant treatment, especially for those who are well-responded in the antidepressant treatment, but not for non-responders. However, due to the limited number of MDD cohort studies available (17–20), and the fact that the MDD sample was highly heterogeneous with respect to symptom severity and treatment response, the association between changes in TNF-α levels and response to antidepressant treatment in MDD was not clear. In addition, most studies (18–20) only measured the levels of TNF-α before and after treatment, and the treatment duration in those studies was varied, so it was impossible to observe the dynamic changes of TNF-α levels during the treatment process. Therefore, the present study explored by setting up multiple follow-up points to examine the dynamic changes in the serum TNF-α levels in a fairly homogeneous group of MDD patients treated with antidepressants in clinical settings. The primary aim of the current study was to examine the difference in the serum TNF-α levels in Chinese inpatients with MDD in the acute phase with healthy controls and, more importantly, to explore the changes in TNF-α levels and their predicting value for effective antidepressant treatment.

METHODS

Overview

The current study included in patients with MDD from the Shanghai Nanhui Mental Health Center, the largest mental health service in Shanghai Nanhui district. The Research Ethics Committees of the Shanghai Pudong Nanhui Mental Health Center approved the study in 2018. The study was conducted in accordance with the tenets of the Declaration of Helsinki. A key element of this study was that all patients were drug-free (at least 2 weeks) prior to admission to the hospital.

Sample and Study Design

All participants provided written informed consent at the recruitment stage of the study. A total of 87 subjects were included, 57 patients with MDD (MDD group) and 30 healthy controls (HC group), aged 29–82 years. The inclusion criteria for the patients with MDD were as follows: (1) diagnosis of MDD according to the criteria defined in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (21)—no additional diagnostic tool was applied—(2) an acute exacerbation of the symptoms of depression; and (3) a baseline score of at least 14 points on the 24-item Hamilton Rating Scale for Depression (HAM-D-24) (22). Each patient with MDD was asked to spend 1 week as an inpatient and undergo a clinical assessment and screening procedures (including physical examination, electrocardiography, and clinical laboratory investigations) before receiving antidepressant treatment. Other inclusion criteria were taking only one type of antidepressant during the study and willingness and ability to complete the 12-week follow-up assessment.

The exclusion criteria were as follows: (1) medical illness, such as chronic diseases, including diabetes, and any infectious disease in the month prior to enrollment; (2) pregnancy; (3) substance abuse; and (4) a history of use of relevant medications, such as antibiotics and antioxidants, within the previous 4 weeks. The physical examinations and routine standard laboratory tests for inpatients helped to exclude the subjects with medical comorbidities and concomitant medication use. At baseline, 30 healthy controls, volunteers from the community, were recruited. After they provided written consent, they were assessed with the Structured Clinical Interview for DSM-IV Axis I Disorders-Non-patient version (SCID-NP) to confirm that they had no past or present mental disorders. They underwent the same procedure of enrollment screening as did the patients with MDD, except the HAM-D-24 assessment.

Clinical Assessment

The primary measure of the study was the HAM-D-24 scores. HAM-D-24 had been widely used in Chinese clinical practice and related studies for over 30 years. It was administered to the inpatients with MDD at baseline, and at 2 and 12 weeks

follow-up. The 2-week follow-up time point was used to evaluate the short-term antidepressant effect, and the 12-week (average length of stay for inpatients in our hospital) follow-up time point was used to evaluate the mid-term antidepressant effect. HAM-D-24 assesses seven factors: (1) anxiety/somatization, (2) weight loss, (3) cognitive disturbance, (4) retardation, (5) diurnal variation, (6) sleep disorder, and (7) desperation. For example, the anxiety/somatization factor of the HAM-D-24 includes six items: anxiety-psychic, anxiety-somatic, somatic symptoms-gastrointestinal, somatic symptoms-general, hypochondriasis, and insight. All inpatients and outpatients were screened and assessed by a face-to-face interview by two senior psychiatrists. The agreement rate between the two psychiatrists was 0.8, expressed as a kappa value. The same clinical assessments, blood collection, and laboratory procedures were performed again at week 2 and 12 for all patients.

Antidepressant Treatment

All the patients were taking one type of antidepressant during the follow-up: 40 of them were taking a SSRI (26 of them took escitalopram with a mean dose of 23.8 mg, 14 of them took paroxetine with a mean dose of 47.9 mg); 12, venlafaxine with mean dose of 187.5 mg; and 5, duloxetine with mean dose of 40 mg.

Experimental Procedure

Five milliliters of blood was drawn by venipuncture into an anticoagulant-free tube between 6:00 a.m. and 7:00 a.m. after an overnight fast. The blood was centrifuged at 3,000 × g for 20 min at 4°C within 2 h of collection. Subsequently, the serum was separated and stored at −80°C until it was assayed.

Serum TNF-α levels were measured in duplicate using enzyme-linked immunosorbent assay (ELISA), with a commercial human ELISA kit (MULTISCIENCES(LIANKE) BIOTECH, CO., LTD), sensitivity 0.16 pg/ml (mean of 6 independent assays), in accordance with the manufacturer's instructions. The intra- and inter-assay coefficients of variation were <7 and 9%, respectively. No cross-reactivity was detected.

We average the duplicate readings for each standard and sample and subtract the average zero standard optical density. The data are linearized by plotting the log of the TNF-α concentrations vs. the log of the optical density (OD), and the best-fit line can be determined by regression analysis, the log of the TNF-α concentrations as horizontal axis, the log of the OD as the vertical axis. Then, we substitute the data into the standard curve and multiply by the dilution factor which is 5 to get the final concentration.

In all remaining study subjects, blood samples were tested for TNF-α concentration, with minimum level of 2.0 pg/mL and maximum of 25.2 pg/mL. There were no results that were undetected or below the sensitivity threshold. The concentration of TNF-α was expressed as pg/mL.

Statistical Analysis

SPSS version 16.0 (IBM Corporation, Armonk, NY, USA) statistical software was used for all statistical computations. The sociodemographic and clinical characteristics of the participants were presented as descriptive statistics, such as percentages and mean scores. The daily antidepressant dose was converted to fluoxetine equivalent (23). A box-plot diagram was created using GraphPad Prism software to analyze the differences in the TNF-α levels between group HC and the baseline, week-2, and week-12 levels in group MDD. Independent *t* tests were conducted to measure group differences in continuous variables, and Chi-square statistics were used to examine categorical variables. The normality of the sample was confirmed by the Kolmogorov–Smirnov test, and the homogeneity was confirmed by the Levene test, after which the independent *t* tests were used to determine the group difference. Patients with MDD whose HAMD scores dropped below 8 were classified as the antidepressant-sensitive (AS) group, and others were classified as the antidepressant-insensitive (AIS) group (24). Two groups were compared by demographic and clinical characteristics and the TNF-α levels at baseline and follow-up points. The correlation between the baseline TNF-α levels and the percentage decrease in the HAM-D-24 scores after 2 and 12 weeks of antidepressant treatment was calculated using Spearman's non-parametric rank-correlation analysis. Subsequently, the relationship was explored via a partial correlation test for the seven factors of the HAM-D-24, controlling for the fluoxetine-equivalent dose of antidepressants. Finally, multivariate analysis with a linear regression model was performed to determine the regression coefficient (β) for the TNF-α levels, age, educational level, age at MDD onset, course of MDD, and equivalent antidepressant dosage for predicting HAM-D-24 score improvements at the week-2 and week-12 follow-up points. All reported results were two-tailed; significance was assumed at $p < 0.05$.

RESULTS

Demographic and Clinical Characteristics

As shown in **Table 1**, the baseline age, education, and marriage data did not differ between the HC and MDD groups. The proportion of females was significantly higher in the MDD group than in the HC group. The majority of patients with MDD had significant reductions in the total HAM-D-24 scores at the 2 and 12-week follow-up evaluations. The clinical features, improvements, and antidepressant exposure in the MDD group are listed in **Table 1**.

Differences in Serum TNF-α Levels Between HC and MDD Groups

The MDD group had significantly higher baseline TNF-α levels (mean = 7.7 pg/ml [SD = 2.6]) than those of the HC group (mean = 3.5 pg/ml [SD = 0.7]). However, in both the 2-week (mean = 4.1 pg/ml [SD = 3.4]) and 12-week (mean = 4.3 pg/ml [SD = 2.7]) follow-up points of the trial, this difference between the groups was no longer significant (**Figure 1**). There was a significant decrease in the TNF-α levels from the baseline values at the 2 and 12-week follow-up points.

TABLE 1 | Comparison of the demographic, clinical, and treatment characteristics between healthy controls and patients with MDD.

Variables	Healthy control	MDD	Comparisons	
			t/χ^{2a}	*p*
Cases [*n*]	30	57	-	-
Age (years) [mean (SD)]	58.1 (15.1)	57.0 (10.6)	0.383	0.671
Age range (years)	29–82	29–77	-	-
Female [*n* (%)]	16 (53.3)	46 (80.7)	$\chi^2 = 7.189$	**0.007**
Education (years) [mean (SD)]	7.3 (3.4)	7.7 (3.0)	$t = 0.521$	0.604
Marriage_Single [*n* (%)]	1 (3.3)	6 (10.5)	$\chi^2 = 1.998$	0.573
Marriage_Married [*n* (%)]	25 (83.3)	42 (73.7)		
Marriage_Divorce [*n* (%)]	3 (10.0)	5 (8.8)		
Marriage_Widowed [*n* (%)]	1 (3.3)	4 (7.0)		
Family History [*n* (%)]	0 (0)	6 (10.5)	$\chi^2 = 1.951$	0.163
Age of MDD onset (years) [mean (SD)]	-	48.4 (14.2)	-	-
No. of MDD episode [mean (SD)]	-	2.6 (1.8)	-	-
No. of hospitalization [mean (SD)]	-	1.8 (1.5)	-	-
Course (months) [mean (SD)]	-	8.5 (8.6)	-	-
Baseline HAM-D [mean (SD)]				
HAM-D total score [*n* (%)]	-	30.8 (4.8)	-	-
HAM-D factor-1: anxiety/somatization	-	8.5 (3.0)	-	-
HAM-D factor-2: weight	-	1.1 (0.9)	-	-
HAM-D factor-3: cognitive disturbance	-	3.6 (2.4)	-	-
HAM-D factor-4: retardation	-	2.1 (0.9)	-	-
HAM-D factor-5: diurnal variation	-	6.4 (1.6)	-	-
HAM-D factor-6: sleep disorder	-	4.9 (1.5)	-	-
HAM-D factor-7: desperation	-	4.2 (1.9)	-	-
Antidepressants (mg per day) [mean (SD)]				
Duloxetine	-	5 (8.8)	-	-
Escitalopram	-	26 (45.6)	-	-
Paroxetine	-	14 (24.6)	-	-
Venlafaxine	-	12 (21.1)	-	-
Fluoxetine-equivalent dose	-	75.8 (94.7)	-	-
Week 2			-	-
HAM-D total score [mean (SD)]	-	15.8 (3.2)	-	-
Δ1 HAM-D [mean (SD)][b]	-	15.0 (3.1)	-	-
Δ1 HAM-D improvement rate (%) [mean (SD)][c]	-	48.8 (6.5)	-	-
Week 12			-	-
HAM-D total score [mean (SD)]	-	7.5 (2.0)	-	-
Δ2 HAM-D [mean (SD)][d]	-	23.3 (4.8)	-	-
Δ2 HAM-D improvement rate (%) [mean (SD)][e]	-	75.2 (6.7)	-	-

[a] *t/χ^2: t for t test, χ^2 for kappa test.*
[b] *Δ1. HAM-D: baseline HAM-D—week 2 HAM-D.*
[c] *Δ1 HAM-D improvement rate (%): (baseline HAM-D—week 2 HAM-D)/baseline HAM-D.*
[d] *Δ2. HAM-D: baseline HAM-D—week 12 HAM-D.*
[e] *Δ2 HAM-D improvement rate (%): (baseline HAM-D—week 12 HAM-D)/baseline HAM-D.*
Significant P values are bolded.

Differences in Demographic and Clinical Characteristics, Serum TNF-α Levels Between as and AIS Groups

After 2 weeks of treatment with antidepressants, none of the patients' HAMD score dropped below 8. After 12 weeks of treatment, 42 of 57 (73.7%) patients with MDD were included in the AS group, and 15 patients were included in the AIS group. There were no significant differences in age, sex, age of MDD onset, course of disease, and baseline HAMD scores between the AS and AIS groups. The decreased HAMD score was higher in the AS group than in the AIS group ($p = 0.044$). The serum TNF-α levels at baseline in the AIS group were higher than those in the AS group ($p = 0.015$). However, there were no differences in the serum TNF-α levels at the 12-week follow-up points between the two groups ($p = 0.218$).

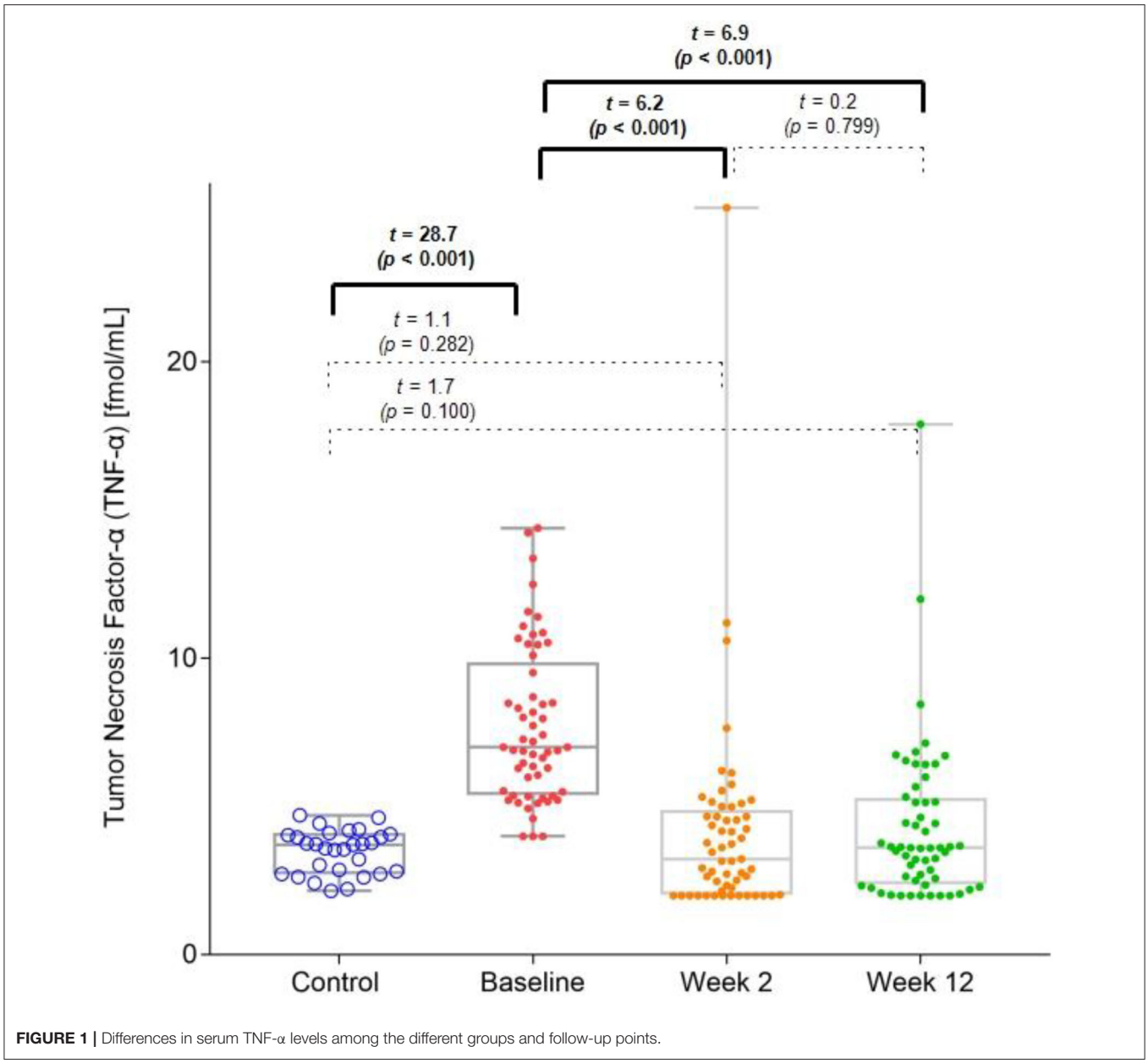

FIGURE 1 | Differences in serum TNF-α levels among the different groups and follow-up points.

Relationship Between Baseline TNF-α Levels and HAM-D-24 Score Improvement

We further analyzed whether the baseline TNF-α levels correlated with the decreased HAM-D-24 scores. The percentage decrease in the HAM-D-24 scores after 2 and 12 weeks of antidepressant treatment were negatively correlated with the baseline TNF-α levels (**Figure 2**).

Correlation Between the Baseline TNF-α Levels and Changes in the Depressive Subdomains After 2 and 12 Weeks of Treatment

At baseline, a significant correlation was found between the HAM-D-24 subdomains of anxiety/somatization and weight loss and the TNF-α levels. **Table 2** highlights the observed significant correlation between the TNF-α levels and the weight loss subdomain score at week 2 and week 12 when controlling for fluoxetine-equivalent antidepressant dose.

Prediction of the HAM-D-24 Score Improvement

Liner regression was used to evaluate the effect of the demographic and clinical variables and TNF-α levels on the HAM-D-24 score improvement rate at week 2, including age, educational level, age at MDD onset, course of MDD, equivalent antidepressant dosage, and baseline TNF-α levels. Consistently, only the baseline TNF-α level was found to significantly predict the week-2 HAM-D-24 score improvement rate in this model

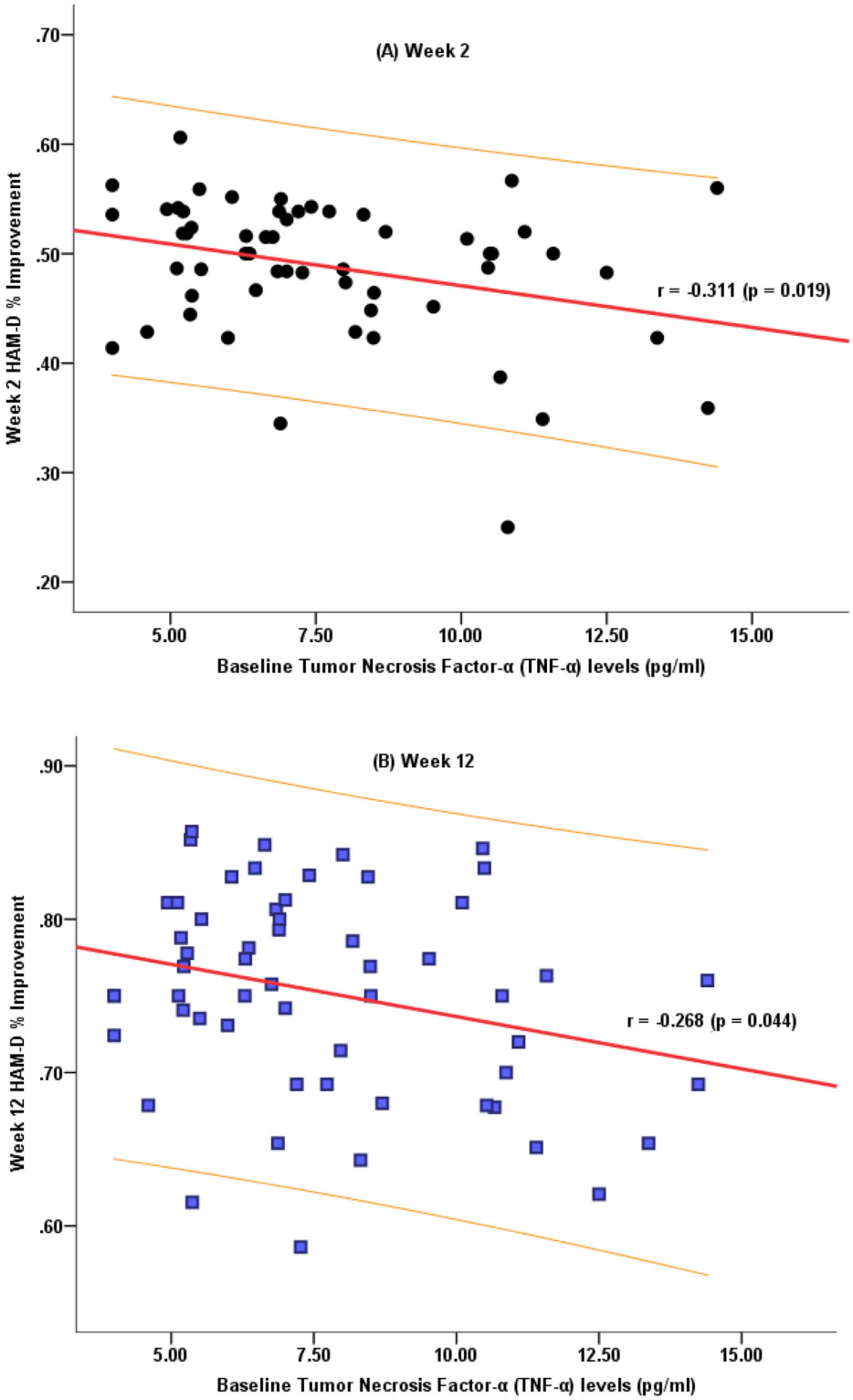

FIGURE 2 | Correlation characteristics between the decrease in the HAM-D-24 total scores after 2 weeks **(A)** and 12 weeks **(B)** of treatment and the baseline TNF-α levels.

TABLE 2 | Correlation of the HAM-D-24 subdomains at baseline and after 2 and 12 weeks of treatment with the TNF-α levels.

BASELINE							
	Anxiety/somatization	**Weight loss**	**Cognitive disturbance**	**Retardation**	**Diurnal variation**	**Sleep disorder**	**Desperation**
r(TNF-α)	0.324	0.313	0.222	0.053	0.023	−0.150	0.020
p(TNF-α)	0.014	0.018	0.097	0.693	0.867	0.265	0.881
WEEK 2 (PARTIAL CORRELATIONS WERE APPLIED BY CONTROL VARIABLE OF ANTIDEPRESSANT DOSAGE)							
Δ1(% Improvement)	**Anxiety/somatization**	**Weight**	**Cognitive disturbance**	**Retardation**	**Diurnal variation**	**Sleep disorder**	**Desperation**
r(TNF-α)	0.005	0.290	0.106	0.174	−0.238	0.048	−0.196
p(TNF-α)	0.975	0.039	0.458	0.222	0.093	0.738	0.167
WEEK 12 (PARTIAL CORRELATIONS WERE APPLIED BY CONTROL VARIABLE OF ANTIDEPRESSANT DOSAGE)							
Δ2(% Improvement)	**Anxiety/somatization**	**Weight**	**Cognitive disturbance**	**Retardation**	**Diurnal variation**	**Sleep disorder**	**Desperation**
r(TNF-α)	−0.148	0.331	0.173	0.085	−0.019	0.028	0.046
p(TNF-α)	0.298	0.018	0.224	0.555	0.896	0.845	0.749

Δ1 (% improvement): the percentage decrease in the HAM-D-24 factors from week 2 to baseline; Δ2 (% improvement): the percentage decrease in the HAM-D-24 factors from week 12 to baseline.

($\beta = -0.410$, $t = 2.487$, $p = 0.017$). For the week-12 HAM-D-24 score improvement rate, two variables were added in the prediction model, the week-2 TNF-α levels and the improvement in the HAM-D-24 score at 2 weeks. Only the variable of improvement in the HAM-D-24 score at 2 weeks ($\beta = 0.413$, $t = 2.970$, $p = 0.005$) was found to be significant.

DISCUSSION

The main finding of the present study is that the serum TNF-α levels were significantly higher in patients with MDD than in healthy controls. Interestingly, after 2 and 12 weeks of antidepressant treatment, a significant improvement in the depressive symptoms with significant changes in the TNF-α levels were detected among the patients. The results of this study suggested that the serum TNF-α levels were sensitive to the depressive state, supporting the cytokine theory in the inflammatory hypothesis for the development of depression.

Several clinical studies have consistently reported that the serum TNF-α levels are increased in patients with depression (12, 25–27). Considering this, it is worthy to examine whether the TNF-α levels are associated with the antidepressant medication treatment and whether they could serve as a putative biomarker for the clinical response. However, owing to the heterogeneity of MDD and its treatments, the results of those studies are not consistent. Namely, TNF-α levels had been reported as increased (28), decreased (29), or unchanged (15) after effective antidepressant treatment. Since the antidepressants applied in current and other studies varied significantly, one possible reason for the inconsistent results is that different types of antidepressants may have different effects on TNF-α levels. For example, Chen et al. (18) found that venlafaxine and paroxetine have different immunomodulatory properties in the treatment of patients with MDD. The present finding of the effects of antidepressants on normalizing the serum TNF-α levels in patients with MDD adds to the growing body of literature that suggests that TNF-α may not only be capable of causing depression, but that it also has a role in the modulation of emotional processes.

Consequently, our results demonstrated that serum TNF-α levels at baseline were significantly lower in the AS group than in the AIS group, which is consistent with previous studies (24, 28). Meanwhile, we also found that the higher the baseline serum TNF-α levels, the lesser the improvement in depressive symptoms in this sample. Together with the decreasing TNF-α levels after an effective treatment, this finding has important clinical implications for the novel treatment of patients with MDD, particularly treatment-resistant patients. The existing evidences show that TNF-α blockers are effective in the treatment of chronic inflammatory disorders, such as psoriasis. A recent study has shown that TNF-α blockers were also effective in decreasing depressive symptoms associated with psoriasis (30). In view of the association pattern between TNF-α levels and treatment improvement, it should be verified whether inhibiting the TNF-α levels will have a therapeutic potential in patients with MDD.

Interestingly, we found that two subdomains (anxiety/somatization and weight loss) of the depressive symptoms showed significant correlations with TNF-α levels. Those symptoms can be interpreted as somatization, which is also commonly reported in somatic diseases. First, depression is highly related with other inflammatory diseases (such as inflammatory bowel disease) (31), gastrointestinal problems, appetite changes, and aches and pains of a diffuse nature as common features of depression and inflammatory disease. This raises the possibility that TNF-α, which is activated in several somatic diseases and leads to somatization, may also be involved in the development of depression (3).

Our study has several limitations that should be noted. First, the premise of this study lies in the nature of the assessments conducted in real clinical practice. However, the raters were not

informed that the aim of this study was to explore the changes in the serum TNF-α levels before and after treatment. The naturalistic aspect would realistically reflect the changes in the TNF-α levels of these patients in an unbiased fashion. Another limitation is that psychotic symptoms and cognitive impairments were not assessed in this study. The current study relied only on the assessment of depressive symptoms, which is inadequate. In addition, there was a gender difference between the patients and healthy controls. In order to ensure that the TNF-α tests are performed using the same batch of human TNF-α ELISA kit, the healthy controls were recruited at the same time as the patients with MDD. Besides, we did not investigate the body mass index, smoking status, and C-reactive protein; all those variables are important parameters related to inflammation. Finally, this study was limited to small sample size and only the serum TNF-α level was included; other inflammatory and anti-inflammatory cytokines were not assessed due to limited resources.

CONCLUSION

In summary, our results revealed that the serum TNF-α levels were higher in patients with MDD than in healthy controls. Following the antidepressant treatment, the TNF-α levels were significantly decreased and comparable to those in the healthy controls. The baseline TNF-α level was correlated with the improvement in depressive symptoms, particularly the symptoms of anxiety/somatization and weight loss. Our findings indicate that TNF-α may be involved in the pathophysiology of depressive symptoms and that it has a predicting value for the treatment response in patients with depressive mood state.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Research Ethics Committee of the Nanhui Pudong New Area Mental Health Center of Shanghai. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TZ, LY, LP, JW, and MQ conceptualized the study, wrote the first draft of the manuscript, and conducted the statistical analyses. WS and CG helped in the design of the study and edited the manuscript. LH, LC, XT, YH, and LX interviewed the participants and collected and organized the primary data. YW managed the literature searches and statistical analyses and edited the manuscript. TZ and JW designed the study and provided supervision in the implementation of the study. All authors have approved the final manuscript.

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Bibliometric and Visual Analysis of Research on the Links Between the Gut Microbiota and Depression From 1999 to 2019

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Background: There is a crucial link between the gut microbiota and the host central nervous system, and the communication between them occurs *via* a bidirectional pathway termed the "microbiota-gut-brain axis." The gut microbiome in the modern environment has markedly changed in response to environmental factors. These changes may affect a broad range of host psychiatric disorders, such as depression, by interacting with the host through metabolic, immune, neural, and endocrine pathways. Nevertheless, the general aspects of the links between the gut microbiota and depression have not been systematically investigated through bibliometric analysis.

Aim: This study aimed to analyze the current status and developing trends in gut microbiota research in the depression field through bibliometric and visual analysis.

Methods: A total of 1,962 publications published between 1999 and 2019 were retrieved from the Web of Science Core Collection. CiteSpace (5.6 R5) was used to perform collaboration network analysis, co-citation analysis, co-occurrence analysis, and citation burst detection.

Results: The number of publications has been rapidly growing since 2010. The collaboration network analysis revealed that the USA, University College Cork, and John F. Cryan were the most influential country, institute, and scholar, respectively. The most productive and co-cited journals were *Brain Behavior and Immunity* and *Proceedings of the National Academy of Sciences of the United States of America*, respectively. The co-citation analysis of references revealed that the most recent research focus was in the largest theme cluster, "cytokines," thus reflecting the important research foundation in this field. The co-occurrence analysis of keywords revealed that "fecal microbiota" and "microbiome" have become the top two research hotspots since 2013. The citation burst detection for keywords identified several keywords, including "Parkinson's disease," "microbiota-gut-brain axis," "microbiome," "dysbiosis," "bipolar disorder," "impact," "C reactive protein," and "immune system," as new research frontiers, which have currently ongoing bursts.

Conclusions: These results provide an instructive perspective on the current research and future directions in the study of the links between the gut microbiota and depression, which may help researchers choose suitable cooperators or journals, and promote their research illustrating the underlying molecular mechanisms of depression, including its etiology, prevention, and treatment.

Keywords: gut microbiota, depression, bibliometric analysis, citespace, developing trends, microbiota-gut-brain axis, cytokines, microbiome

INTRODUCTION

Depression is a common mental illness that can affect both mental and physical health (1). It affects ∼350 million of the world's population, as reflected in limited functioning and diminished quality of life (2). According to the Global Burden of Disease Study, in 2017, depression disorders ranked as the third leading cause of global years lived with disability in females, and the fifth leading cause in males (3). However, the pathophysiology of depression disorders has not been fully elucidated, and no single mechanism can adequately explain all aspects of this disease (4). The key molecular mechanisms related to depression disorders include "the monoamine hypothesis," "hypothalamic-pituitary-adrenal (HPA) axis changes," "inflammation," "neuroplasticity and neurogenesis," "structural and functional brain changes," "genes," "environmental milieu," and "epigenetics (gene-environment interactions)" (4). Therefore, multiple biological and psychosocial determinants are known to be important factors influencing depression (4). Among the factors associated with depression pathology, the gut microbiota can affect a broad range of host psychiatric disorders, such as depression, by interacting with the host through metabolic, immune, neural, and endocrine pathways (5, 6). The communication between the host central nervous system (CNS) and the human gut microbiota occurs *via* a bidirectional pathway, termed the "microbiota-gut-brain axis," in which the microbiota and its metabolism are major components (7, 8). The communication pathways linking the gut microbiota with the CNS are incompletely understood but appear to have four main routes (6) (**Figure 1**). Microbial metabolism affects mediators of gut-brain-communication, including neurotransmitters [e.g., serotonin (5-HT) and γ-aminobutyric acid (GABA)], short-chain fatty acids (SCFAs) (e.g., butyrate), hormones (e.g., cortisol), and immune system modulators (e.g., quinolinic acid) (5). The metabolites modulated by the gut microbiota can influence mood, possibly through the activation of peripheral receptors on immune, neural, or endocrine pathways (8). In addition to the peripheral stimulation of these mediators, other direct and indirect mechanisms have been proposed to explain how metabolites affect depressive behavior, such as through direct stimulation of central receptors and epigenetic regulation of histone acetylation or DNA methylation (8).

Compared with our ancestral microbiome, the gut microbiome in the modern environment has been markedly altered because of some environmental factors, such as diet, antibiotic exposure, and proton pump inhibitors. These differences may have important effects on host brain health (9). Fortunately, the rapid development of culture-independent molecular methods (e.g., 16S ribosomal RNA and metagenomic sequencing analysis) (10), metabolomics (11), available animal models of depression (12), and modern neuroimaging and computational biology (13, 14), has enabled quantitative analysis of the components of the gut microbiome and microbial metabolism, and exploration of the pathophysiological roles of the gut microbiota in depression and microbiome-gut-brain interactions. All aspects of these techniques are contributing to gut microbiota research, thus making the gut microbiota a hotspot in depression research. In recent decades, the relationships between the gut microbiota and depression in animals and humans have been widely studied (6). For instance, many probiotic strains known as "psychobiotics," such as *Lactobacillus paracasei* PS23 and *Lactobacillus helveticus* NS8 (15, 16), as well as multi-species probiotics (17), have been shown to have antidepressant-like effects in rodent studies. Multiple studies in animal models have presented evidence that depression leads to microbiome changes or microbiota changes lead to depression, and indicated a causal relationship between them (6). Many human studies have also shown that probiotics are associated with a significant reduction in depression (18), possibly through an anti-inflammatory mechanism (19). Additionally, some clinical trials have been conducted to explore the feasibility of probiotics as adjuvants for traditional antidepressant therapies (20, 21). Although research on the connection between the gut microbiota and depression is widely available worldwide, the general aspects of this Research Topic, to our knowledge, have not been systematically analyzed through bibliometric analysis. Therefore, our study aimed to fill this gap in the literature.

Bibliometrics involves the use of a series of defined metrics and enables researchers to assess the published research output, impact, and developing trends by using statistical and quantitative methods (22). It can allow researchers to extract quantitative information on distribution by country/region, institution, author, and journal, and can aid in identifying research hotspots and frontiers in a particular field in a short time. Over the past 10 years, bibliometrics has been widely used to analyze scientific research in medical research worldwide (23). Previous bibliometric studies have focused on the gastrointestinal microbiome (24), the intestinal microbiota in obesity (25), or the microbiome-gut-brain axis (26), but have not addressed the gut microbiota in depression. Our bibliometric analysis

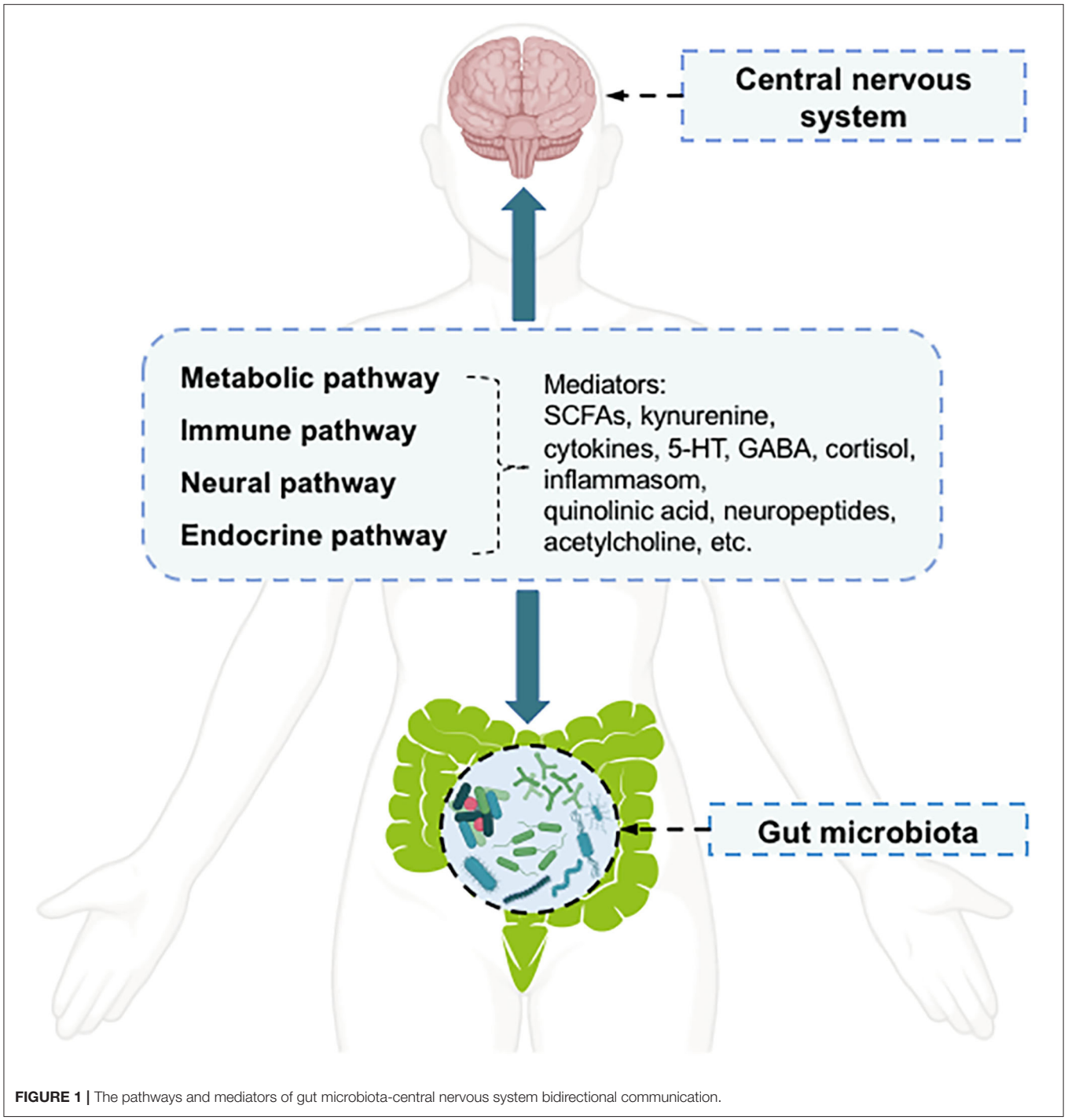

FIGURE 1 | The pathways and mediators of gut microbiota-central nervous system bidirectional communication.

involves this new and important field. The results should be helpful for researchers examining the gut microbiota in the depression field, aiding them in identifying journals to publish in and collaborators. The timely review and analysis of the hotspots and research trends may also promote the development of this field and advance research illustrating the molecular mechanisms underlying depression, such as its etiology, prevention, and treatment.

Therefore, this study aimed to comprehensively analyze the current status and developing trends in publications between 1999 and 2019 in gut microbiota research in the depression field through a bibliometric and visual analysis. The Web of Science™ Core Collection (WoSCC) database and CiteSpace software were used to conduct the bibliometric and visual analysis. Our focus was to: (i) investigate the outputs and growth trends in publications; (ii) construct international scientific collaboration

networks among countries/regions, institutions, and authors; (iii) determine the core countries/regions, institutions, researchers, and journals; and (iv) explore the key topics, hotspots, and research trends, to guide future research and applications.

MATERIALS AND METHODS

Introduction to CiteSpace

Science mapping, one of the main approaches used to explore a field of research in bibliometrics, is a general process of domain analysis and visualization. Its research scope includes a scientific discipline, and a research field or topics of particular research questions (27). CiteSpace, the Java application chosen to perform the bibliometric analysis, was developed in 2004 by Professor Chaomei Chen (College of Computing and Informatics, Drexel University, Philadelphia, PA, USA), an international expert in the information visualization field (28). It is an interactive analytic tool enabling visualization tasks in science mapping through a combination of bibliometrics, visual analytic methods, and data mining algorithms. CiteSpace supports several types of bibliometric studies, including collaboration network analysis, co-citation analysis, and co-occurrence analysis, and can generate visual maps such as geospatial visualizations (27). Collaboration network analysis can identify the core countries/regions, institutions, and authors in a particular field as well as their cooperative relationships. Additionally, co-citation analysis and co-occurrence analysis can reflect the research foundation and hotspots of the field, respectively. To date, it has been continually developed and widely used in the field of medical research (29, 30).

Data Acquisition and Search Terms

Bibliometric analysis relies on literature databases. One of the best-known databases is the WoSCC database, a curated collection of high-quality scholarly content on the Web of Science™ platform (WoS; previously known as Web of Knowledge), an online subscription-based scientific citation indexing service maintained by Thomson Reuters (31). To avoid the bias due to the daily database updates, because the database remains open, we performed the literature retrieval from WoSCC on a single day, May 15, 2020. The search index used in WoSCC was standardized to include all the relevant publications to ensure the comprehensiveness of the bibliographic data.

Synonyms for gut microbiota and depression were included in the search strategy, as follows: TS = (((gut OR intestin* OR gastrointestin* OR gastro-intestin*) AND (microbiot* OR microbiome* OR flora OR microflora OR bacteria)) OR prebiotic OR probiotic OR antibiotic OR dysbiosis) AND TS = (depression OR depressed OR depressions OR depressive OR despondent OR gloomy). In the present study, the inclusive and exclusion criteria were as follows: (i) the timespan ranged from 1999 to 2019, encompassing 20 years in total, (ii) only articles and reviews were included, whereas other document types (e.g., letters, meeting abstracts, retracted publications, and book chapters) were excluded, (ii) no species limitations were set, (iv) the publication language was restricted to English, and (v) duplicate publications were excluded. The search results were directly analyzed with the WoSCC literature analysis wire and exported for further analysis in CiteSpace. The present study used published data from secondary sources and did not involve any interactions with human subjects; hence, the requirement for institutional review board approval was waived (32).

Analysis Tools

Features such as the publication outputs, subject categories of WoS, *h*-index, and impact factor (IF) were analyzed with the WoSCC literature analysis wire (33). The *h*-index is a measure of productivity and the impact of a researcher who has published *h* articles cited at least h times each; that is, if the *h*-index of a scientist is 10, the scientist published 10 articles with at least 10 citations per article (34). The IF from Thomson Reuters indicates the impact of journals, referring to the number of citations to a given journal in a specific year (35).

CiteSpace (5.6 R5) (http://cluster.cis.drexel.edu/~cchen/citespace/) was used to perform collaboration network analysis (authors, countries/regions, and institutions), co-citation analysis (journals, authors, and references), analysis of keyword co-occurrence, and citation burst detection for keywords and references. The specific parameters used in CiteSpace were set as follows: time slicing (from 1999 to 2019, years per slice = 1), term source (title, abstract, author, keyword, and keywords plus), node type (one option chosen at a time from author, institution, country, keyword, cited reference, cited author, and cited journal), link strength (cosine), link scope (within slices), selection criteria (top 50 per slice), and pruning (none).

The visualizations generated by CiteSpace consisted of nodes and link lines. The nodes in the network maps represented the type of study being analyzed, such as the authors, countries/regions, institutions, cited references, and keywords; link lines between the nodes indicated cooperative, co-cited, or co-occurring relationships (36). The size of a node indicated the number of citation or occurrence, and the width of a line indicated the strength of the relationship (36, 37). The color of a node, represented by a series of annual rings, indicated the distribution time (36, 38). The importance of the publication was identified and we measured its betweenness centrality. A key node or pivot point in the scientific collaboration or reference co-citation network was marked with a purple ring if its centrality was ≥ 0.1 (39, 40). The calculated Q-value and silhouette-value were indicators representing the modularity and homogeneity of the cluster network, respectively. The larger the Q-value, the better the modularity. The closer the silhouette-value to zero, the higher the homogeneity. A Q-value > 0.3 identified the cluster structure as significant, and a mean silhouette > 0.5 or > 0.7 indicated that the clustering result was reasonable or highly credible, respectively (40). A citation burst had two attributes: the intensity and the duration of the burst. The burst detection revealed abrupt changes in terms or citations over a specified period, thereby identifying emerging research trends (41).

IBM SPSS Statistics, version 25.0 (SPSS Inc., Chicago, IL, USA) was used to depict and visualize the growth trend in annual publication outputs by using a polynomial regression model. We tested for possible correlation relationships between the total number of publication outputs and the betweenness centrality

for each country/region, institution, and author that reached a centrality threshold above zero. Pearson's correlation coefficient (r) was calculated in SPSS. A p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Analysis of Publication Outputs and Growth Trend Prediction

The search retrieved 1,962 publications that met the inclusion and exclusion criteria (**Figure 2**), and 1,520 and 442 articles and reviews, respectively, were identified. Over the past 20-year period, the development track showed two stages: one was the initial period (1999–2009), which had a very slow development speed, and the other was a rapid development period (2010–2019). The number of publication outputs increased from 31 publications in 1999 to 407 publications in 2019, with an average of 98 publications per year (**Figure 3A**). Compared with those in the year 1999, the cumulative growth rate reached 1212.903, 920.690, and 5450.000% in 2019 for total publications, articles, and reviews, respectively, and the compound annual growth rate (CAGR) was 13.740, 12.317, and 22.240%, respectively (**Figure 3B**). The CAGR was the annualized average rate of growth between the years 1999 and 2019, calculated as follows: CAGR = [(value in year 2019/value in year 1999) $^{(1/20)}$-1]. As presented in **Figure 3C**, the publication trend remained relatively limited and stable before the 2010s, whereas continual growth of research on gut microbiota in the depression field has occurred since then. The model fitting curves of growth in document number showed a strongly positive correlation with the year of publication ($R^2 = 0.976$, 0.964, and 0.980 for total publications, articles, and reviews, respectively). We conservatively estimated that the total publication, article, and review outputs will exceed 450, 325, and 125, respectively, in 2020. The top five WoS categories of the analyzed publications were neurosciences ($n = 322$, 16.412%), psychiatry ($n = 240$, 12.232%), pharmacology/pharmacy ($n = 194$, 9.888%), clinical neurology ($n = 123$, 6.269%), and gastroenterology/hepatology ($n = 119$, 6.065%).

Analysis of Scientific Collaboration Network

Fifty-two countries/regions contributed to the publications on gut microbiota research in the depression field. The top 10 countries/regions according to publications and centrality are listed in **Table 1**. The top five countries/regions, in order of the number of publications, were the USA, People's Republic of China, Canada, Australia, and England. The top five countries/regions by centrality were the USA, Germany, Australia, England, and Italy. Pearson's correlation analysis revealed a significant correlation between publications and centrality at the country/region level ($r = 0.861$, $p < 0.001$). Comprehensive analyses of publications and centrality indicated that the USA (publications: 580, centrality: 0.35), Australia (publications: 120, centrality: 0.16), and England (publications: 118, centrality: 0.16) were the most influential in the field. The collaboration network map among countries/regions is presented in **Figure 4A**, in which there were 52 nodes and 319 link lines. The nodes and the link lines between them represent the countries/regions and their cooperative relationships, respectively. The larger the node, the more publications. The wider the line, the stronger the relationships. Changes in node color represent the distribution time of publications. The node marked with a purple ring represents centrality ≥ 0.1. Overall, the USA (collaborators: 38), England (collaborators: 29), Germany (collaborators: 29), Canada (collaborators: 28), and Australia (collaborators: 28) had the largest number of national partners.

From 1999 to 2019, a total of 266 institutions published in this field. **Table 1** illustrates the top ten institutions according to publications and centrality. The top five institutions with the greatest contribution to this field were University College Cork, McMaster University, Deakin University, Harvard University, and University of Toronto. In terms of centrality, the top five institutions were University College Cork, McMaster University, Catholic University of Louvain, University of Copenhagen, and Harvard University. A significant correlation between publications and centrality was also observed at the institution level (Pearson $r = 0.734$, $p < 0.001$). On the basis of the analyses of publications and centrality, University College Cork (publications: 74, centrality: 0.19), McMaster University (publications: 47, centrality: 0.16), and Harvard University (publications: 34, centrality: 0.11) were the major research institutions. The generated institution network map identified 266 nodes and 581 link lines, which represented the institutions and their cooperative relationships, respectively; thus extensive collaborations between institutions were found (**Figure 4B**). For instance, University College Cork, represented by the largest node marked with a purple ring, had the most publications and the most extensive cooperation, with more than 25 institutions, such as TEAGASC, McMaster University, Harvard University, Deakin University, University of Auckland, University of Gothenburg, University of California-Los Angeles, and Catholic University of Louvain. Although Catholic University of Louvain did not have as many publications, it had many research partners, such as Medical University of Graz, University of Reading, University College Cork, McMaster University, University of Copenhagen, and University of Auckland.

In total, 368 authors published in this field in the past two decades. **Table 1** presents the top 10 authors according to publications and centrality. Eight authors published more than 10 publications in this field. Among these active authors, John F. Cryan was ranked first, with 60 publications, followed by Timothy G. Dinan and Michael Maes. The top three authors ranked by centrality were John F. Cryan, Jane A. Foster, and Michael Maes, whereas none had a centrality ≥ 0.1, thus indicating that international cooperation among top scientific researchers was insufficient. Furthermore, there was no significant correlation between publications and centrality at the author level (Pearson $r = 0.380$, $p = 0.249$). The co-authorship network map is shown in **Figure 4C**, containing 368 nodes and 924 collaboration lines. The nodes and the link lines between them represent the authors and their cooperative

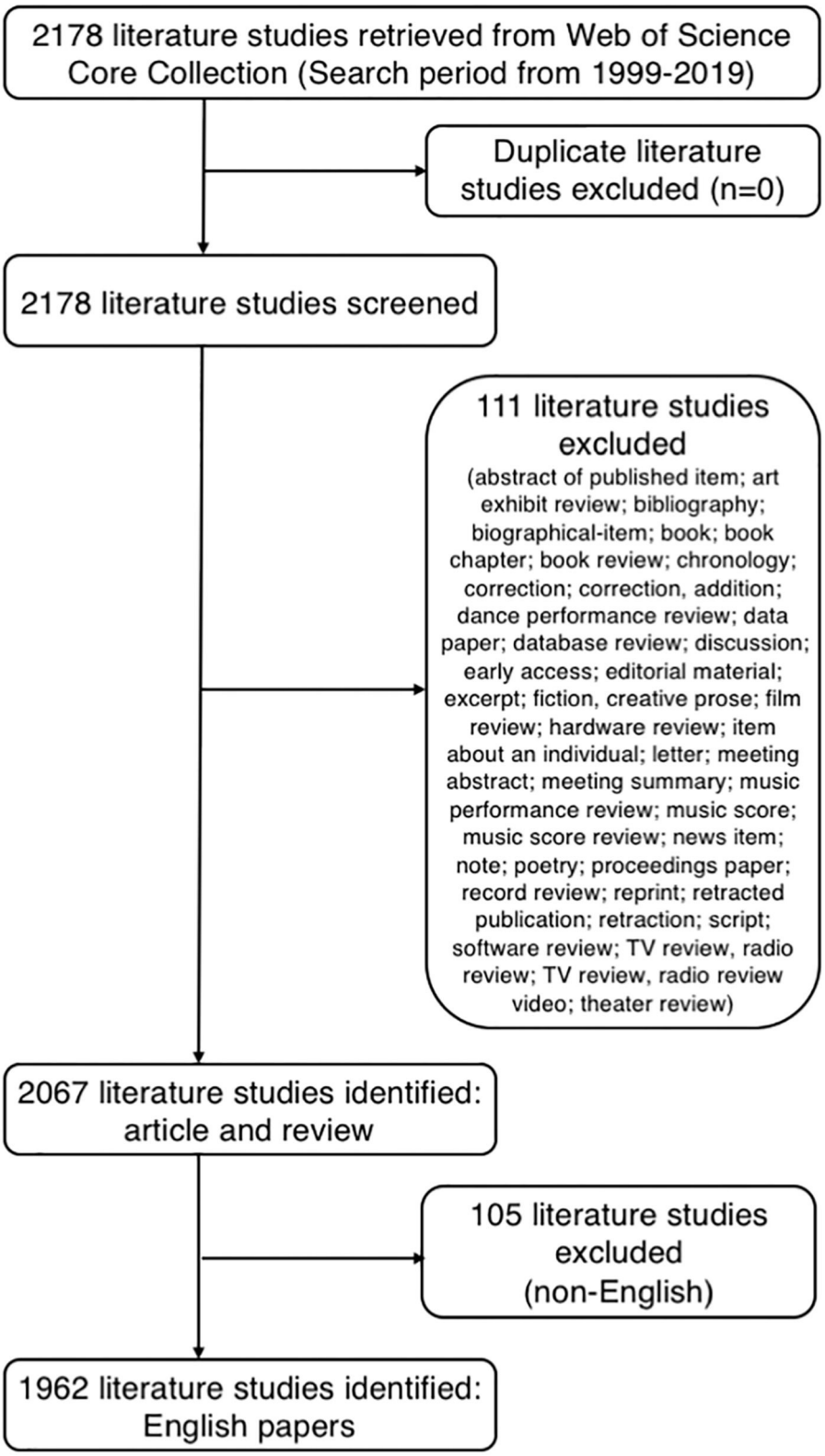

FIGURE 2 | Flowchart for including and excluding literature studies.

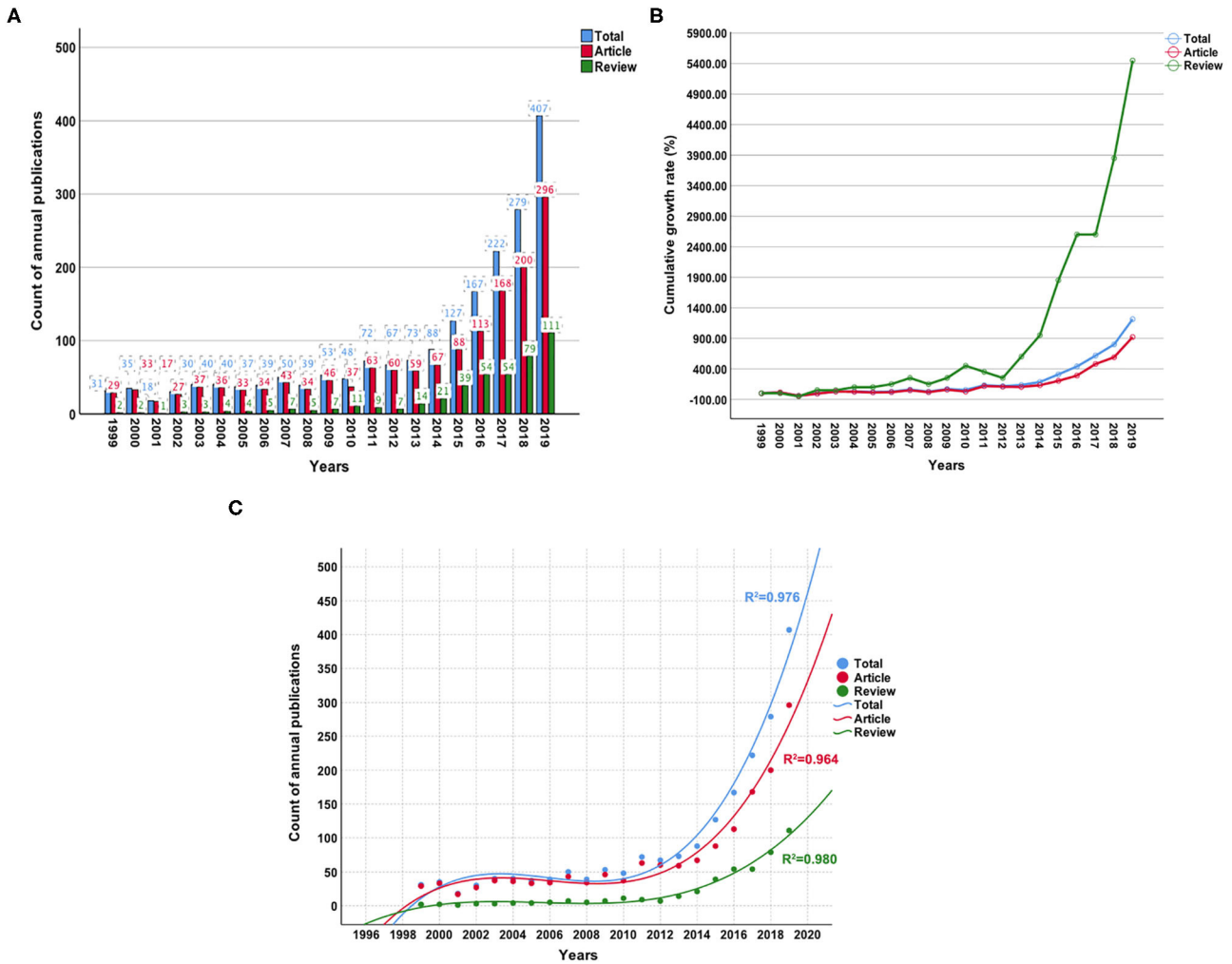

FIGURE 3 | Trends in the number of publications from 1999 to 2019 on gut microbiota research in the depression field. **(A)** The annual number of publications. **(B)** The cumulative growth rate for publications. **(C)** The polynomial fitting curves of growth trends.

relationships, respectively. None of the nodes are marked with a purple ring, owing to their very low centrality (<0.1). As presented in the map, Michael Maes, represented by the third largest node, had restricted relationships with John F. Cryan or Timothy G. Dinan. Notably, Jane A. Foster played a central role in international collaborations but did not have considerable publication productivity.

Analysis of Journals and Co-cited Journals

The articles included were published in 1,038 different journals, many of which were specialized journals. As one of the most important indicators, co-citation analysis has been widely used in bibliometrics. **Table 2** presents the top 10 journals and co-cited journals for gut microbiota research in the depression field. The most prolific journal in this field was *Brain Behavior and Immunity*, followed by *PLoS One*, *Poultry Science*, and *Scientific Reports*. All the highly productive journals had an IF above 2.0. Co-cited journals were those cited together by other researchers. The top three journals according to co-citation count were *Proceedings of the National Academy of Sciences of the United States of America* (*PNAS*), *PLoS One*, and *Nature*. The journals with betweenness centrality ≥ 0.1 included *PNAS* (centrality: 0.20), *British Journal of Nutrition* (citations: 432, centrality: 0.16), *Science* (centrality: 0.12), and *Nature* (centrality: 0.11). The published references of those top journals reflect the foundation of this field.

Analysis of Co-cited Authors

Co-cited authors are those cited together by other researchers. The co-citation analysis by author included 678 cited authors, 14 of whom were cited at least 200 times. The top 10 co-cited authors and their affiliates, major research fields, and *h*-indexes are listed in **Table 3**. The top three co-cited authors all came from Ireland. Lieve Desbonnet ranked first, with 315 citations, followed by Timothy G. Dinan, John F. Cryan, and Javier A. Bravo, whereas the remaining authors had fewer than 300

TABLE 1 | The top 10 countries/regions, institutions, and authors in terms of publications and centrality.

Items	Publications			Centrality		
	Ranking	Name	Number	Ranking	Name	Number
Country/Region	1	USA	580	1	USA	0.35
	2	Peoples R China	257	2	Germany	0.17
	3	Canada	158	3	Australia	0.16
	4	Australia	120	4	England	0.16
	5	England	118	5	Italy	0.12
	6	Italy	89	6	Canada	0.11
	7	Germany	86	7	Peoples R China	0.10
	8	Ireland	84	8	Belgium	0.07
	9	Japan	84	9	Brazil	0.05
	10	France	61	10	Ireland	0.04
Institution	1	University College Cork	74	1	University College Cork	0.19
	2	McMaster University	47	2	McMaster University	0.16
	3	Deakin University	37	3	Catholic University of Louvain	0.13
	4	Harvard University	34	4	University of Copenhagen	0.12
	5	University of Toronto	24	5	Harvard University	0.11
	6	University of Melbourne	24	6	University of Toronto	0.09
	7	Chulalongkorn University	23	7	University of Queensland	0.08
	8	Chinese Academy of Sciences	20	8	University of Auckland	0.08
	9	University of Copenhagen	15	9	University of Adelaide	0.08
	10	University of California, San Diego	13	10	Jiangnan University	0.08
Author	1	John F. Cryan	60	1	John F. Cryan	0.03
	2	Timothy G Dinan	57	2	Jane A Foster	0.03
	3	Michael Maes	30	3	Michael Maes	0.02
	4	Gerard Clarke	20	4	Timothy G Dinan	0.01
	5	Michael Berk	16	5	Michael Berk	0.01
	6	André F Carvalho	15	6	André F Carvalho	0.01
	7	George Anderson	13	7	John Bienenstock	0.01
	8	John Bienenstock	12	8	Paul Forsythe	0.01
	9	Gregers Wegener	9	9	Michael G Surette	0.01
	10	Catherine Stanton	8	10	Felice N Jacka	0.01

citations. The uppermost *h*-index value was for Michael Maes, followed by John F. Cryan and Timothy G. Dinan. Their research areas were mainly neurosciences, psychiatry, and pharmacology. These authors contributed to the work in this field, which provided the research basis.

Analysis of Co-cited References

References cited are often considered a core component of bibliometric research. Co-cited references are those co-cited in the reference lists of other articles. **Figure 5A** shows a cluster visualization of the reference co-citation network generated by CiteSpace software, which was divided into 90 clusters, of which only the largest six extracted from the references, on the basis of indexing terms and identified by a log-likelihood ratio algorithm. They are shown with different convex hulls in the figure, including cytokines (cluster #0), inflammation (cluster #1), forced swim test (cluster #2), chronic fatigue syndrome (cluster #4), lipopolysaccharide (LPS) (cluster #12), and gastrointestinal (cluster #21). The different nodes in the map represent cited references, and their authors are labeled in black. The representative authors in the largest six clusters are shown in **Figure 5A**. The details of the largest six clusters of references in the co-citation network are shown in **Table 4**. The total Q-value was 0.6901, and each cluster had a mean silhouette above 0.5, indicating that the cluster quality was reasonable. **Figure 5B** shows the top six clusters in a timeline view, which depicts clusters along with horizontal timelines and has a vertical arrangement according to descending size, thus indicating the scientific relevance of the published articles. **Figure 5B** and **Table 4** show that the most recent research focus was in cluster #0, "cytokines" (mean year 2014). The cluster and timeline visualization of the reference co-citation map contained

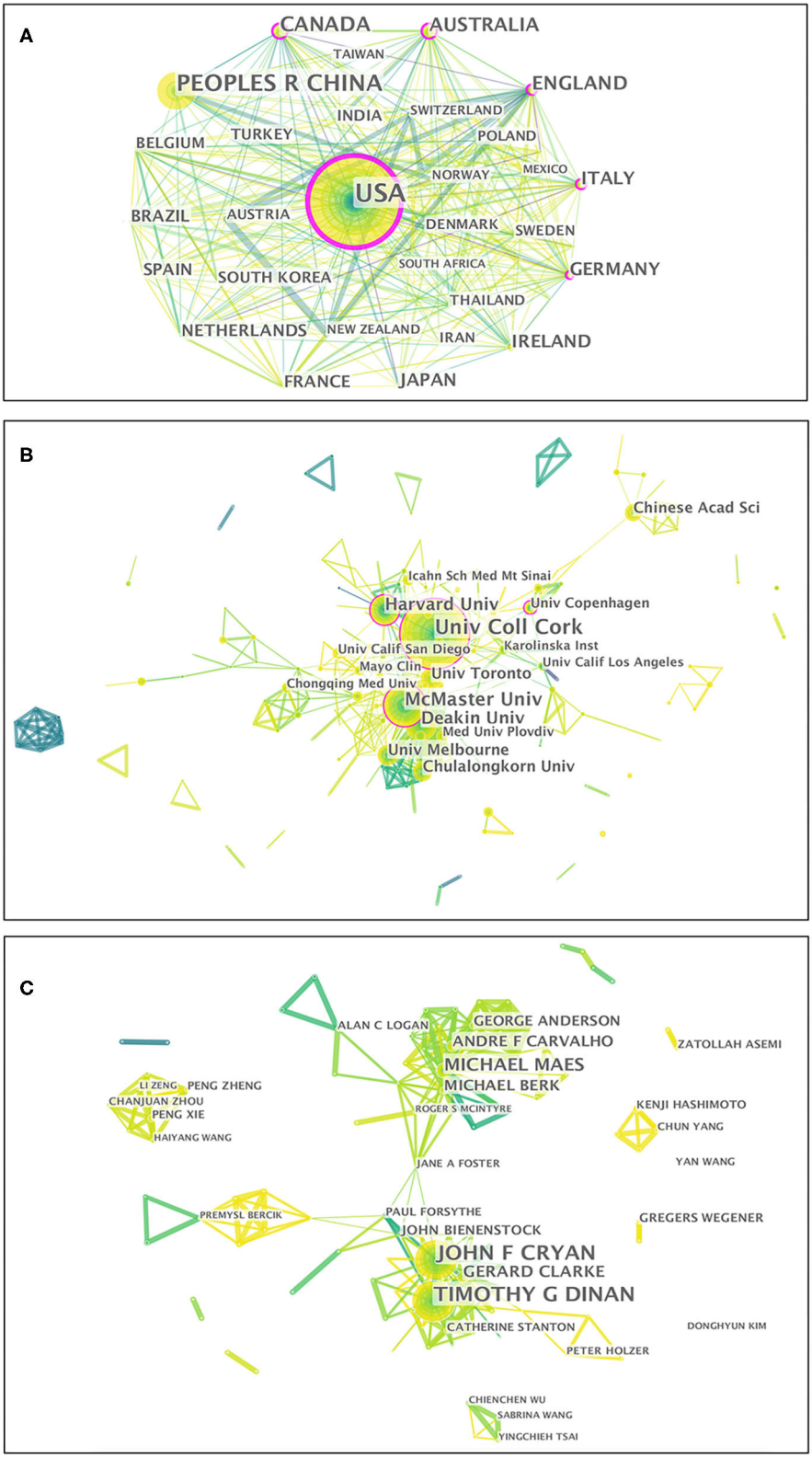

FIGURE 4 | Visualization map of the scientific collaboration network analysis of gut microbiota research in the depression field from 1999 to 2019. Collaboration among countries/regions **(A)**, institutions **(B)**, and authors **(C)**. The nodes in the map denote elements such as an author, country/region, or institute, and link lines between nodes denote collaborative relationships. The larger the circle, the more articles published. The wider the line, the stronger the relationship. The outermost purple ring denotes the centrality level, and highly central nodes are considered pivotal points in the research field.

TABLE 2 | The top 10 journals and co-cited journals for gut microbiota research in the depression field.

Items	Ranking	Name	Country	Counts	IF (2019)
Journal	1	*Brain Behavior and Immunity*	USA	34	6.633
	2	*Plos One*	USA	34	2.740
	3	*Poultry Science*	USA	23	2.659
	4	*Scientific Reports*	England	21	3.998
	5	*Journal of Affective Disorders*	Netherlands	16	3.892
	6	*Nutrients*	Switzerland	15	4.546
	7	*Neurogastroenterology and Motility*	England	14	3.008
	8	*Translational Psychiatry*	USA	14	5.280
	9	*Frontiers in Psychiatry*	USA	13	2.849
	10	*Journal of Animal Science*	USA	13	2.092
Co-cited Journal	1	*Proceedings of the National Academy of Sciences of the United States of America*	USA	797	9.412
	2	*Plos One*	USA	786	2.740
	3	*Nature*	England	683	42.778
	4	*Gastroenterology*	USA	623	17.373
	5	*Brain Behavior and Immunity*	USA	603	6.633
	6	*Science*	USA	566	41.845
	7	*Biological Psychiatry*	USA	566	12.095
	8	*Neurogastroenterology and Motility*	England	547	3.008
	9	*Gut*	England	532	19.819
	10	*Molecular Psychiatry*	England	464	12.384

TABLE 3 | The top 10 co-cited authors with the most citations.

Ranking	Times cited	Author	Institution (Country)	Major research fields	*h*-index
1	315	Lieve Desbonnet	National University of Ireland Galway/ Royal College of Surgeons (Ireland)	Neurosciences/Neurology, Psychiatry, Pharmacology/Pharmacy	16
2	314	Timothy G. Dinan	University College Cork (Ireland)	Neurosciences/Neurology, Psychiatry, Pharmacology/Pharmacy	93
3	312	John F. Cryan	University College Cork (Ireland)	Neurosciences/Neurology, Pharmacology/Pharmacy, Psychiatry	95
4	301	Javier A. Bravo	Pontificia Universidad Católica de Valparaíso (Chile)	Neurosciences/Neurology, Pharmacology/Pharmacy, Gastroenterology/Hepatology	19
5	276	Premysl Bercik	McMaster University (Canada)	Gastroenterology/Hepatology, Neurosciences/Neurology, Microbiology	43
6	260	Nobuyuki Sudo	Kyushu University (Japan)	Neurosciences/Neurology, Endocrinology Metabolism, General Internal Medicine	29
7	260	Michael Maes	Chulalongkorn University (Thailand)	Neurosciences/Neurology, Psychiatry, Pharmacology/Pharmacy	95
8	255	Michael Messaoudi	ETAP Appl Ethol (France)	Nutrition/Dietetics, Pharmacology/Pharmacy, Biochemistry Molecular Biology	19
9	222	Siobhain M. O'Mahony	University College Cork (Ireland)	Neurosciences/Neurology, Psychiatry, Pharmacology/Pharmacy	26
10	207	John R. Kelly	Trinity College Dublin (Ireland)	Psychiatry, Neurosciences/Neurology, Pharmacology/Pharmacy	9

410 nodes and 1,796 links, representing the cited references and their co-cited relationships, respectively.

The characteristics of the top 10 highly co-cited references concerning gut microbiota research in the depression field are summarized in **Table 5**. All were found in cluster #0, and the top-ranked reference was published by Javier A Bravo (42), with a co-citation count of 283. The articles with the maximum co-citations are generally important foundational studies in this field.

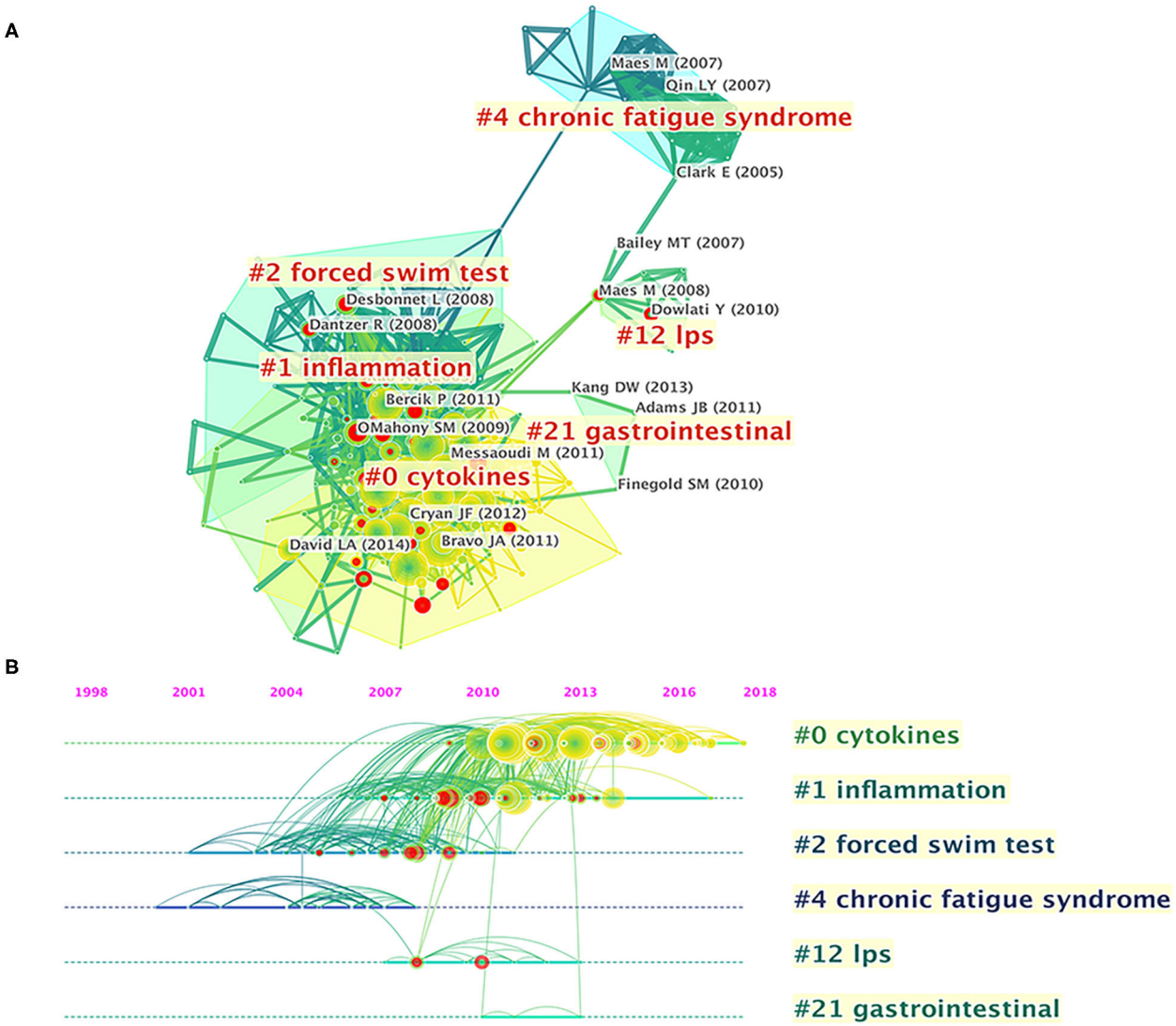

FIGURE 5 | Reference co-citation network analysis of publications on gut microbiota research in the depression field between 1999 and 2019. **(A)** Cluster visualization of the reference co-citation map. **(B)** Timeline visualization of the reference co-citation map. Top clusters and respective landmark references are labeled. The nodes in the map denote co-cited references, and links between nodes denote co-citation relationships. The citation rings denote the citation history of a reference. Large nodes or nodes with red tree-rings are either highly cited or have citation bursts in a given time slice.

TABLE 4 | The largest six clusters of references in the co-citation network.

Cluster	Size	Mean silhouette	Mean year	Label (LLR algorithm)	Representative reference
0	70	0.725	2014	Cytokines	Bravo et al. (42)
1	51	0.658	2010	Inflammation	Bercik et al. (43)
2	40	0.816	2007	Forced swim test	Desbonnet et al. (44)
4	21	0.981	2004	Chronic fatigue syndrome	Maes et al. (45)
12	7	0.989	2010	Lipopolysaccharide (LPS)	Maes et al. (46)
21	3	0.968	2011	Gastrointestinal	Finegold et al. (47)

"LLR" means log-likelihood ratio.

TABLE 5 | The top10 co-cited references related to gut microbiota research in the depression field between 1999 and 2019.

Ranking	Cited by	Authors	Title	Source title	Year of publication	Type of document
1	283	Bravo et al. (42)	Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse *via* the vagus nerve.	*Proceedings of the National Academy of Sciences of the United States of America*	2011	Article
2	235	Cryan and Dinan (48)	Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior.	*Nature Reviews Neuroscience*	2012	Review
3	207	Messaoudi et al. (49)	Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects.	*British Journal of Nutrition*	2011	Article
4	200	Diaz Heijtz et al. (50)	Normal gut microbiota modulates brain development and behavior.	*Proceedings of the National Academy of Sciences of the United States of America*	2011	Article
5	196	Jiang et al. (51)	Altered fecal microbiota composition in patients with major depressive disorder.	*Brain Behavior and Immunity*	2015	Article
6	177	Bercik et al. (52)	The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice.	*Gastroenterology*	2011	Article
7	169	Foster and McVey Neufeld (53)	Gut-brain axis: how the microbiome influences anxiety and depression.	*Trends in Neurosciences*	2013	Review
8	167	Clarke et al. (54)	The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner.	*Molecular Psychiatry*	2013	Article
9	164	Neufeld et al. (55)	Reduced anxiety-like behavior and central neurochemical change in germ-free mice.	*Neurogastroenterology and Motility*	2011	Article
10	156	Naseribafrouei et al. (56)	Correlation between the human fecal microbiota and depression.	*Neurogastroenterology and Motility*	2014	Article

Analysis of Co-occurring Keywords

A map of keyword co-occurrence reflects research hotspots. **Figure 6** presents a time-zone view of the keyword co-occurrence network to map the knowledge structure of research, resulting in 350 nodes and 2,168 links, representing the keywords and their co-occurring relationships, respectively. The larger the node, the greater the occurrence the keyword. In terms of co-occurrence frequency, the top 10 keywords were "depression," "gut microbiota," "anxiety," "inflammation," "microbiota," "stress," "irritable bowel syndrome," "gut-brain axis," "intestinal microbiota," and "brain" (**Table 6**). However, the keywords "fecal microbiota" and "microbiome" have become the top two research hotspots since 2013 (**Figure 6**), appearing in 98 and 94 citing studies, respectively (**Table 6**).

Analysis of Burst Detection

References with citation bursts, defined as those that have frequent citations over time, can be used to indicate the evolution of a knowledge domain (57). A total of 59 references with strong citation bursts were detected (**Figure 7**). The duration of the burst is displayed by a red line segment. References with citation bursts first appeared in 2007 (58, 59), and the most recent references with citation bursts appeared in 2017 (60–65). The strongest burst (strength: 18.7714) appeared in 2010 for a 2008 article (44). Nine references had a burst that lasted until 2019 (60–68). The references with citation bursts between 2010 and 2017 accounted for 93.22%.

Burst keywords can be used to predict new frontier topics in research in a particular field. **Figure 8** shows the top 68 keywords with the strongest citation bursts. "Antibiotics" was the strongest

TABLE 6 | The top 20 keywords in terms of frequency for gut microbiota research in the depression field.

Ranking	Keyword	Frequency	Ranking	Keyword	Frequency
1	Depression	532	11	Probiotics	163
2	Gut microbiota	370	12	Behavior	151
3	Anxiety	225	13	Double blind	103
4	Inflammation	201	14	Fecal microbiota	98
5	Microbiota	195	15	Microbiome	94
6	Stress	185	16	Health	93
7	Irritable bowel syndrome	169	17	Disease	93
8	Gut-brain axis	167	18	Expression	89
9	Intestinal microbiota	167	19	Quality of life	88
10	Brain	164	20	Antibiotics	82

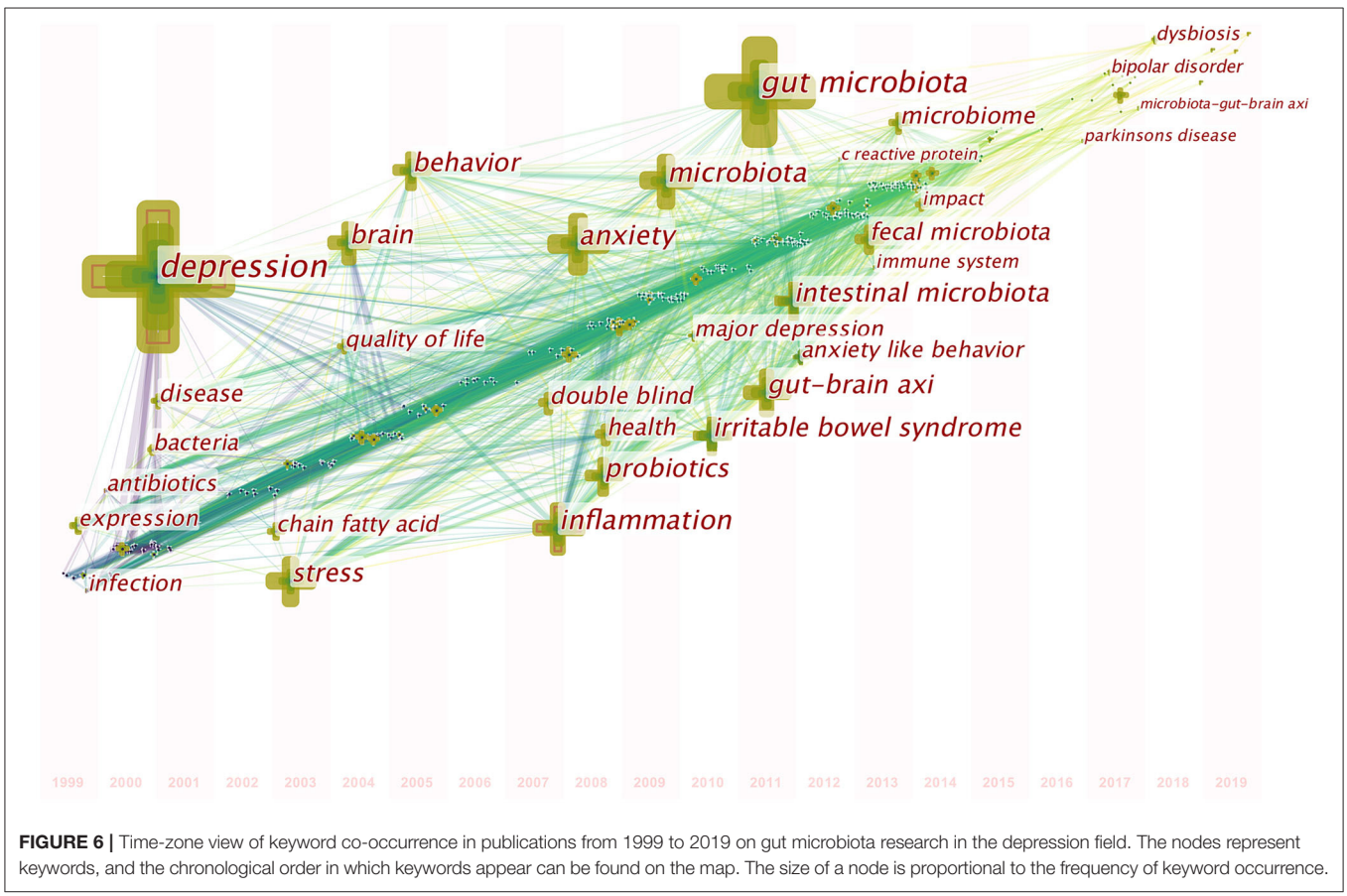

FIGURE 6 | Time-zone view of keyword co-occurrence in publications from 1999 to 2019 on gut microbiota research in the depression field. The nodes represent keywords, and the chronological order in which keywords appear can be found on the map. The size of a node is proportional to the frequency of keyword occurrence.

burst keyword (strength: 16.4535) in this field from 1999 to 2012, and was followed by "infection (strength: 15.5516)," "anxiety-like behavior (strength: 13.0703)," and "performance (strength: 11.1118)." The keywords with a burst lasting until 2019 included "Parkinson's disease," "microbiota-gut-brain axis," "microbiome," "dysbiosis," "bipolar disorder," "impact," "C reactive protein (CRP)," and "immune system," reflecting the most recent research trends. The bursts in certain keywords can also be used to analyze the evolution of research. For instance, **Figure 8** reveals that rodents such as mice and rats have replaced non-rodent animals such as piglets, cattle, and horses, as the preferred animal models in gut microbiota-depression research. The results also suggest that studies in recent decades, have increasingly focused on the relationships between the gut microbiota and potential depression-related disorders (e.g., Alzheimer's disease, inflammatory bowel disease, irritable bowel syndrome, Parkinson's disease, and bipolar disorder), as well as the underlying mechanisms, involving the immune system, intestinal permeability, bacterial translocation, cytokines such as necrosis factor-alpha, CRP, and the microbiota-gut-brain axis.

References	Year	Strength	Begin	End	1999 - 2019
Rothstein JD, 2005, NATURE, V433, P73	2005	3.4304	**2007**	2013	
Logan AC, 2005, MED HYPOTHESES, V64, P533	2005	9.166	**2007**	2013	
OMahony L, 2005, GASTROENTEROLOGY, V128, P541	2005	6.3262	**2008**	2013	
Benton D, 2007, EUR J CLIN NUTR, V61, P355	2007	11.3528	**2008**	2015	
Desbonnet L, 2008, J PSYCHIATR RES, V43, P164	2008	18.7714	**2010**	2016	
Goehler LE, 2005, BRAIN BEHAV IMMUN, V19, P334	2005	3.715	**2010**	2012	
Sudo N, 2004, J PHYSIOL-LONDON, V558, P263	2004	9.9268	**2010**	2012	
Dantzer R, 2008, NAT REV NEUROSCI, V9, P46	2008	12.1758	**2010**	2016	
Collins SM, 2009, GASTROENTEROLOGY, V136, P2003	2009	3.9433	**2010**	2011	
Gareau MG, 2011, GUT, V60, P307	2011	6.2803	**2011**	2016	
Goehler LE, 2008, BRAIN BEHAV IMMUN, V22, P354	2008	7.3379	**2011**	2014	
Maes M, 2008, NEUROENDOCRINOL LETT, V29, P117	2008	10.4851	**2011**	2016	
Li W, 2009, PHYSIOL BEHAV, V96, P557	2009	4.7925	**2011**	2013	
Forsythe P, 2010, BRAIN BEHAV IMMUN, V24, P9	2010	7.8636	**2011**	2014	
Lyte M, 2006, PHYSIOL BEHAV, V89, P350	2006	5.762	**2011**	2014	
Gareau MG, 2007, GUT, V56, P1522	2007	7.3379	**2011**	2014	
Verdu EF, 2006, GUT, V55, P182	2006	7.3379	**2011**	2014	
Bercik P, 2010, GASTROENTEROLOGY, V139, P2102	2010	14.3072	**2011**	2016	
Cani PD, 2008, DIABETES, V57, P1470	2008	3.1352	**2012**	2013	
Rousseaux C, 2007, NAT MED, V13, P35	2007	8.6929	**2012**	2015	
OMahony SM, 2011, PSYCHOPHARMACOLOGY, V214, P71	2011	4.415	**2012**	2014	
McKernan DP, 2010, NEUROGASTROENT MOTIL, V22, P1029	2010	8.6929	**2012**	2015	
Kunze WA, 2009, J CELL MOL MED, V13, P2261	2009	6.3989	**2012**	2015	
Maes M, 2009, METAB BRAIN DIS, V24, P27	2009	3.763	**2012**	2013	
Rao A Ravishankar, 2009, ..., V0, P1	2009	8.423	**2013**	2015	
Rhee SH, 2009, NAT REV GASTRO HEPAT, V6, P306	2009	7.868	**2013**	2017	
Cryan JF, 2011, NEUROGASTROENT MOTIL, V23, P187	2011	3.926	**2013**	2015	
Neufeld Karen-Anne M, 2011, COMMUN INTEGR BIOL, V4, P492	2011	4.9478	**2013**	2015	
Barrett E, 2012, J APPL MICROBIOL, V113, P411	2012	4.0289	**2013**	2015	
OMahony SM, 2009, BIOL PSYCHIAT, V65, P263	2009	10.8672	**2013**	2017	
Jeffery IB, 2012, GUT, V61, P997	2012	7.8615	**2014**	2015	
Davari S, 2013, NEUROSCIENCE, V240, P287	2013	5.395	**2014**	2016	
Collins SM, 2013, CURR OPIN MICROBIOL, V16, P240	2013	5.7605	**2014**	2015	
De Filippo C, 2010, P NATL ACAD SCI USA, V107, P14691	2010	6.2854	**2014**	2015	
Ohland CL, 2013, PSYCHONEUROENDOCRINO, V38, P1738	2013	8.8789	**2014**	2016	
Dominguez-Bello MG, 2010, P NATL ACAD SCI USA, V107, P11971	2010	5.7605	**2014**	2015	
Mayer EA, 2011, NAT REV NEUROSCI, V12, P453	2011	3.9692	**2014**	2015	
Park AJ, 2013, NEUROGASTROENT MOTIL, V25, P733	2013	9.2668	**2014**	2016	
Arseneault-Breard J, 2012, BRIT J NUTR, V107, P1793	2012	6.8106	**2014**	2015	
Maes M, 2012, J AFFECT DISORDERS, V141, P55	2012	6.3812	**2015**	2019	
Ait-Belgnaoui A, 2014, NEUROGASTROENT MOTIL, V26, P510	2014	3.8716	**2015**	2016	
Rao AV, 2009, GUT PATHOG, V1, P0	2009	9.4145	**2015**	2017	
Moloney RD, 2014, MAMM GENOME, V25, P49	2014	8.3047	**2015**	2016	
Borre YE, 2014, TRENDS MOL MED, V20, P509	2014	5.8807	**2015**	2017	
Wikoff WR, 2009, P NATL ACAD SCI USA, V106, P3698	2009	9.6931	**2015**	2016	
Desbonnet L, 2014, MOL PSYCHIATR, V19, P146	2014	9.4165	**2015**	2017	
Dinan TG, 2013, NEUROGASTROENT MOTIL, V25, P713	2013	12.236	**2015**	2017	
Crumeyrolle-Arias M, 2014, PSYCHONEUROENDOCRINO, V42, P207	2014	6.6599	**2015**	2016	
Mayer EA, 2014, J NEUROSCI, V34, P15490	2014	6.1365	**2015**	2017	
Braniste V, 2014, SCI TRANSL MED, V6, P0	2014	9.1098	**2016**	2019	
De Palma G, 2015, NAT COMMUN, V6, P0	2015	9.2126	**2016**	2017	
Stilling RM, 2014, GENES BRAIN BEHAV, V13, P69	2014	8.2562	**2016**	2019	
Schmidt K, 2015, PSYCHOPHARMACOLOGY, V232, P1793	2015	9.2126	**2016**	2017	
Steenbergen L, 2015, BRAIN BEHAV IMMUN, V48, P258	2015	5.1799	**2017**	2019	
Dowlati Y, 2010, BIOL PSYCHIAT, V67, P446	2010	7.4351	**2017**	2019	
Carabotti M, 2015, ANN GASTROENTEROL, V28, P203	2015	7.1275	**2017**	2019	
Sampson TR, 2015, CELL HOST MICROBE, V17, P565	2015	6.5639	**2017**	2019	
Qin JJ, 2010, NATURE, V464, P59	2010	8.161	**2017**	2019	
Kelly JR, 2015, FRONT CELL NEUROSCI, V9, P0	2015	3.6232	**2017**	2019	

FIGURE 7 | References with the strongest citation bursts in publications from 1999 to 2019 on gut microbiota research in the depression field. The strength values reflect the frequency of citation. The red bars indicate references cited frequently; the green bars indicate references cited infrequently.

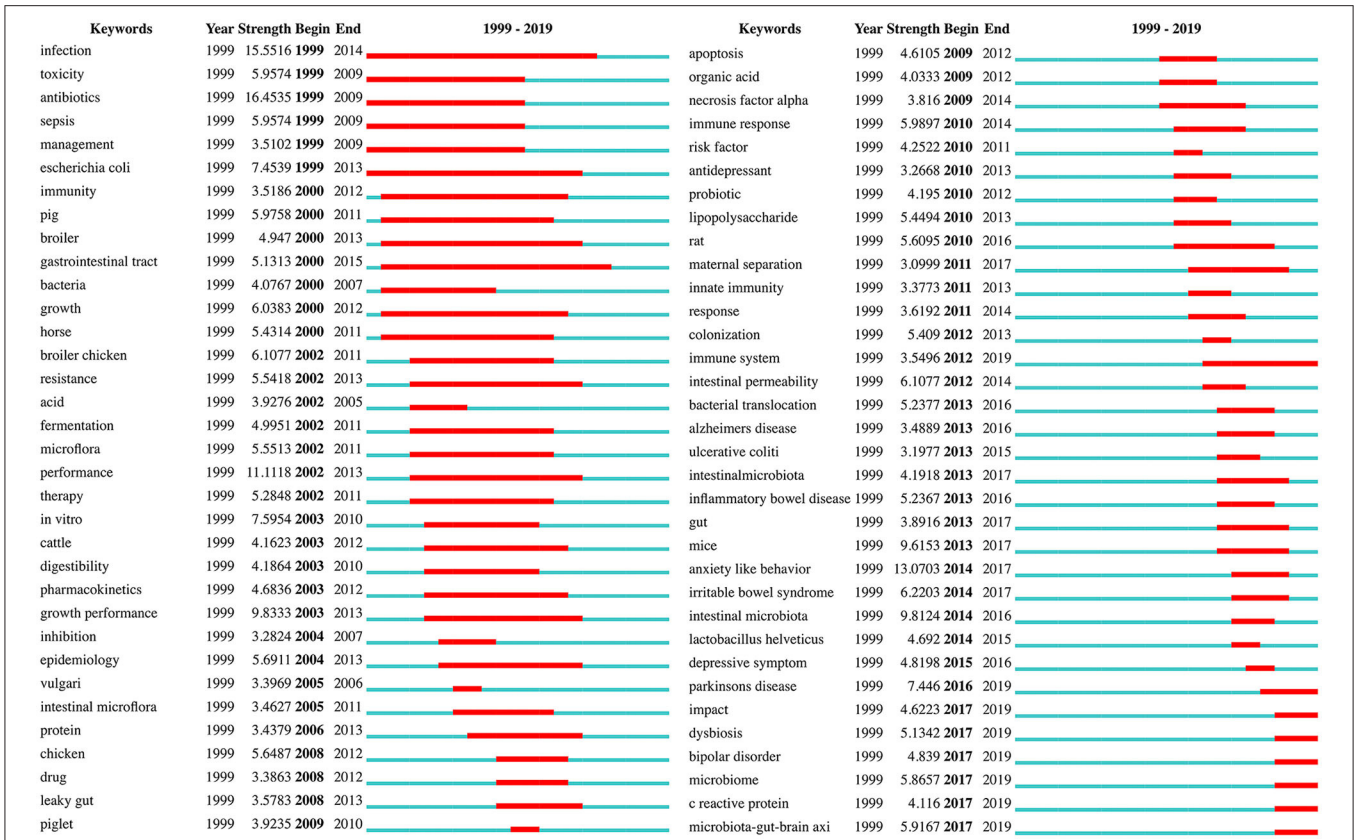

FIGURE 8 | Keywords with the strongest citation bursts in publications from 1999 to 2019 on gut microbiota research in the depression field.

DISCUSSION

This is the first application of bibliometric and visual analysis methods to gut microbiota research in the depression field. A total of 1,962 publications originating from WoSCC were analyzed, and we present a comprehensive overview of the worldwide hotspots and trends in gut microbiota research in the field of depression over the past two decades. Our analysis revealed the rapid growth in the number of publications since 2010 and the close international scientific cooperation in this field. The USA, University College Cork, and John F. Cryan were the most influential country, institute, and scholar, respectively. *Brain Behavior and Immunity* and *PNAS* were the most productive and co-cited journals, respectively. The most recent research focus was on the largest theme cluster "cytokines," thus reflecting the important research foundation in this field. Since 2013, the top two research hotspots have been "fecal microbiota" and "microbiome." Additionally, "Parkinson's disease," "microbiota-gut-brain axis," "microbiome," "dysbiosis," "bipolar disorder," "impact," "CRP," and "immune system" reflected the most recent research trends in the field. Thus, our results provided insights and valuable information regarding gut microbiota research in depression; these findings may help researchers choose suitable cooperators or journals, and promote their research through our timely review and analysis of the hotspots and research trends.

Collaboration network analysis can provide detailed information for evaluating research collaborations and identifying the key cooperators. Among countries/regions, the USA had an absolute advantage in this field, possibly because of its better economics and expenditure in scientific research; for example, a special research project on the gut microbiota-brain axis was launched in 2013 (69), thus resulting in a positive influence on research productivity and quality (70). Remarkably, China, as a developing country, has shown vast progress in this field over the past two decades. Although China was the second most productive country, its influence in this field was inferior to that of the USA because of its insufficient international cooperation. Among institutions, University College Cork was a key player, possibly because the main researchers worldwide (including John F. Cryan, Timothy G. Dinan, Gerard Clarke, and Siobhain M. O'Mahony) in this area worked at this institute, whereas the institutions and researchers in the USA were quite scattered. Overall, close international scientific cooperation was found in this field. However, we discovered that the cooperation between top researchers appears to be insufficient, a finding that may be associated with their research being comparatively full-fledged (40). For example, we found that John F. Cryan and Timothy G. Dinan, who were academic leaders at the Alimentary

Pharmabiotic Centre, University College Cork, Ireland, had the most productive publications and highest citations in this field. They were the first to define the term "psychobiotic," which refers to a live organism that produces positive effects on the mental health of people with psychiatric illness when ingested in adequate amounts (71). In addition, their research team has conducted many studies in the field of psychobiotics, funded by Science Foundation Ireland, the Health Research Board of Ireland, and the European Community's Seventh Framework Programme, in close collaboration with several companies including GlaxoSmithKline, Pfizer, Cremo, Wyeth, and Mead Johnson (26, 72).

Journal analysis and co-cited journal analysis can provide important information that can help researchers select appropriate journals for article submission. Through our research, we discovered that the top 10 most active journals published less than a quarter (10.04%) of the total publications on gut microbiota research in the depression field, thus indicating a clearly dispersed distribution of the literature distribution across journals, possibly because of the diversified research directions involving neurosciences, psychiatry, pharmacology/pharmacy, clinical neurology, and gastroenterology/hepatology. Thus, on the one hand, researchers may have many journal choices, and on the other hand, they may have difficulty in choosing the most appropriate journal because of a lack of knowledge or experience (38). In addition, there was only a 30% concordance rate between the top 10 most active journals and the top 10 co-cited journals, thus suggesting that the quality of research in this field still must be improved, and the international cooperation among researchers should be strengthened to produce high-quality research (38).

According to reference co-citation network analysis, all the top 10 highly co-cited references were located in the largest theme cluster #0, "cytokines," which formed an important basis for studies in this field. Mounting evidence suggests that the levels of some pro-inflammatory cytokines [e.g., interleukin (IL)-1β, IL-6, IL-18, and tumor necrosis factor (TNF)-α] are increased in depressive disorder, whereas some anti-inflammatory cytokines [e.g., IL-10 and transforming growth factor (TGF)- β1] are decreased (73, 74). Some meta-analyses have provided evidence that the alterations in the levels of peripheral cytokine (e.g., IL-8, TNF-α, IL-6, and IL-10) are associated with the antidepressant treatment response in patients with major depressive disorder (MDD) (75, 76). IL-1β, IL-6, and TNF-α are the three main cytokines that play a key role in mediating signaling *via* the gut microbiome-to-brain communication pathways (77). Other cytokine signaling pathways have also been widely explored. For example, a previous study has demonstrated that live *Lactobacillus rhamnosus* (*JB-1*) can modulate the immune system by inhibiting the synthesis of TNF-induced IL-8 in the human epithelial cell lines T84 and HT-29 (78). Among the top ten co-cited references, the first was an article published by Bravo et al. in *PNAS* in 2011 (42), in which the authors further found that *L. rhamnosus* (*JB-1*) modulates the GABAergic system in mice *via* the vagus nerve, thereby exerting beneficial effects in the treatment of stress-related psychiatric disorders such as depression and anxiety. The second most co-cited reference was a review published by Cryan and Dinan in *Nature Reviews Neuroscience* in 2012 (48), in which the authors describe how the gut microbiota influences brain function and behavior through neural (vagus and enteric nervous system), endocrine (cortisol), and immune (cytokines) pathways involving bidirectional communication between the gut microbiota and the brain. Those publications have laid important research foundations in this field.

Furthermore, the dynamics of this field was partly characterized by references with citation bursts (57, 79). The reference with the strongest burst, occurring in 2010, was an article published by Desbonnet et al. in the *Journal of Psychiatric Research* in 2008 (44), in which the authors revealed that *Bifidobacteria* treatment attenuates pro-inflammatory immune responses and elevates serotonergic precursors and tryptophan, thus suggesting that depressed people, particularly those with accompanying gastrointestinal inflammation, may benefit from this probiotic, owing to its potential antidepressant properties. An article published by Maes et al. in the *Journal of Affective Disorders* in 2012 (66), had a citation burst starting in 2015 that is still ongoing. Their findings suggest that in mesenteric lymph nodes, "translocated" commensal bacteria activate immune cells and elicit IgA and IgM responses, and increased bacterial translocation may play a role in the pathophysiology of chronic depression by causing progressive amplification of inflammatory and cell-mediated immune pathways. The most recent citation bursts occurred in 2017, among which the strongest burst was due to an article published by Qin et al. in *Nature* in 2010 (64). This was the largest metagenomic survey study to date of human gut microbial genes established by metagenomic sequencing, thus setting the stage for comparing gut microbiome profiles between healthy and diseased individuals (80, 81).

According to the co-occurring keyword analysis, we identified some of the most important hotspots in this field over the past two decades, including depression, gut microbiota, anxiety, and inflammation. We found that the publication outputs have increased rapidly since 2010, partly because of the key hotspot, the second highest frequency keyword "gut microbiota," occurring in 2011. Since 2013, "fecal microbiota" and "microbiome" have become the new top hotspots in this field. The fecal microbiota, as a proxy for the gut microbiota, have been shown to be significantly associated with depression (51, 56). Moreover, fecal microbiota transplants (FMT) technology, an ancient administration route traced back to fourth century China (82), is receiving increasing attention. In this method, stool is transferred from a healthy donor into a recipient, with the goal of replacing the recipient's "bad" bacteria with "good" bacteria to normalize the gut microbiota composition and gain therapeutic benefits (83). Accumulating clinical studies suggest that FMT has potential therapeutic effects on depression-related disorders, owing to the increase in microbiota diversity (84, 85). FMT is also called stool transplantation and is an emerging tool used in animal models in gut microbiota research. For example, Li et al. (86) have used an antibiotic cocktail to deplete the gut microbiota in mice, and then colonized the gut, *via* FMT, with the microbiota from mice exposed to chronic unpredictable

mild stress. Subsequently, similar anxiety- and depression-like behavior was observed, possibly because of the significant elevation in pro-inflammatory cytokines modulated by the gut microbiota. Another hotspot is "microbiome." The amalgam of microorganisms in a particular habitat (such as the skin, mouth, vagina, or gut) is defined as a microbiota (87). Although the term microbiome refers specifically to the collective genomes of all microorganisms in a microbiota, it is sometimes used as a synonym for microbiota (88). Microbiome-host interaction, although not a novel concept, has recently been revisited in a surge of studies, particularly those focusing on the role of the gut microbiome in regulating the maturation and functionality of the host immune system (89). Notably, the gut microbiome composition is also under the influence of multiple intrinsic and extrinsic factors, such as host genetics (90), prolonged physiological stress (91), alcohol (92), dietary habits (93), and other lifestyle factors. Additionally, an integrated analysis of gut microbial community profiling and the host metabolomic signatures can help to provide a comprehensive understanding of microbiome-host interaction, thus providing a basis for studying the pathogenesis of depression (94). Notably, the traditional concepts will probably need to be revised by considering the reciprocal interactions between the gut microbiome and xenobiotics, termed the "microbiome-xenobiotic interactions" (95). For example, a recent study has shown that administration of psychotropics (such as fluoxetine, lithium, valproate, and aripiprazole) significantly alters the gut microbiome composition (96). Despite the increasing recognition of these hotspots, as these research directions develop, further innovations and breakthroughs may be hindered. Therefore, there is a need to pay more attention to frontier Research Topics identified by the burst keyword analysis in the years ahead.

Of note, many strong burst keywords associated with emerging areas of gut microbiota-depression-related research have appeared in recent years, including anxiety-like behavior, intestinal microbiota, and mice. Moreover, some keywords, such as the abovementioned hotspot "microbiome," had bursts that are currently ongoing, among which the top keyword was "Parkinson's disease."

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease, with a median incidence in developed countries of 14 per 100,000 people in the total population, and 160 per 100,000 people at least 65 years of age (97). Apart from classical motor symptoms such as tremors, bradykinesia, and rigidity, some non-motor symptoms, including depression, anxiety, apathy, and psychosis, also affect quality of life in people with Parkinson's disease (98). Depression occurs in approximately 35% of people with Parkinson's disease, and the alterations in neurotransmitter systems, as well as neurotrophic factors, neuropeptides, immunomodulatory mediators, and stress hormones, may be associated with the depression observed in these individuals (99). Previous evidence supports the antidepressant properties of minocycline (a broad-spectrum tetracycline antibiotic), owing to its suppression of microglial activation, and neuro-protective or anti-inflammatory actions (100, 101). A population-based cohort study in older people (HEllenic Longitudinal Investigation of Aging and Diet) has found that adherence to the Mediterranean diet is associated with a lower probability of non-motor markers of prodromal Parkinson's disease, mainly depression and constipation (102). This finding may be due to decreased matrix metalloproteinase 9 activity (103), thereby maintaining the intestinal microbiota diversity and decreasing the severity of depression (104, 105). All the above findings suggest that manipulation of the microbiota may be beneficial in alleviating mood disorders in Parkinson's disease (104). Thus, modifying the microbiota to modulate depression in Parkinson's disease may be a future research direction. Moreover, depression, as a prodromal biomarker, can be used to identify the risk of developing Parkinson's disease, and assessment of the gut microbiota may also be considered an early biomarker of this disease in the future (104).

Other frontier topics in this field should also be noted, such as "microbiota-gut-brain axis," "dysbiosis," "CRP," and "immune system," which have currently ongoing bursts. Depression is a form of mood disorders that probably results from immunological deregulation (89). The immune system (including both the innate and adaptive immune system) is an important part of the microbiota-gut-brain axis that mediates the communication between the gut microbiota and CNS (106). The communication between the brain and the immune system occurs through different pathways, including neural and humoral pathways (107). The gut microbiota can activate epithelial cell gene expression *via* molecules such as Toll-like receptor (TLR), an innate immune system protein (108), thus inducing the production and release of pro-inflammatory cytokines (107). These cytokines then cross the blood brain barrier (BBB), possibly *via* a volume diffusion mechanism (109). Increased gut dysbiosis can also increase mucosal barrier permeability, thereby allowing bacteria and their pro-inflammatory products into the circulatory system and contributing to a chronic pro-inflammatory state (110). In individuals with MDD compared with healthy controls, higher elevated peripheral levels of CRP, an immune-inflammatory marker, have been confirmed by meta-analysis (111). CRP has also been demonstrated to have a relationship with depressive symptoms in obese people (112, 113). The probable pathway linking inflammation to these symptoms is an obesity-depression-inflammation cycle, involving systemic low-grade inflammation, partly owing to the changes in the gut microbiota (113). Thus, future clinical studies should focus on the feasibility of using anti-inflammatory strategies for patients with obesity and MDD, who might possibly benefit from reducing systemic inflammatory biomarkers such as CRP.

Our study has several limitations. First, publications were retrieved from only the WoSCC database, which may have led to bias and incompleteness in the included studies. In addition, our retrieval strategy may not have identified all the relevant references, owing to the limited terms, types of literature, and language; thus, our findings may not be comprehensive. Second, we cannot ensure that each publication retrieved was completely relevant to the topic meeting the search criteria. Third, some authors have the same name, and some keywords have different expressions, for example, "gut-brain axis" and "brain-gut axis"; thus, bias may have still existed despite our normalization

procedures. Future studies should consider more databases, such as Scopus, more accurate and comprehensive search terms, and the latest software versions, in which bugs are addressed. However, we believe that our findings reflect the overall state of, and general trends in, this field, because the number of publications in our analysis was sufficiently large. We also believe that these limitations may be noted in future similar studies and addressed wherever possible.

CONCLUSIONS

This study comprised a bibliometric and visual analysis of gut microbiota research in the field of depression over the past two decades. The number of publications has been rapidly growing since 2010. Overall, close international scientific cooperation was observed in this field, but more cooperation among researchers may be necessary. There were multiple journals for researchers to choose from because of the diversified research directions. The most recent research focus was on "cytokines," thus reflecting the important research foundation in this field. "Fecal microbiota" and "microbiome" have become the top two research hotspots since 2013. However, "Parkinson's disease," "microbiota-gut-brain axis," "microbiome," "dysbiosis," "bipolar disorder," "impact," "CRP," and "immune system" were identified as new frontiers of research, whose bursts are currently ongoing. Therefore, our timely review and analysis of the hotspots and research trends may promote the development of this field.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DS and YW conceived and designed the study. XZ wrote the original draft preparation. HL assisted in literature searching. JH conducted analysis based on WoS. XN, LL, and MZ conducted the CiteSpace analysis. SH, HC, and TX provided the figures and tablets. CQ, ZW, SD, and YT reviewed and edited the manuscript for content and style. All authors contributed to the article and approved the submitted version.

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Blunted Expansion of Regulatory T Lymphocytes Is Associated With Increased Bacterial Translocation in Patients With Major Depressive Disorder

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Background: Major Depressive Disorder (MDD) is associated with both proinflammatory and adaptive immune response abnormalities. Regulatory T lymphocytes (Tregs), a subtype of CD4+ T cells, are relevant for maintaining immune-inflammatory system homeostasis and control of inflammation such as the kind potentially induced by the interactions between the intestinal microbiome and gut mucosa. We investigated the Treg population and its distribution along their stages of differentiation/activation, as well as its function in MDD patients without concomitant diseases. We also studied the potential association between Treg alterations, intestinal barrier damage, and bacterial translocation.

Methods: 30 MDD patients and 20 healthy controls were studied. The levels of circulating CD25FoxP3^{+} Tregs and their distribution on the naïve (T_N), effector (T_E), central (T_{CM}), and effector memory(T_{EM}) differentiation/activation stages were analyzed using polychromatic flow cytometry. Chemokine receptors (CCR) 2, 5, and 6, and the intracytoplasmic IL-10 expression by the Tregs were also analyzed. The serum IL-10 was measured using Luminex. The serum levels of zonulin and the intestinal fatty acid-binding protein (I-FABP), both markers of gut barrier function, and the LPS-binding protein (LBP), a marker of bacterial translocation, were measured using an enzyme-linked immunosorbent assay.

Results: MDD patients had increased number of circulating Tregs cells with enhanced number of Tregs at the T_N, T_E, T_{CM}, and T_{EM} stages. The percentage of Tregs cells at T_N stage was significantly higher in MDD patients. The percentage of Tregs that expressed CCR2 and CCR6 was increased as well as those expressing IL-10. MDD patients had significantly increased levels of circulating I-FABP

and LBP. MDD patients with high LBP levels had a significant reduction in the number of circulating Tregs compared to normal-LBP MDD patients.

Conclusions: MDD patients showed an expansion of circulating Tregs and their $CD25^{high}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets throughout the different stages of CD4+ T lymphocyte differentiation/activation. Tregs also showed an increased frequency of cells expressing CCR6 and CCR2. IL-10 Treg production was also enhanced in MDD patients that concurrently had increased serum IL-10 levels. However, this Treg expansion was blunted in MDD patients with gut barrier damage and increased bacterial translocation.

Keywords: major depressive disorder, regulatory T lymphocytes (Treg), chemorreceptors, LPS-binding protein, gut barrier, CD4+ lymphocytes, interleukin 10 (IL10)

INTRODUCTION

Major depressive disorder (MDD) is a disease with high prevalence. The lifetime risk of depression is 15–18%, meaning that almost one in five people will experience at least one episode at some point in their lifetime (1). It is considered to be the third leading cause of disability worldwide (2). There is increasing interest in developing a more effective MDD treatment in order to improve the poor response experienced in approximately a third of the patients (3). Many neurobiological systems have been implicated in the etiopathogenesis and pathophysiology of MDD (1). New therapeutic approaches that target MDD pathogenic mechanisms beyond monoamine modulation are needed, in part, because of the shortcomings of selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitor, and tricyclic antidepressant treatments.

Experimental and human data have highlighted the importance of aberrant immune-inflammatory response in the development of depression (4). The main finding supporting the abnormal function of the immune system in MDD patients is the consistency among articles reporting increased circulating levels of proinflammatory cytokines (5). The risk of depression is increased in many disorders with an inflammatory component (6–9). Much of the research has focused on the activation of the innate immune system (10). However, intriguing new evidence suggests that the disease profile of MDD patients includes an immunosuppressive component, especially when involving the adaptive immune system (11). This pathogenic perspective is supported by epidemiological data of greater susceptibility to viral infections, reduced immune responses to vaccines and the slowed wound healing in MDD patients (12–14). In addition, depressed individuals with infectious diseases and tumors show a worse prognosis (15, 16). The understanding of the cellular mechanisms involved in the pathogenesis of the MDD immune dysfunction remains partially understood.

Regulatory T lymphocytes (Tregs) are a subtype of CD4+ T cells known to be relevant for maintaining immune system homeostasis and self-tolerance (17). The Forkhead box P3 (FoxP3) transcription factor and the alpha chain of the IL-2 receptor (CD25) are the main markers of Tregs (18). Treg cells are a heterogeneous population based on their differentiation, phenotype, functional activity, and activation status (19). Depending on the intensity of CD25 expression two Tregs subsets can be identified, those with high or low expression ($CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Tregs, respectively) with different commitment to Treg cell fate (20, 21). Two $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Tregs subsets may be identified by the intensity of CD25 expression known as "resting" and effector Treg cells.

Tregs are highly heterogeneous (22). One subdivision is based on the history of antigen activation that distinguishes naïve (T_N) Tregs from effector (T_E), central (TCM), and effector memory (TEM) Tregs that can be identified by the expression of the RO and RA isoforms of the CD45 common leukocyte antigen family and the CCR7 antigen (23). These Treg subsets have different distinctive patterns of activation and effector functions (22). In summary, the T_N Treg subset exhibits a non-effector function while T_{CM} Tregs can rapidly proliferate and express effector molecules after being stimulated by an antigen and diminished activation requirements. T_{EM} Tregs produce effector cytokines but have limited proliferative capacity while T_E Tregs are at a final differentiation stage and possess high levels of cytokine production. Tregs can exert their immune suppression capacity by utilizing different mechanisms. The production of interleukin (IL) 10 by Tregs is a pivotal mechanism for inhibition of cells of the innate and adaptive immune responses (24). Tregs display different homing properties, and their appropriate compartmentalization is crucial for their *in vivo* activity. Several chemokine receptors (CCR) regulate Tregs tissue traffic including the migration to inflamed tissues (25). For instance, loss of CCR6 prevents directing Treg migration to inflamed tissues whereas CCR5 expression helps in directing Treg migration to inflamed tissues. Abnormalities in CCR 2, 5, and 6 expression by Tregs have shown to have pathogenic relevance in different inflammatory disorders (26).

Quantitative and functional alterations of Tregs have been implicated in the development of several common autoimmune and inflammatory diseases such as systemic lupus erithematosus and rheumatoid arthritis (27). The pathogenic relevance of Tregs has also been demonstrated in patients with cancer or infectious diseases. The potential alteration of Tregs in MDD patients has been investigated but contradictory numbers (increased, normal, and decreased) of Tregs have been reported (28–32).

Tregs play a key role in the control of harmful inflammation such as the kind potentially induced by the interactions between the intestinal microbiome and gut mucosa (33). Damage of

the intestinal barrier with increased gut permeability and bacterial translocation has been found in MDD patients (34). Furthermore, increased levels of the LPS-binding protein (LBP), a bacterial translocation biomarker, have been associated with a higher systemic proinflammatory stage and monocyte activation in MDD patients (35).

Along these lines, we hypothesized that a heterogenous Treg alteration in MDD patients could explain the contradictory results previously described. We also investigated a potential association of gut barrier damage and bacterial translocation with Treg alterations in MDD patients. We considered it critical that the phenotypical and functional study of Tregs and gut barrier permeability had to be performed in MDD patients without concomitant diseases that could otherwise have potential interactions with the immune system or with the gut barrier.

Our work focused on the study of a homogeneous population of 30 patients with refractory MDD and no concurrent diseases that could be associated with immune system abnormalities. The patients were not homogenous in terms of pharmacological treatment. In parallel, we analyzed 20 age- and sex-matched healthy controls (HCs). We studied the numbers of T_N, T_E, T_{CM}, and T_{EM} of Tregs along with their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets. We also analyzed the pattern of CCR 2, 5, and 6 receptor expression and IL-10 production by activated Tregs, as well as the serum levels of IL-10. In addition, the serum levels of zonulin and the intestinal fatty acid-binding protein (I-FABP), both markers of gut barrier function, and the LPS-binding protein (LBP), a marker of bacterial translocation, were also measured.

MATERIALS AND METHODS

Patients and Study Protocol

In this study, we included 30 patients with MDD from the Department of Psychiatry of the Clinica Universidad de Navarra and from the Hospital Universitario Príncipe de Asturias. The inclusion criteria included the following: (a) psychiatrist-confirmed diagnosis of MDD, single or recurrent, according to the Diagnostic and Statistical Manual of Mental Disorders criteria, Fifth Edition (DSM-V) (36); (b) a minimum score of 14 points on the 17-item Hamilton Rating Scale for Depression (HRSD); and (c) age, 18–65 years. Potential subjects were excluded for the following reasons: (1) acute infection in the last 3 months; (2) chronic bacterial or viral infection; (3) the use of steroids or immunomodulatory drugs in the last 3 months; (4) an autoimmune disease; (5) a cardiovascular disease, including hypertension and ischemic heart disease; (6) a hematopoietic, lung, hepatic, or renal disorder; (7) an endocrine or metabolic disease, including diabetes mellitus and hypercholesterolemia, or a body-mass index (BMI) higher than 30; (8) a history of malignancy; (9) immunodeficiency and malnutrition; (10) pregnancy or lactation; and (11) concomitant psychiatric illness, assessed with the MINI International Neuropsychiatric Interview (37). The patients with refractory MDD included in the suty were not homogenous in terms of psychotropic drugs on board. The patients were studied in parallel with 20 sex-, age-, and BMI-matched HCs from the same epidemiological area.

This study was approved by the ethics committee of the University of Navarra and the Hospital Universitario Príncipe de Asturias. After the study procedures had been fully explained, the subjects provided written informed consent before study enrollment.

Blood samples were drawn from all subjects via standard venipuncture using an established aseptic technique. Samples were obtained at the time of the evaluation. Serum samples were also included for analysis. After collection, the samples were centrifuged, and the serum was isolated, aliquoted, and stored at −80°C until analysis.

Isolation of Peripheral Blood Mononuclear Cells

Blood was collected by antecubital puncture from patients and healthy controls. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood by Ficoll-Hypaque (Lymphoprep™, Axis-Shield, Oslo, Norway) gradient centrifugation. They were then resuspended in RPMI 1640 (Biowhittaker Products, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum, 25 mM Hepes (Biowhittaker Products) and 1% penicillin-streptomycin (Biowhittaker Products). Cell enumeration was performed by conventional light microscopy using a Neubauer chamber following trypan blue dead cell exclusion criteria The viability of fresh PBMC was checked by both trypan blue (light microscopy) and flow cytometry exclusion.

In vitro Culture

Stimulated Tregs expression of IL-10 was assessed *in vitro* intro intracytoplasmic staining in the presence of brefeldin (38). T cells were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma Aldrich Quimica, Spain) plus 1 μg/ml ionomycin (Calbiochem-Novabiochem, La Jolla, CA) for 6 h. The study of regulatory T lymphocytes was determined in parallel at 4°C in the absence of exogenous stimuli.

Surface and Intracellular Lymphocyte Staining

T cells were phenotypically analyzed in PBMC by nine-colors polychromatic in flow cytometry in a FACSAria cytometer using FACSDiva software (Becton-Dickinson). For surface staining, 1 million cells PBMC were incubated in four FACS tubes with monoclonal antibodies combinations of fluorescein IsoTioCyanate (FITC)-anti-CCR2/CCR5/CCR6 (Biolegend), peridinin chlorophyll protein (PercP)-anti-CD3 (Biolegend), phycoerythrin-cyanine seven (PE-Cy7)-anti-CD25 (BD), allophycocyanin-alexa-780 (APC-Alexa780)-anti-CCR7 (eBioscience), brilliant violet-405-anti-CD4 (Biolegend), and brilliant violet-605-anti-CD45RA (Biolegend). The cells were fixed and permeabilized with Fix and Perm solution (Anti-Human Foxp3 Set, eBioscience) during 35 min, and then, cells were washed with phosfate saline buffer (PBS) plus FBS (fetal bobine serum), and incubated with Permeabilitation Buffer and Normal Rat Serum (eBioscience) during 15 min. Finally, the cells were incubated 30 min with intracellular monoclonal

antibodies: phycoerythrin (PE)-anti-IL-10 (Becton-Dickinson) and allophycocyanin (APC)-anti-FoxP3 (eBioscience).

Quantification of Serum Proteins

To study the concentrations of IL-10 in the serum, the Milliplex MAP Kit (MERCK, Darmstadt, Germany) was employed using the protocol recommended by MERCK. The plate was read in a Luminex MAGPIX with xPONENT software. The concentration of the cytokine was calculated from the standard curve using mean fluorescence intensity (MFI) analysis with Analyst software (MERCK).

To study intestinal barrier damage, the concentration of I-FABP and zonulin in the serum were measured by enzyme-linked immunosorbent assay (ELISA). I-FABP was purchased from Hycult Biotech (Hycult Biotech, PA, USA), and zonulin was purchased from R&D Systems (R&D Systems, MN, USA). #e tests were carried out according to the protocols provided in the kits. #e plate was read in an iMark Microplate Reader at 450 nm with Microplate Manager So(ware (Termo Fisher Scientific, MA, USA).

To study bacterial translocation, the concentration of LBP in the serum was measured by ELISA (Abnova, Taipei, Taiwan). We performed 1:800 dilutions of the samples from the MDD patients and HCs. The test was carried out according to the protocol provided in the kit. The plate was read in an iMark Microplate Reader at 450 nm with Microplate Manager Software (Thermo Fisher Scientific).

Statistical Analysis

Analyses were performed using SPSS-19 software (SPSS-IBM, Armonk, NY). Since most variables did not fulfill the normality hypothesis, the Mann Whitney *U*-test for non-parametric data was used to analyze differences between independent groups. Significance level was set at $p < 0.05$.

RESULTS

Demographic Patient Characteristics

Table 1 shows the demographic data and clinical characteristics of the 30 MDD patients and 20 HCs included in the study. No significant differences were found between MDD patients and HCs with respect to the variables that were studied, except for employment status. The patient group included 19 females and 11 males, ranging from 27 to 53 years of age. The duration of their depressive episode before recruitment was 16.12 ± 2.85 weeks. Seventeen patients (56.7%) had suffered at least one previous MDD episode. At the time of the study, the mean value of the HRSD was 15.95 ± 1.25. Furthermore, 10% of the patients presented psychotic (delusional) symptoms during the current episode.

All patients received pharmacological treatment at their doctor's discretion: 30 (100%) received antidepressant medications, 28 (93.3%) received anxiolytics or hypnotics, 5 (16.7%) received mood stabilizers, and 10 (33.3%) received atypical antipsychotics. 28 (93.3%) received combination pharmacotherapy, consisting of at least 2 different types of medication in 19 patients (63.3%) and of at least 3 different types of medication in the other 9 patients (30%). None of the patients received electroconvulsive therapy (ECT).

TABLE 1 | Sample characteristics.

	MDD	HC	*p*-value
Socio-demographic			
Age, mean (SD)	43.26 (13.14)	40.45 (12.46)	0.45
Sex (% female)	19 (63.3%)	12 (60%)	0.98
Currently employed and active *n* (%)	13 (59.1%)	18 (90%)	<0.01
College degree *n* (%)	16 (53.3%)	12 (60%)	0.77
Past history			
Family history of depression *n* (%)	17 (56.7%)	8 (40%)	0.38
Family history of other psychiatric disorder *n* (%)	22 (77.3%)	11 (55.5%)	0.22
Health characteristics and somatic morbilities			
BMI, mean (SD)	26.74 (5.41)	25.5 (5.36)	0.51
Smoking *n* (%)			0.13
Never	12 (40%)	6 (30%)	
Occasionally	8 (26.7%)	11 (55%)	
Everyday	10 (33.3%)	3 (15%)	
Drinking *n* (%)			0.43
Never	7 (23.3%)	3 (15%)	
Occasionally	20 (66.7%)	16 (80%)	
Everyday	3 (10%)	1 (5%)	

MDD Patients Show Increased Number of Circulating Tregs at the Different Stages of CD4+ Differentiation

We investigated the circulating counts of Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets defined by the level of CD25 expression in MDD patients and age-, sex-, and ethnically matched HCs. **Figure 1** shows the flow cytometry gating strategy and histograms of Tregs in a representative case of MDD patient. We found a significant increase in the number of circulating Tregs cells in MDD patients with respect to HCs ($p = 0.01$) (**Figure 2**). This Tregs expansion is explained by a significant increase in the counts of both $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Tregs subsets in MDD patients ($p = 0.001$). Furthermore, the percentage of total Tregs ($p = 0.005$) and of their $CD25^{hi}FoxP3^{+}$ ($p = 0.003$) subset in the circulating $CD4^{+}$T population in MDD patients were significantly higher than those found in HCs.

We also investigated the counts and distribution of Treg cells and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets at the T_N, T_{CM} T_{EM}, and T_E stages of CD4+ T lymphocyte differentiation/activation in MDD patients and HCs (**Figure 3**). Significant enhanced numbers of total and both Tregs subsets were found at the four stages of CD4+ T lymphocyte differentiation/activation in MDD patients ($p < 0.001$). We also found a redistribution of the Treg cells and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets along the four stages of activation/maturation. The percentages of $CD25^{+}FoxP3^{+}$ Treg cells ($p = 0.024$) and their $CD25^{hi}FoxP3^{+}$ ($p = 0.01$) and $CD25^{low}FoxP3^{+}$ subsets ($p = 0.01$) at T_N stage

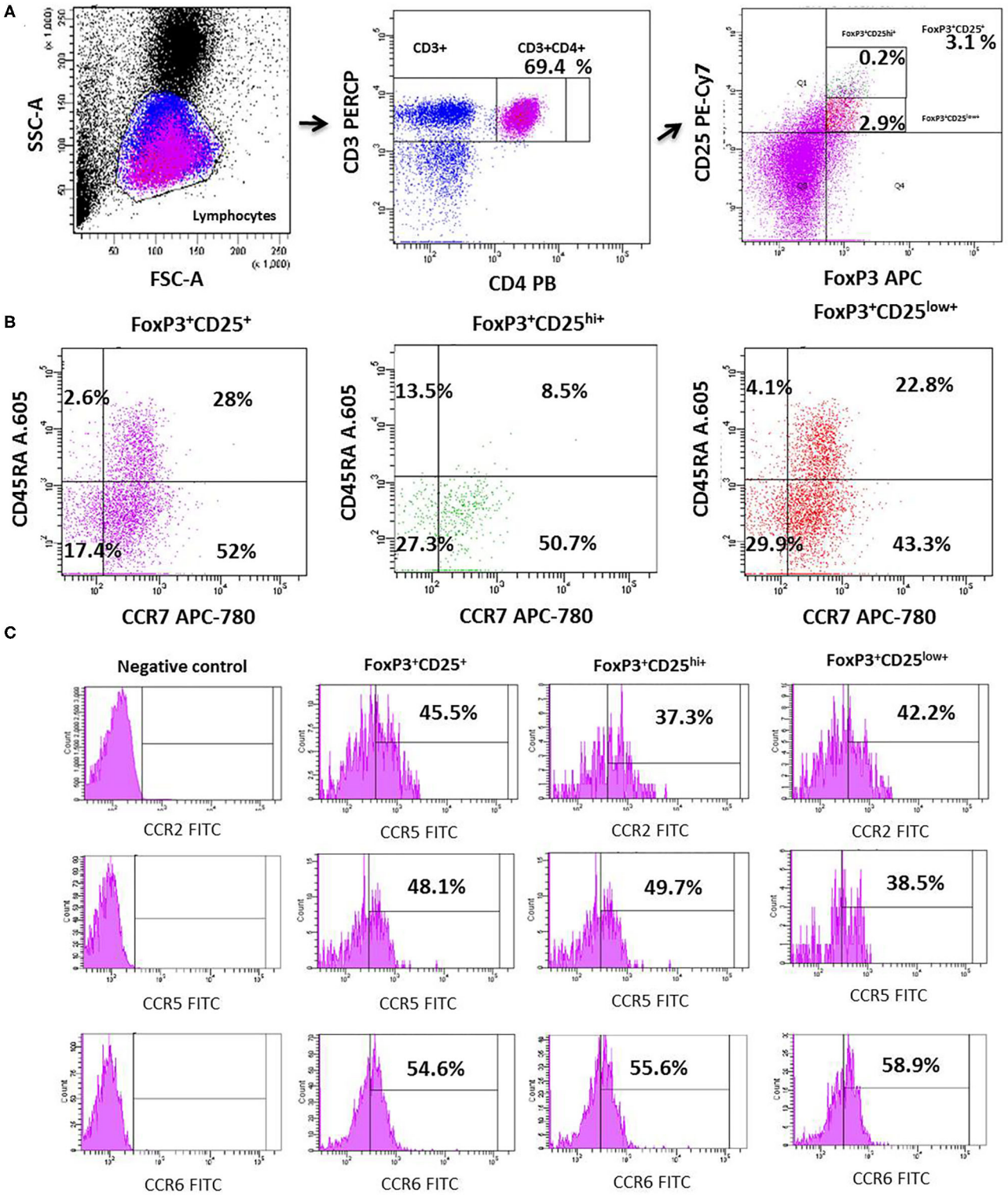

FIGURE 1 | Dot plots represent the flow cytometry gating strategy and histograms of the circulating total Tregs and their $CD25^{high}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in a representative case of MDD. The first row dot plots represent the selected gates and percentages to obtain the total Tregs **(A)**, the second those of the individualized total Tregs and their $CD25^{high}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets (**B**, respectively). The **(C)** shows dot plots of the selected gates and percentages of the CCR2 (superior line), CCR5 (medium line), and CCR6 (lower line) expression by total Tregs and their $CD25^{high}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets.

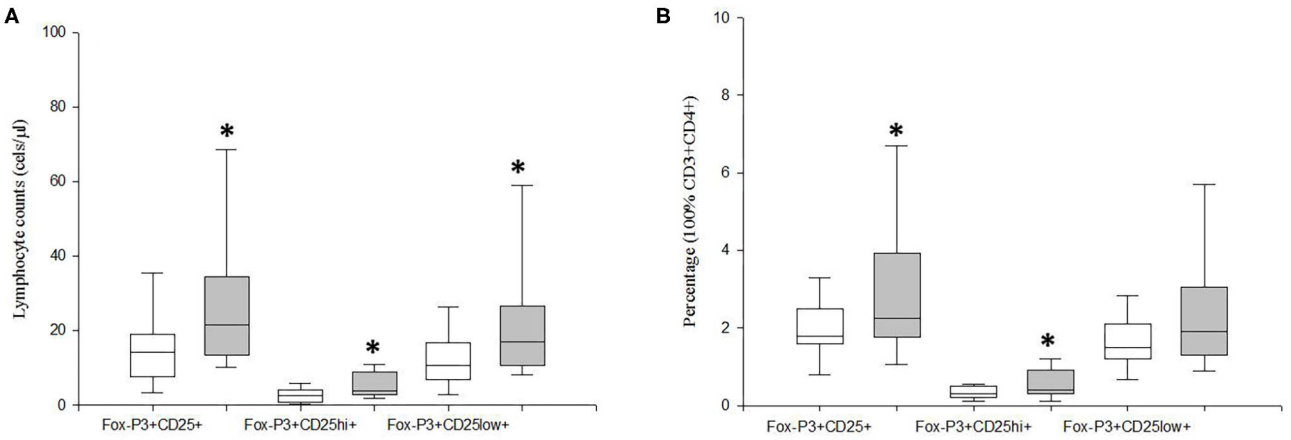

FIGURE 2 | Absolute number and frequency of total circulating Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in MDD patients and healthy controls. Absolute number (cells/μl) (y axis) of $CD25^{+}FoxP3^{+}$ lymphocytes and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in MDD patients (gray box plots) and healthy controls (white box plots) are shown in panel **(A)**. Frequency of the total Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in the circulating CD4+ T lymphocytes in MDD patients (gray box plots) and controls (white box plots) are shown in **(B)**. The top of the rectangle indicates the third quartile, the horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and another vertical line extends from the bottom of the rectangle to indicate the minimum value. *Significant difference between MDD and healthy controls for the indicated variable.

were significantly higher in MDD patients than those found in HCs.

We investigated the potential correlation between the patients' HDRS score and their circulating Treg cells and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets but, we did not find statistically significant correlations. Neither did we find statistically significant differences between the Tregs counts among the patients that suffered their first depressive episode and those with at least a previous episode. In addition, we did not find statistically significant differences neither with respect to patients with or without antipsychotic drugs nor men and women (Data not shown).

Tregs From MDD Patients Show Increased CCR6 and CCR2, but Normal CCR5 Expression

We studied the frequency of Tregs cells and of their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets that express the CCR2, CCR5, and CCR6 chemoreceptors in MDD patients and HCs (**Figure 4**). We found that the percentages of the total Tregs and both subsets that expressed CCR2 and CCR6 were significantly higher in MDD patients than that in HCs ($p < 0.05$). However, there were not significant differences in the percentage of Tregs that express CCR5 between MDD patients and HCs.

MDD Patients Show Increased IL10 Productions by Tregs

We studied the intracellular expression of IL-10 in the Tregs from MDD patients and HCs after PMA stimulation. We found that the percentage of the total Tregs that expressed IL-10 in MDD patients was significantly higher than that in HCs ($p = 0.02$) (**Figure 5**). The increased percentage of Tregs expressing IL-10 in MDD patients was explained by the significantly enhanced percentages found in the T_{EM} and T_E differentiation/activation stages ($p = 0.03$ and $p = 0.02$, respectively). Next, in both groups of subjects, we calculated the potential number of circulating Tregs that could express IL-10 by multiplying their number by the percentage of cells that express the cytokine after PMA stimulation in both groups of subjects (**Figure 5**). We observed that the number of circulating Tregs that could express IL-10 was significantly increased in the MDD patients compared to that found in the HCs ($p = 0.02$).

We also measured the circulating levels of IL-10 in MDD patients and HCs (**Figure 5**). We found that MDD patients had significantly increased serum concentration of IL-10 compared to HCs ($p = 0.04$).

Increased LBP Serum Levels Are Associated With a Reduction of Tregs in MDD Patients

We measured the serum concentrations of LBP, zonulin, and I-FABP in MDD patients and HCs (**Figure 6**). MDD patients showed significantly increased levels of LBP and I-FABP compared to those found in HCs ($p = 0.04$ and $p = 0.01$, respectively). We detected LBP, zonulin and I-FABP in all the serum from patients.

We found that 11 (high-LBP MDD) out of the 30 MDD patients had LBP levels higher than the 95th percentile of those found in HCs (**Figure 6**). In addition, we found that the counts of Tregs and of both $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Tregs subsets in MDD patients with high-LBP MDD were significantly lower than those found in normal-LBP patients ($p = 0.003$, $p = 0.05$, $p < 0.001$, respectively). Interestingly, there were not significant differences in the counts of Tregs and of

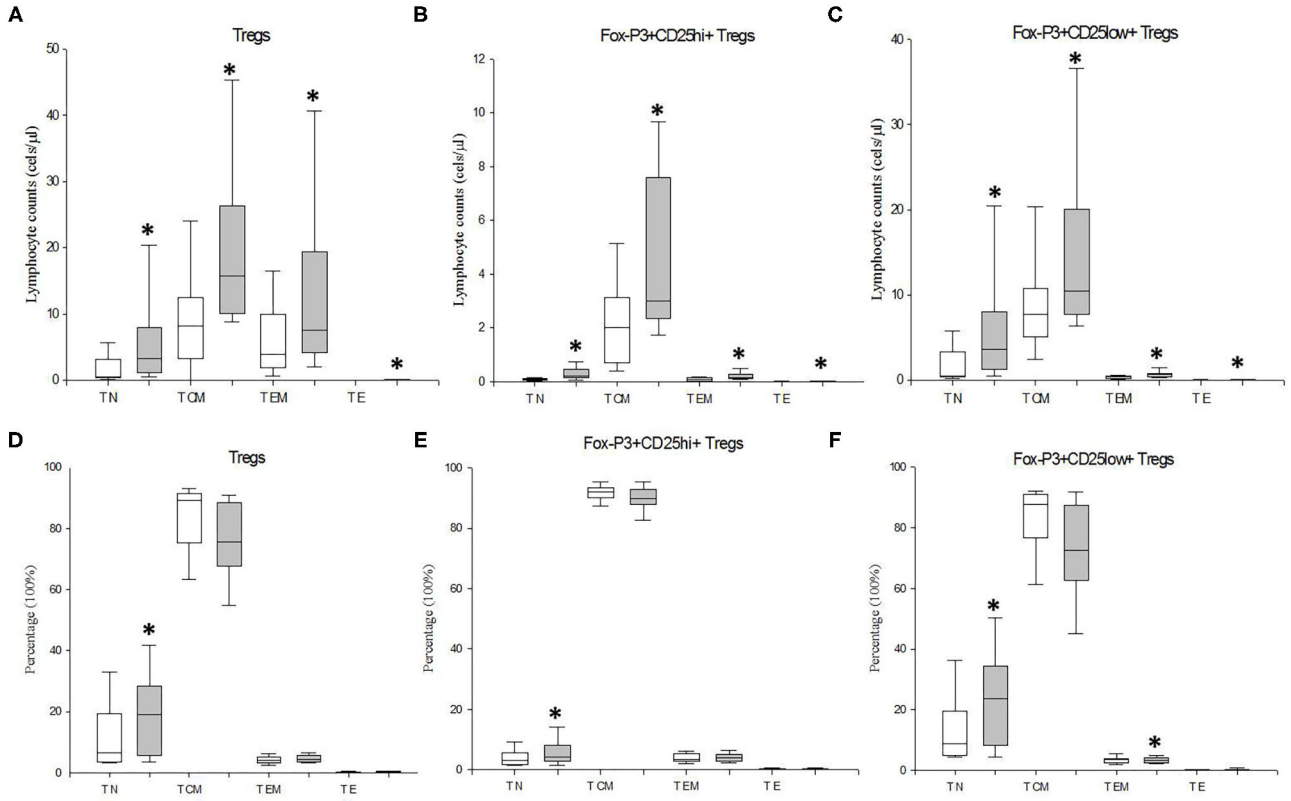

FIGURE 3 | Absolute number and frequency of circulating total Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets at the T_N, T_{CM} T_{EM}, and T_E stages of CD4+ T lymphocyte differentiation/activation in MDD patients and healthy controls. Absolute number (cells/μl) (y axis) of Tregs lymphocytes and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets at the T_N, T_{CM} T_{EM}, and T_E stages of CD4+ T lymphocyte differentiation/activation in MDD patients (gray box plots) and healthy controls (white box plots) are shown in **(A–C)**, respectively. Frequency of the total Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets at the T_N, T_{CM} $T_{EM,}$ and T_E stages of differentiation/activation in MDD patients (gray box plots) and controls (white box plots) are shown in **(D–F)**, respectively. The top of the rectangle indicates the third quartile, the horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and another vertical line extends from the bottom of the rectangle to indicate the minimum value. *Significant difference between MDD and healthy controls for the indicated variable.

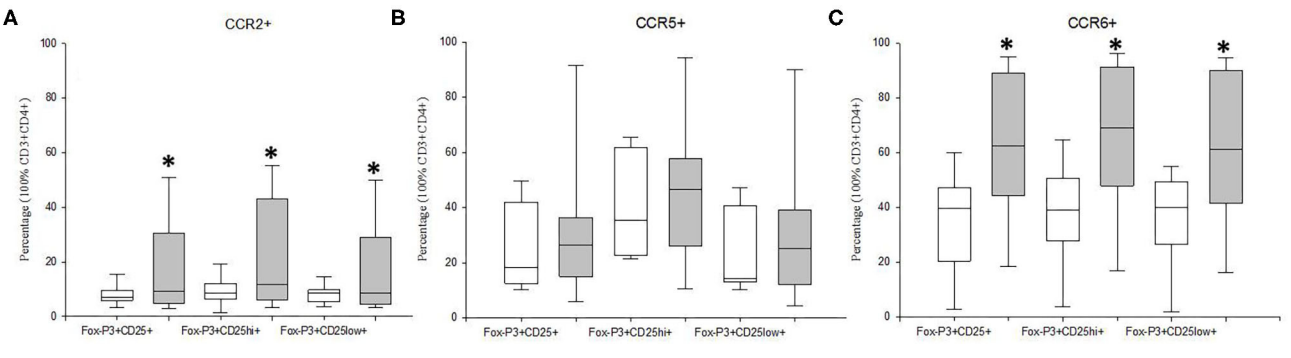

FIGURE 4 | Frequency of CCR2, CCR5 and CCR6 expression by Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in MDD patients and healthy controls, Frequencies of total Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets (y axis) in MDD patients (gray box plots) and healthy controls (white box plots) that express the CCR2 **(A)**, CCR5 **(B)**, and CCR6 **(C)**. The top of the rectangle indicates the third quartile, the horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and another vertical line extends from the bottom of the rectangle to indicate the minimum value. *Significant difference between MDD and healthy controls for the indicated variable.

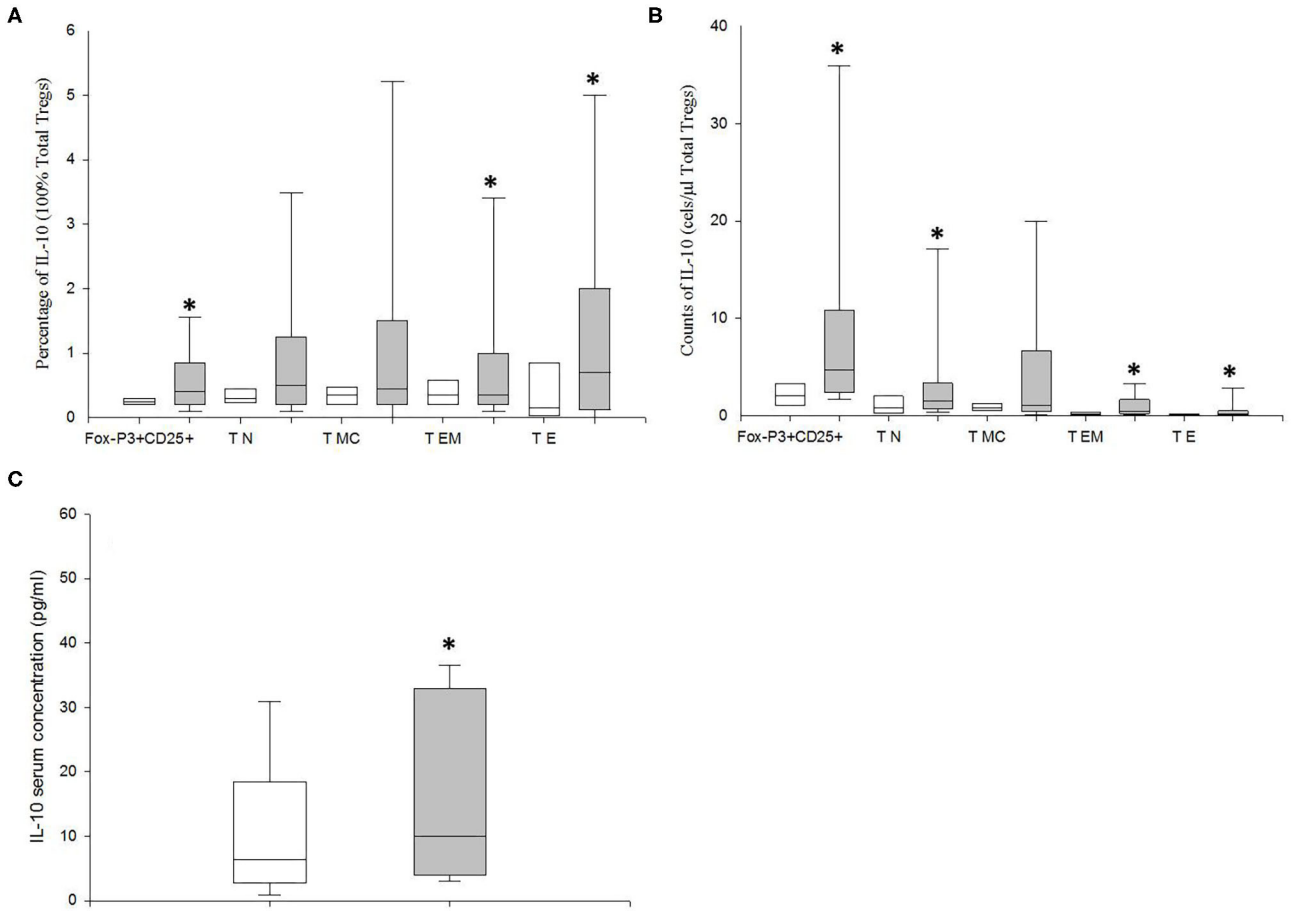

FIGURE 5 | Frequency of IL-10 expression and number of circulating total Tregs and of those at the T_N, T_{CM} T_{EM}, and T_E stages of differentiation/activation able to produce this cytokine in MDD patients and healthy controls. Frequencies of total Tregs and of those at the T_N, T_{CM} T_{EM}, and T_E stages of differentiation/activation (y axis) in MDD patients (gray box plots) and healthy controls (white box plots) that express IL10 (x axis) after *in vitro* PMA (50 ng/ml) stimulation for 4 h in total **(A)**. Absolute number (cells/μl) (y axis) of circulating total Tregs and of those at the T_N, T_{CM} $T_{EM,}$ and T_E stages of differentiation/activation in MDD patients (gray box plots) and healthy controls (white box plots) that are able to express the indicated cytokine (x axis) after *in vitro* PMA stimulation for 4 h **(B)**. Serum concentrations (pg/μl) (y axis) of IL-10 MDD patients (gray box plots) and healthy controls (white box plots) **(C)**. The top of the rectangle indicates the third quartile, the horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and another vertical line extends from the bottom of the rectangle to indicate the minimum value. *Significant difference between MDD and healthy controls for the indicated variable.

both $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Tregs subsets between high-LBP MDD patients and HCs. However, we did not observe significant differences in the expression of IL-10 in the Tregs between both groups of MDD patients (data not shown).

DISCUSSION

In this paper, we have demonstrated that compared to healthy controls, MDD patients show a marked expansion of circulating Tregs and their $CD25^{high}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets through the different T_N, T_E, T_{CM}, and T_{EM} stages of CD4+ T lymphocyte activation/differentiation. Tregs also show an increased frequency of cells that express CCR6 and CCR2. Furthermore, the percentage of $CD25^{+}FoxP3^{+}$ Tregs that produce IL-10 is also enhanced in MDD patients that concurrently have increased enhanced serum IL-10 levels. However, this Tregs expansion was blunted in MDD patients with gut barrier damage and increased bacterial translocation.

MDD is associated to a systemic inflammatory response that has been defined by the established increased concentrations of circulating proinflammatory cytokines and chemokines found in these patients (39). In addition, clinical findings and cellular studies support the knowledge of abnormal function of the natural and adaptative immune responses (14). The understanding of cellular mechanisms involved in the MDD immune system dysfunction remains elusive.

Tregs are a cornerstone of the immune system playing a critical role in the regulation of the activation and effector activity of cells of the innate and adaptive immune response (40). Tregs appear to be pivotal in the control and suppression of

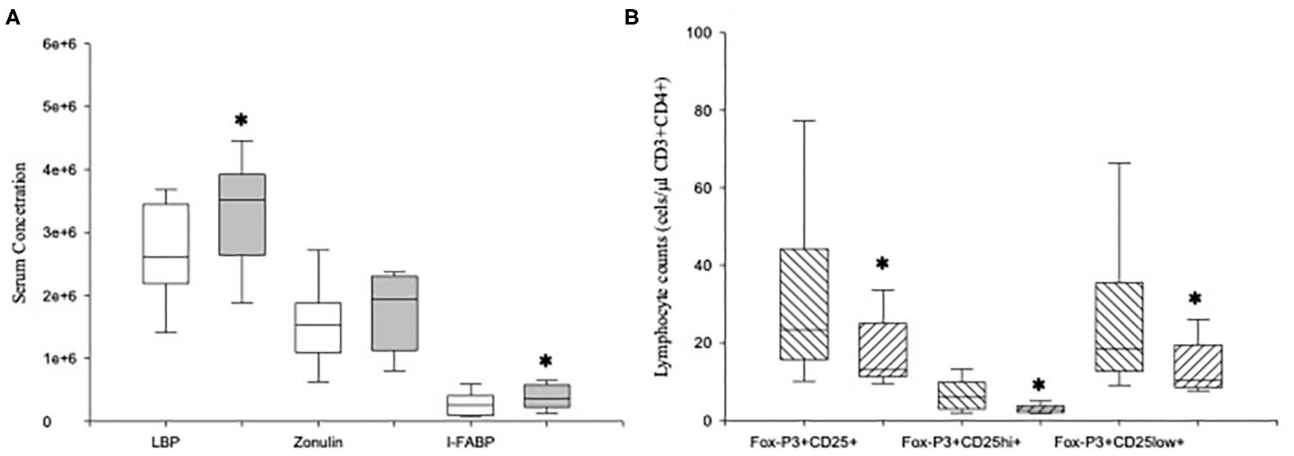

FIGURE 6 | Serum levels of LBP, zonulin and I-FABP in MDD patients and absolute number of circulating Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets in high-LBP and normal-LBP MDD patients. Serum concentrations (y axis) of LBP (ng/ml), zonulin (mg/ml) and I-FABP (pg/ml) in MDD patients (gray box plots) and healthy controls (white box plots) **(A)**. Absolute number of circulating Tregs and their $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ subsets (y axis) in high-LBP (ascending lined box plots) and normal-LBP (descending lined box plots) MDD patients subsets and healthy controls (white box plots) **(B)**. The top of the rectangle indicates the third quartile, the horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and another vertical line extends from the bottom of the rectangle to indicate the minimum value. *Significant difference between MDD and healthy controls for the indicated variable.

the immune responses against non-self and self-antigens (41). Our data show a clear increase in the number of Tregs and in its frequency in the $CD4^{+}$ T lymphocyte circulating population in MDD patients. Circulating Tregs represent a versatile and dynamic cell population that is composed of different functionally heterogeneous subsets (42). In MDD patients, our data demonstrate an expansion of both effector $CD25^{hi}FoxP3^{+}$ and resting $CD25^{low}FoxP3^{+}$ Tregs subsets compared to those of the HCs supporting the observed increase in the numbers of total circulating Tregs. Conflicting results regarding the Tregs numbers have been previously described in MDD patients (28–30, 43). Several reasons that are not mutually exclusive may explain the heterogeneity in the numbers of circulating Tregs found in MDD patients. First, The differences in methodology for identification of Tregs may explain the observed discrepancies between studies. We have applied in this study a current stringent and precise cytometric strategy of Tregs characterization. Second, The clinical characteristics of the study samples. We have analyzed a homogenous population of patient with MDD with persistent symptomatology for an interval between 10 and 20 weeks. We included as controls a balanced sex, age, and BMI group of HCs from a similar epidemiological area. We studied patients with persistent MDD symptoms in spite of pharmacological treatment because we aimed to discover the relevance of the impact of the severe disease excluding those with rapid response to treatment. Furthermore, with this inclusion criteria, we avoid the reported increase in Tregs numbers during effective antidepressant treatment, whatever antidepressant is prescribed (44). To prevent potential interference of concomitant or previous disease and/or treatment with the immune system function, we applied precise exclusion criteria supporting the homogeneity of the population and the absence of potential causes of interference. Third. A new light of explanation of the heterogeneity in the numbers of circulating Tregs reported in MDD patients might be inferred from our finding of two groups of patients, defined by the serum high- and normal-LBP levels, with normal or increased counts of circulating Tregs, respectively. Differences in the frequency of subjects from both groups of patients in a cohort may explain discrepancies in the counts of the Tregs observed between studies. We did not find differences in the Tregs counts between the patients suffering their first episode and those with recurrent disease, which supports linking the expansion of these lymphocytes with the presence of MDD symptomatology.

The activation of Tregs driven by antigen stimulation promotes their progression from the naïve stage to the memory and effector stages characterized by differentiated functional and phenotype properties. Thus, in addition to the quantitative analysis of Tregs in MDD patients, we investigated their distribution along the different activation/differentiation stages. MDD patients show increased numbers of total Tregs and of both $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Treg subsets at the different T_N, T_E, T_{CM}, and T_{EM} stages. These results agree with previous articles reporting increased percentage of memory Treg cells in the MDD patients, but the authors did not describe the other stages of Tregs activation/differentiation (30). We observed a preferential expansion of these Tregs populations at the T_N stage in MDD patients. Although, thymus-derived and peripherally derived Treg have been identified, our results do not favor the claimed impairment in Tregs generation in MDD patients (31, 43). Conversely, this finding suggests that MDD is associated to a host environment, favoring the cell progression

to further activation/differentiation advanced T_{CM} T_{EM} and T_E stages (45, 46). The observed enhanced expression of IL-10 by Tregs from MDD patients also support the relevance of an environment promoting Tregs activation in these patients.

The pattern of CCR expression by Tregs is involved in the regulation of their homing and inflammatory response patterns. We have found that Tregs from MDD patients show increased frequency of cells that express CCR6 and CCR2. It has been reported than CCR2 and CCR 5 are necessary to ensure efficient homing of Treg to inflammatory tissue and migration toward the antigen site (25). These findings suggest that Tregs from MDD patients may have an anomalous pattern of tissue migration. The acquisition of homing receptor phenotypes probably occurs after the activation of periphery Tregs (23). Thus, the potential relevance of environment promoting abnormal Tregs activation in MDD may be also suggested.

Different mechanisms may be involved in the pathophysiology of the Treg abnormalities found in MDD patients. As previously discussed, the observed expansion of functionally active Tregs in approximately two thirds of the MDD patients might be favored by a promoting environment. The consequences of this Treg expansion may be relevant for the understanding of the pathogenesis of the disease. There is increasing evidence of the pathophysiological relevance of Treg heterogeneity and plasticity (46). $CD25^{low}FoxP3^{+}$ Tregs can lose FoxP3 expression and acquire effector Th cell function, such as the Th1 and Th17 phenotypes, under certain conditions, whereas $CD25^{hi}FoxP3^{+}$ Tregs are rather stable on mice. Furthermore, human Treg cells seem to be rather unstable. $CD25^{hi}FoxP3^{+}$ Treg cells may differentiate into Th17 producer cells in the presence of inflammatory cytokines (47). Former $FoxP3^{+}$ T cells are also found to be particularly increased in inflamed tissues (48, 49). Thus, it is possible to speculate that a Th1 and Th17 plasticity of the increased numbers of Tregs found in MDD patients might occur and contribute to a systemic pro-inflammatory disbalance. Furthermore, the expansion of active Tregs with suppressor activity on the adaptive immune response may be involved in the pathogenesis of increased susceptibility to viral infections, reduced immune responses to vaccines and the slowed wound healing observed in MDD patients (12–14).

A third of MDD patients have not developed Treg expansion. In these patients, we found marked gut barrier damage and increased bacterial translocation. There is evidence that the microbiota influences the immune system and vice versa. More specifically, there are close interactions between the gut microbiota and the Tregs (50). The balance of effector lymphoid cells and Treg cells can have a profound influence on how the gut mucosa responds to stressors that elicit damage (51). Furthermore, MDD is associated with an enhanced intestinal permeability, or "leaky gut," and increased bacterial translocation (52, 53). In this subset of MDD patients, we have confirmed enhanced I-FABP serum levels, a proven peripheral blood marker of gut barrier function (54). This abnormal intestinal mucosa barrier favors bacterial, including gram -, translocation. Circulating LPS promotes the hepatic synthesis of LBP, an acute phase reactant, and LPS-LBP complexes bind to CD14 on the monocyte membrane with a subsequent activation of the cell. In several clinical settings, the long- term (72 h) plasma levels of LBP induced by transient endotoxemia appear to better reflect long-term exposure to LPS than does measuring LPS itself (55). Of interest, LBP is just one of several markers of bacterial translocation. In agreement with previous reports, our findings show that as a group, MDD patients have increased LBP serum levels, with a third also showing a markedly high concentration of this bacterial translocation marker (56). Interestingly, we observed a significant reduction in the counts of Tregs and in both the $CD25^{hi}FoxP3^{+}$ and $CD25^{low}FoxP3^{+}$ Treg subsets in high-LBP MDD patients in comparison to those found in normal-LBP MDD patients. The clinical characteristics of the MDD patients included in this study exclude chronic viral or bacterial infections, as well as any existence of a concomitant or recent (at least 3 months prior to the study) bacterial infection. Thus, these results strengthen the connection between intestinal barrier dysfunction, bacterial translocation, and the immune-inflammatory system in MDD patients, with an expansion of activated monocytes in MDD patients (53). It is possible to suggest that the reduction of the expansion of Tregs might limit the gut migration of these cells. This potential reduction of Tregs of systemic origin could compromise the total number of intestinal Tregs (57). Treg restriction in the gut mucosa might favor gut barrier damage and the increased bacterial translocation found in this previously referenced third of MDD patients. Although there is no direct evidence of causality, our results support the relevance of the so-called gut-brain axis, linking gastrointestinal function and the immune system with the emotional and cognitive brain centers (58, 59).

This work has limitations. It is a cross sectional study and the course of the disease on natural Tregs need to be formally tested and replicated in longitudinal studies. The clinical homogeneity of the MDD patients studied combined with their very similar HDRS score, undermines the ability to study the potential association of the Treg expansion with the severity of the disease. Furthermore, the characteristics and size of the sample included in this pathophysiological study also limits the analysis of the potential association of the Tregs abnormalities with the clinical features of the disease. We have analyzed a homogenous population of MDD patients presenting persistent symptomatology for 10–20 weeks. However, all patients were medicated, and it might be a cofound. Furthermore, in this study, we were not able to control for additional factors that might also be associated with dysregulated cellular immunity, such as hormonal status, e.g., during menstrual cycle phases, physical activity, or other lifestyle- or environment-associated factors.

Taking these results together, two different MDD subsets can be established according to the analysis of circulating Tregs. Two thirds of them have an expansion of Tregs whereas the remaining third has normal Treg numbers, which were associated with severe gut barrier damage and increased bacterial translocation. These results support the heterogeneity of the mechanisms of immune-inflammatory dysfunction in MDD patients, as well as a need for the individualized biological study of patients for the development of new immunoregulatory therapeutic strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the University of Navarra and the Ethics committee of the Hospital Universitario Principe de Asturias. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to this work and have approved the manuscript content.

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ORIGINAL RESEARCH
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Study of the SCL-90 Scale and Changes in the Chinese Norms

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Objective: This study aimed to investigate the Chinese norms for the Symptom Checklist 90 (SCL-90) scale and its application.

Methods: In total, 7,489 adults from Tianjin and Qingdao in China were included. Their data were compared with the norm data of 1,388 people published by Jin et al., the combined norms published by Tang et al., the data of 2,808 adults published by Chen and Li, and the data of 1,890 adults from Tong in China.

Results: In five different periods, notable changes were observed in each factor of the SCL-90 that significantly differed from the previous norms. The scores of each factor showed an increasing annual trend. Compulsion consistently obtained the highest scores, and phobia consistently obtained the lowest scores. The scores tended to decrease from compulsion to anxiety, and psychosis scored lower than paranoia. There was a significant difference in the detection rate between the critical screening value of two points and the standard score. Using the standard score as the critical value, the detection rate ranged between 13 and 16% and was relatively concentrated. Using two points as the critical value, the detection rate ranged between 38 and 50%.

Conclusion: The usual model in China is not consistent with social development. Using two points as the critical value is no longer suitable for the SCL-90. New Chinese norms and measurement standards should be developed. The mean value plus one standard deviation could be used as the new measurement standard.

Keywords: SCL-90, Chinese norms, critical value, scores of each factor, norm changes

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INTRODUCTION

The Symptom Checklist 90 (SCL-90) is a psychosomatic screening scale proposed by Derogatis that is widely used in China and elsewhere (1). The SCL-90 can be used to distinguish between patients with and without psychosomatic diseases and has good reliability and validity (2, 3). However, the SCL-90 lacks widely accepted norms (4). The SCL-90 scale currently used in China was translated by Wang (5). For the translated version of the scale, Jin and Wu published a set of data of Chinese norms in 1986 that were based on data from 1,388 patients (6). Subsequently, many application studies have been performed in China, and these studies mainly included surveys of the general population and studies concerning the obstacles to large-scale screening.

In 1999, Wang et al. (7) proposed a norm suitable for Chinese middle school students. In 1999, Tang et al. (8) proposed a combined norm based on 47,354 people by integrating the Chinese literature. Chen and Li (9) examined a sample of 2,808 people in 2003 and discussed the combined norm.

Regarding its application, an important problem with the scale is its relatively lack of time-effectiveness, which is not only a concern in populations with mental health concerns but also results in varying levels of performance at different times and in populations with different cultural backgrounds. Many studies concerning this issue were published as much as a decade ago. The norm for Wang's translation, which is currently used in China, was proposed in 1986 and is more than 30 years old. Since the Chinese economic reform policy was implemented in the 1980s, significant social changes have occurred in China, making it necessary to update the norms of the SCL-90. This study was performed to compare the differences between data from a current sample population and previously reported sample population data or norms and study the changes in different factors of the SCL-90.

MATERIALS AND METHODS

Ethics

These centers do not have ethics committees. Their role is to perform checkups for consumers but not patients, and they have no diagnostic responsibility. However, there is an electronic consent form (written in Chinese) that each subject (the consumer in the context of these physical examination centers) signs before the administration of the psychological tests. In section 10.1 of the electronic consent form, the following is presented: "The owner of this testing system has the right to perform analyses of non-sensitive data."

Subjects

The subjects were 7,489 people aged 20–45 years who were selected from 10 medical centers, including four commercial medical examination centers in Tianjin five commercial medical examination centers and one public hospital medical examination center in Qingdao China, from January to August 2019. These visitors all received health checkups and had no clear psychiatric problems. A health checkup was performed by a psychological counselor.

Instruments

The version of the SCL-90 translated by Wang was used (5).

Statistical Analysis

The SCL-90 data of 7,489 persons evaluated in 2019 were compared with previously published norms. The data used for the comparisons were obtained from previously published Chinese studies and consisted of the following: (1) norm data of 1,388 people published by Jin et al. (6); (2) combined norm data of 47,354 people summarized in the literature by Tang et al. (8) in 1999; (3) data of 2,808 people from Hangzhou, China reported by Chen and Li (9) in 1999; and (4) a nationwide sample of 1,890 people in China presented by Tong in 2006 (4).

TABLE 1 | Comparison between the 2019 data and Chinese norms in 1986.

Subscales	1986 (n = 1,388)		2019 (n = 7,489)		Z
	M	SD	M	SD	
Somatization	1.37	0.48	1.961	0.639	−39.83***
Obsessive compulsive	1.62	0.58	2.396	0.657	−44.80***
Interpersonal sensitivity	1.65	0.51	2.105	0.687	−28.74***
Depression	1.5	0.59	2.018	0.639	−29.65***
Anxiety	1.39	0.43	1.942	0.607	−40.87***
Hostility	1.48	0.56	2.044	0.661	−33.47***
Phobic anxiety	1.23	0.41	1.514	0.544	−22.38***
Paranoid ideation	1.43	0.57	1.936	0.653	−29.65***
Psychoticism	1.29	0.42	1.744	0.594	−34.41***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; notably, the same applies below.*

TABLE 2 | Comparison of the data published by Tang et al. (8), Chen and Li (9) and 2019.

Subscales	Tang (1999; n = 47,354)		Chen (1999; n = 2,808)		Z	
	M	SD	M	SD	Tang 2019	Chen 2019
Somatization	1.48	0.54	1.36	0.39	−61.93***	−57.74***
Obsessive compulsive	1.83	0.64	1.47	0.45	−69.52***	−81.29***
Interpersonal sensitivity	1.79	0.65	1.44	0.45	−37.13***	−57.20***
Depression	1.70	0.65	1.33	0.39	−39.93***	−66.00***
Anxiety	1.55	0.55	1.30	0.37	−52.59***	−64.87***
Hostility	1.64	0.63	1.36	0.41	−49.52***	−62.96***
Phobic anxiety	1.40	0.50	1.17	0.30	−16.98***	−40.62***
Paranoid ideation	1.69	0.62	1.32	0.42	−30.47***	−56.26***
Psychoticism	1.53	0.56	1.25	0.34	−29.20***	−52.58***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

Python software was used for the data analysis. Due to the large sample size, a z-test was used. A P-value <0.05 was considered statistically significant.

RESULTS

Comparative Analysis of Data From Five Studies

We compared the data of 7,489 people in 2019 with the 1986 norms, the data of 47,354 people published by Tang in 1999, the data of 2,808 people published by Chen and Li (9) and the data published by Tong in 2006. We found that the scores of all factors in the 2019 Chinese data were significantly higher than those in previous years and that the data were more concentrated (see **Tables 1**–**3**).

Figure 1 shows the differences in the scores across the five different sample populations. **Figure 1** shows that the Chinese norms in 2006 are close to those in 1986, although there are differences. The scores of each factor increased annually. The

TABLE 3 | Comparison between the 2019 and 2006 data.

Subscales	2006 (n = 1,890)		2019 (n = 7,489)		Z
	M	SD	M	SD	
Somatization	1.42	0.44	1.961	0.637	−43.09***
Obsessive compulsive	1.66	0.52	2.396	0.657	−52.30***
Interpersonal sensitivity	1.51	0.49	2.105	0.687	−42.82***
Depression	1.50	0.47	2.018	0.639	−39.68***
Anxiety	1.34	0.39	1.942	0.607	−52.66***
Hostility	1.50	0.51	2.044	0.661	−39.29***
Phobic anxiety	1.27	0.39	1.514	0.544	−22.50***
Paranoid ideation	1.44	0.47	1.936	0.653	−37.93***
Psychoticism	1.33	0.39	1.744	0.594	−37.14***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

comparison between the 2019 data and Tang's 1999 data shows the most obvious changes. The trends in the factor scores across the different sample datasets were generally consistent. All data sets showed that compulsion factors had the highest scores and that phobic anxiety factors had the lowest scores. The scores showed a tendency to decline from compulsion to anxiety, and the scores of psychosis were lower than those of paranoid ideation.

Different Meanings of Subjective Perception Evaluation and Norm Evaluation

The authors of the studies expressed different opinions regarding the critical value for each dimension of the SCL-90. Some studies used two points as the critical value for each factor for screening purposes (10). Whether this critical value of two points is valid urgently needs to be determined. Therefore, in this experiment, the hypothesis was examined based on the norms of China. The detection rate was calculated based on the mean value in 2019 plus one standard deviation as the critical value and compared with the detection rate calculated based on two points as the critical value. We found significant differences in all factors, except for phobic anxiety. Using the mean score as the critical value, the detection rate ranged between 13 and 16% and was relatively concentrated. However, using two points as the critical value, the detection rate was concentrated between 38 and 50%, except for compulsion (70.09%), phobic anxiety (13.98%) and psychosis (26.21%) (see **Table 4**).

DISCUSSION

The SCL-90 scale is widely used worldwide (1, 11). The SCL-90 is a screening scale and needs to be revised regularly. China is currently using Wang's translated version from 1984 and the norms proposed in 1986, which are currently more than 30 years old. The rapid development of society has inevitably led to psychological changes in the Chinese population. If we continue to use the norms published 30 years ago for research, these data will be inconsistent with the current psychological status of the population. Therefore, this study compared the data of five large sample populations collected over time and concluded that the current population has notable differences from previous populations in several factors. Moreover, the scores of all factors tended to increase annually, which is consistent with the research by Tang, Chen and others. In this general upward trend, the differences between the neurotic and psychopathic factors were particularly prominent in the 2019 study. The neurotic features of the population, such as depression, anxiety, obsessive compulsive, and somatization, were significantly higher than the psychotic symptoms, such as paranoid ideation and psychoticism. The individuals included in this study were young people aged between 20 and 45 years who experienced high levels of pressure, which may have contributed to the high scores of various factors. The scores of each factor increased annually. Thus, it is inadvisable to continue to use the previously established norms, which are not consistent with how Chinese society developed, and new Chinese norms are urgently needed.

In previous studies, the sample sizes used to generate norms were not large and mostly ranged between 1,000 and 3,000 (4, 6, 9). Although the norms proposed by Tang et al. were based on data from 47,354 people, the data were obtained over the course of 7 years (8). However, the sample size in this study was 7,000, ensuring greater accuracy. The individuals included in this study were mostly enrolled at physical examination centers, enabling the inclusion of a large sample population due to the high degrees of mobility and compliance. This method is a feasible approach to obtaining large sample sizes in future studies. Most previous studies were conducted in hospitals. In recent years, studies conducted in physical examination centers have gradually increased (12, 13), but the sample size has remained small. This study can provide more information for the use of norms in physical examination settings.

The Critical Value

Each item on the SCL-90 is scored on a five-point scale from 1 to 5. One point indicates no symptoms, and two points indicates mild symptoms (14). The authors of the scale did not propose a critical value. Generally, the value of two points is used for screening based on experience rather than the standard score (10–15). In 1999, Tang et al. (8) proposed that the mean value plus one standard deviation should be used as the critical value. This study showed that there is a large difference in the screening results when the mean and two points are used. Using the mean score as the critical value, the detection rate ranged between 13 and 16%, which is reasonable. When the critical value was two points, the detection rate varied from 13 to 70%, which covered a large range. This finding indicates a reduced specificity of detection. Thus, the critical value of two points is no longer suitable as the critical value when the SCL-90 is used for screening purposes. New standards are urgently needed. The mean value plus one standard deviation is a candidate standard.

Comparison With Norm Data From Other Countries

According to the literature (16), in the US population, the average score of each item of the SCL-90 was lower than that reported here with an average score below 0.5 and scores concentrated

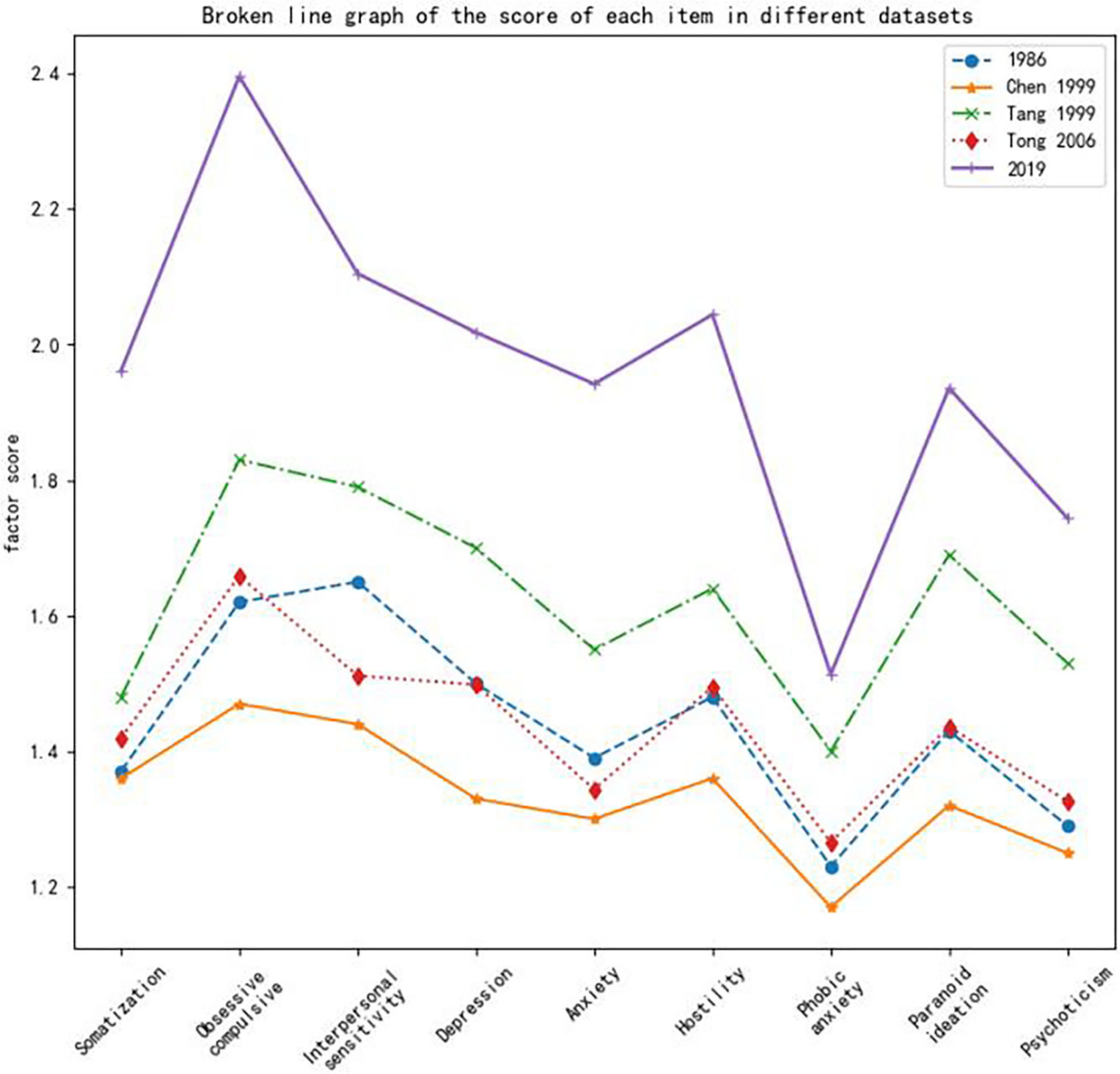

FIGURE 1 | Broken line graph of the score of each item in different datasets.

in the range from 0.2 to 0.4, except for phobic anxiety (0.13) and psychosis (0.14). The average scores of all items among New Zealand college students ranged between 0.7 and 1.2, except for phobic anxiety (0.28). A study showed (17) that the scores of all items in the German population ranged between 0.29 and 0.5, except for phobic anxiety (0.14) and psychosis (0.18). The scores of obsessive-compulsive symptoms were the highest, and there was a downward trend in the scores from compulsion to anxiety. Other studies (16, 18) have shown that the scores of each item slightly increase as the economy continues to develop in the United States, but the average scores of all items in the British population ranged between 0.4 and 0.6, except for phobic anxiety (0.24) and psychosis (0.27). A study involving college students in Spain (19, 20) showed that the scores of all items ranged between 0.4 and 1, with scores of 0.18 for phobic anxiety and 0.36 for psychosis. Furthermore, another study (21) showed that the scores of all items in the Vietnamese population ranged from 0.3 to 0.8, and the scores did not markedly change over time. A previous study (22) compared sample populations from three different regions in Chile. In all three regions, obsessive-compulsive symptoms had the highest scores, and psychosis and phobia had the lowest scores. Another study (15) investigated students from two universities in Hungary and found that the various items of the SCL-90 had scores between 0.37 and 0.8;

TABLE 4 | Comparison of the subjective perception rating and norm detection rate.

Subscales	Mean (detection rate)	two points (detection rate)	*Z*
Somatization	1,132 (15.12%)	3,034 (40.51%)	26.95***
Obsessive compulsive	1,082 (14.45%)	5,249 (70.09%)	55.12***
Interpersonal sensitivity	1,046 (13.97%)	3,810 (50.87%)	38.25***
Depression	1,223 (16.33%)	3,442 (45.96%)	30.37***
Anxiety	1,226 (16.37%)	3,051 (40.74%)	25.14***
Hostility	1,028 (13.73%)	3,191 (42.61%)	34.37***
Phobic anxiety	1,047 (13.98%)	1,047 (13.98%)	0
Paranoid ideation	1,180 (15.75%)	2,870 (38.32%)	24.28***
Psychoticism	1,209 (16.14%)	1,963 (26.21%)	12.71***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

obsessive-compulsive symptoms had the highest scores, and psychosis had a lower score than paranoid ideation. Previous studies (23, 24) have found that the SCL-90 scores of Danes were higher than those of the US population, but the difference was <1 point. The above overall scores were all lower than those in 2019 in China, but they are highly consistent. For example, the score of obsessive-compulsive symptoms is consistently the highest, the score of psychosis is lower than that of paranoid ideation, and the scores of phobic anxiety and psychosis are the lowest. According to the data from these countries, the overall trend in the SCL-90 scores remains the same in different countries. However, the overall score is relatively high in China possibly due to factors related to cultural and economic development. This finding indicates that the SCL-90 should have different evaluation criteria in different countries.

Because the sample populations used in this study were regional, there may be some differences from the norms applicable to China as a whole, and some errors are expected. Further studies will include sample populations from more regions for further analysis and research.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee/IRB of Tianjin Ciming checkup center.

AUTHOR CONTRIBUTIONS

WDa, YX, WDo, XY, and YM contributed to the conception and design of the study. JL and JJ organized the database. JJ and YM performed the statistical analysis. WDa and YX wrote the first draft of the manuscript. YM, SZ, and WW wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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REVIEW
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Resting State Functional Connectivity Biomarkers of Treatment Response in Mood Disorders: A Review

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There are currently no validated treatment biomarkers in psychiatry. Resting State Functional Connectivity (RSFC) is a popular method for investigating the neural correlates of mood disorders, but the breadth of the field makes it difficult to assess progress toward treatment response biomarkers. In this review, we followed general PRISMA guidelines to evaluate the evidence base for mood disorder treatment biomarkers across diagnoses, brain network models, and treatment modalities. We hypothesized that no treatment biomarker would be validated across these domains or with independent datasets. Results are organized, interpreted, and discussed in the context of four popular analytic techniques: (1) reference region (seed-based) analysis, (2) independent component analysis, (3) graph theory analysis, and (4) other methods. Cortico-limbic connectivity is implicated across studies, but there is no single biomarker that spans analyses or that has been replicated in multiple independent datasets. We discuss RSFC limitations and future directions in biomarker development.

Keywords: resting state functional connectivity, fMRI, depression, bipolar disorder, mood disorder, biomarker, treatment response, neuroimaging

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INTRODUCTION

Psychiatric disorders are currently defined by symptom clusters in the Diagnostic and Statistical Manual of Mental Health Disorders, 5th Edition (DSM-5) (1). These clinical constructs are fairly reliable (2–4), but they lack biological validity. Research Domain Criteria (RDoC) aims to address this issue by providing a neuroscientific framework in which to study psychiatric symptoms independent of DSM classifications (5–9). Attempts to create new clinical phenotypes by mapping symptoms onto RDoC constructs have had limited success (10), highlighting the need to identify biological substrates and biomarkers.

A biomarker is a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" (11). As such, biomarkers can represent clinically relevant intermediate outcomes or endpoints that are difficult to measure. Several branches of medicine have used biomarkers to identify pathology in presymptomatic or asymptomatic individuals, elucidate treatment mechanisms of action, predict or monitor treatment response, improve existing treatments, and develop new treatments (12). There are currently no established biomarkers in psychiatry, a field with few measurements besides scales for reported or observable symptoms.

Neuroimaging is a critical tool for developing psychiatric biomarkers (6). Traditional functional neuroimaging examines the spatial and temporal characteristics of blood oxygen level-dependent (BOLD) signal during alternating blocks of task and rest. What happens during rest is essentially treated as noise, a spontaneous signal drift to be subtracted from task block data. Newer paradigms question this traditional perspective on signal vs. noise, especially since task-based BOLD signal only accounts for a fraction of overall neural metabolism (13). Connectivity analyses of rest blocks has revealed spatial and temporal patterns that effectively launched the field of resting state functional connectivity (RSFC) (13, 14).

The ability of RSFC to identify known connections between brain regions has been empirically validated (15–18). Functional connectivity does not necessarily imply anatomical connectivity, although this relationship can be useful to explore if present (19–21). There are several advantages to RSFC vs. task-based activation, including simpler data acquisition and greater capacity to detect individual- and group-level differences (22). Despite these advantages, RSFC is inherently noisier because signal fluctuations are highly dynamic yet low in amplitude (23). There are also issues such as scanner drift, motion artifact, and limited signal-to-noise in ventral brain regions that are prominent in but not unique to RSFC (24–29). These factors make it challenging to measure intra- and inter-scan reliability within or between studies. Furthermore, the wide variety of RSFC analytic techniques complicates interpretation between studies.

The present study is a review of RSFC biomarkers of treatment response in mood disorders, which are prevalent and often debilitating conditions associated with chronic illnesses, suicide, and all-cause premature death (30–40). There are effective treatments for mood disorders, but at least one-third of patients do not remit despite multiple treatment trials (41, 42). Furthermore, little is known about treatment mechanisms of action, making it difficult to select specific treatments for specific symptom profiles or patients (5, 7, 9, 43–45). Several existing reviews have successfully highlighted task-based and RSFC biomarkers of mood disorders (46–55). We aim to expand this knowledge base by focusing on the evidence for biomarkers of treatment response across diagnoses, theoretical brain network models, and treatment modality. To this end, our review is organized around the following primary analytic techniques: (1) reference region (seed-based) analysis, (2) independent component analysis (ICA), (3) graph theory analysis, and (4) other methods. We hypothesized that there will be no single treatment biomarker validated for mood disorders across diagnoses, models, treatment modalities, and independent datasets.

METHODS

We followed general Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (56, 57) in this review of TSFC biomarkers of treatment response in mood disorders. HGK searched PubMed for studies with various combinations of the following terms: "resting state," "functional connectivity," "fMRI," "treatment effects," "neuroimaging biomarkers," "mood disorders," "depression," and "bipolar disorder." During the first quarter of 2020, HGK identified candidate abstracts of studies reporting RSFC changes following treatment as well as studies attempting to predict treatment response based on RSFC. Studies meeting these criteria were reviewed by JJT, HGK, and AA. Studies were excluded if they were not written in English, if they did not include treatment, or if they reported RSFC changes in healthy participants. Results are organized by RSFC analysis method. See **Supplementary Material** for a table summarizing the main studies discussed in the text.

RESULTS

Reference Region (Seed-Based) Analysis

Reference region analysis, or seed-based analysis, involves assessing the time series correlation between an *a priori* region of interest (ROI) and all other voxels in the brain (17). Whole-brain, voxel-wise functional connectivity maps of co-variance with the seed region are usually generated from general linear model analysis (58). Like many other FSFC analyses, seed-based analysis assumes that temporal correlation between two or more regions implies a functional connection between them, potentially revealing a brain circuit or network (59). Reference region analysis is considered univariate because voxelwise data are regressed against the broader model in an independent manner (58).

RSFC between two brain regions is typically bidirectional. The following section is organized based on which ROI connectivity was defined as the primary measure in each particular study.

Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) is of particular interest in mood disorders. It is typically divided into dorsal/pregenual and rostral/subgenual regions based on cytoarchitecture, connectivity, and putative function in healthy controls.

Dorsal/Pregenual Anterior Cingulate Cortex

The dorsal/pregenual region has been consistently implicated in conflict resolution (60) and in several processes associated with depression (18, 61, 62). RSFC between dorsal/pregenual ACC and limbic regions such as amygdala, striatum, and medial thalamus is decreased in depression (18), and this connectivity increases after successful treatment with sertraline (63). Clinical symptom improvement correlates with increased dorsal/pregenual ACC-amygdala connectivity and decreased amygdala activation (64). ACC connectivity changes have also been documented after successful electroconvulsive therapy (ECT) for unipolar and bipolar depression, with specific changes in connectivity to orbitofrontal cortex, caudate, dorsolateral prefrontal cortex, and posterior cingulate cortex (65).

The data on whether dorsal/pregenual connectivity can predict treatment response are limited and mixed. One study of late-life depression reported that decreased RSFC between dorsal/pregenual ACC and other regions of the cognitive control network predicted low remission rates after 12 weeks

of escitalopram (61). In a different study, increased RSFC of the dorsal/pregenual ACC to the cognitive control network predicted non-response to 12 weeks of escitalopram, duloxetine, or venlafaxine (62). These conflicting reports are difficult to reconcile, and more data are needed.

Rostral/Subgenual Anterior Cingulate Cortex

The rostral/subgenual ACC, which encompasses Brodmann area 25 and is also known as subcallosal ACC, has been frequently implicated in the pathophysiology of major depression (66, 67). Rostral/subgenual ACC is generally found to be anticorrelated with the dorsal/pregenual ACC. In other words, rostral/subgenual ACC RSFC to limbic regions is increased in depression (68, 69).

Several studies have explored whether RSFC connectivity of the rostral/subgenual ACC can predict treatment outcomes across treatment modalities (70–78). One study found a positive correlation between baseline RSFC of right rostral/subgenual ACC to right dorsolateral prefrontal cortex (DLPFC) and improvement in depressive symptoms following group cognitive-behavioral therapy (74). A different study found a negative correlation between depression improvement and baseline RSFC between left rostral/subgenual ACC and the broader left ACC after 8 weeks of monotherapy with bupropion, escitalopram, or aripiprazole (70).

Some studies have used RSFC of rostral/subgenual ACC to explain differential outcomes following cognitive behavioral therapy vs. medication. In a study of 122 patients with major depressive disorder, positive RSFC of rostral/subgenual ACC with left anterior ventrolateral prefrontal cortex, insula, dorsal midbrain, and left ventromedial prefrontal cortex was associated with remission after CBT and non-response to escitalopram or duloxetine monotherapy. Negative connectivity was associated with the opposite response outcome. Regardless of treatment modality, response was positively correlated with rostral/subgenual ACC RSFC to right post-central gyrus and negatively correlated with rostral/subgenual ACC RSFC to right superior frontal gyrus. By contrast, remission was negatively correlated with rostral/subgenual ACC RSFC to right precentral gyrus and right posterior putamen (71).

RSFC of rostral/subgenual ACC has also been explored in psychedelics and interventional psychiatry. Psilocybin was shown to increase rostral/subgenual ACC RSFC with posterior cingulate cortex and precuneous, but this effect was not correlated with clinical improvement (79). By contrast, increased RSFC between rostral/subgenual ACC and right lateral prefrontal cortex was positively correlated with response to a single ketamine infusion (78).

One of the most robust and convincing lines of research investigating rostral/subgenual ACC is its relationship to transcranial magnetic stimulation (TMS) response. Numerous studies have used baseline rostral/subgenual ACC RSFC to predict or optimize TMS response, although the network itself varies slightly between studies. For example, DLPFC regions (specifically within Brodmann Area 46) that are most anticorrelated to rostral/subgenual ACC tend to be the most effective TMS treatment targets (73). Other studies have taken slight variations on this network, with one showing that TMS responders had stronger baseline anticorrelation between rostral/subgenual ACC and medial and left superior frontal gyrus (specifically Brodmann Area 10) (77). A different group reported that baseline rostral/subgenual ACC RSFC with ventromedial prefrontal cortex, dorsomedial prefrontal cortex, dorsal/pregenual ACC and posterior cingulate cortex predicted TMS response (80). The role of the rostral/subgenual ACC seems to be preserved even if the TMS target or patient population changes. One study of patients with unipolar or bipolar depression showed that response to dorsomedial prefrontal cortex TMS was associated with positive RSFC between rostral/subgenual ACC and DLPFC and negative RSFC between rostral/subgenual ACC and insula, putamen, parahippocampus, and amygdala (76). The rostral/subgenual ACC RSFC also seems to be implicated in accelerated TMS studies as well, with one study showing that responders had stronger rostral/subgenual ACC correlation with medial orbitofrontal cortex after treatment (77).

In the context of these TMS results, it is important to note that there is some convergent evidence for rostral/subgenual ACC across neuromodulation modalities. RSFC of rostral/subgenual ACC has been used to predict ECT response (72), and BA 25 within this region is the primary target for deep brain stimulation for treatment-resistant depression (81–87).

Orbitofrontal Cortex

Orbitofrontal cortex is often implicated in RSFC changes from other seed regions, but there is not a robust literature for it as a primary seed region in mood disorder treatment response. In one study, increased RSFC between right medial orbitofrontal cortex and left amygdala correlated with response to lithium monotherapy in patients with bipolar disorder (75).

Dorsolateral Prefrontal Cortex

DLPFC, a large region spanning Brodmann Area 9 (BA9) and 46 (BA46) (88–90), plays a critical role in higher-order cognition and emotional processing. Numerous PET studies have shown decreased blood flow and oxygen consumption in depression (91), including some that specifically link cognitive symptoms of depression to DLPFC dysfunction (92, 93). Studies have predicted response to escitalopram and duloxetine with RSFC of DLPFC to bilateral middle frontal and inferior parietal regions (94).

As outlined earlier, a significant portion of recent seed-based analyses of DLPFC RSFC are in the context of TMS. Numerous studies have shown that DLPFC RSFC can correlate with or predict TMS response. In one study, higher RSFC between left DLPFC and striatum predicted TMS response (95). Several regions have been shown to exhibit connectivity changes after TMS response when DLPFC is the seed region, including parahippocampus (80) and left caudate (96). More studies are needed to clarify which downstream brain network nodes are most critical to TMS response.

Amygdala

As an integral node of the limbic system, amygdala has been most consistently implicated in response to negative stimuli in healthy controls. Several studies have identified altered amygdala function in depression and mania (18, 75, 97, 98), which likely inspired analyses of whether amygdala RSFC predicts or correlates with treatment response across various modalities. In early-life depression, increased baseline RSFC between amygdala, left DLPFC, and left anterior insula predicted treatment response to cognitive behavioral therapy (74). A similar strategy has been used to examine response to medications, although results are varied. In one study, increased baseline RSFC between amygdala, right central parietal opercular cortex, and Heschl's gyrus predicted response to fluoxetine or sertraline monotherapy in adolescents (99). Predictors of poor response to serotonergic medication for depression have been identified as increased baseline RSFC between amygdala, right precentral gyrus, and left supplementary motor area in one study (99), and increased baseline RSFC between amygdala and bilateral orbitofrontal cortex in a different study (94). Yet another study found that response to fluoxetine or sertraline was associated with increased RSFC between amygdala and right middle and middle frontal gyri, and decreased RSFC between amygdala and right posterior cingulate/precuneus (100). Limited sample sizes and methodological differences make it challenging to integrate these findings across studies.

Only one study investigating the impact of second-generation neuroleptic monotherapy on amygdala RSFC that met inclusion criteria. In this study, response to quetiapine was correlated with increased RSFC between left amygdala, superior and middle occipital gyri, and bilateral mid-cingulate, and between right amygdala and superior and middle occipital gyri and cuneus (101). This study was conducted in patients with unipolar depression and comorbid anxiety. Additionally, there was one study identifying the effect of a mood stabilizer on amygdala RSFC. In this study, increased RSFC between amygdala, rostral/subgenual ACC, and ventromedial prefrontal cortex correlated with improvements in depressive and hypomanic symptoms after lithium monotherapy (75).

There are some interesting studies implicating amygdala RSFC with response to real-time functional magnetic resonance imaging neurofeedback in patients with depression who are not taking medications (102, 103). One study demonstrated that abnormal RSFC between amygdala and several regions was reversed by real-time neurofeedback, with a specific emphasis on hippocampus (103). A different study showed that increased RSFC between amygdala and precuneus was associated with clinical improvement after real-time neurofeedback (102).

Amygdala RSFC does not appear to be thoroughly examined in the context of neuromodulation studies, but one study showed that patients with schizophrenia or major depressive disorder both showed significant RSFC decreases between right amygdala, right temporoparietal junction, medial prefrontal cortex, left posterior insula, and right DLPFC, and increases between right amygdala and hypothalamus after ECT. None of these changes correlated with changes in symptom severity (104).

Striatum

Striatum is thought to process several aspects of cognition in healthy controls, from motor planning to motivation and decision-making. In the context of mood disorders, psychomotor slowing or agitation have been linked to striatal connectivity changes (105). Nucleus accumbens, and ventral striatum more broadly, has been implicated in reward processed. Whereas, decreased RSFC has been linked to unipolar depression (106, 107), increased RSFC has been linked to bipolar disorder (108). One study showed that first episode mania was associated with decreased RSFC in the dorsal and caudal corticostriatal systems, and increased RSFC in the ventral striatal systems. Moreover, these baseline RSFC abnormalities predicted improvement in patients receiving lithium or quetiapine (109).

Insula

Insula is the one of the primary regions in which interoceptive information and emotional salience are processed (110, 111). Most of the mood disorder studies that have attempted to correlate insula RSFC to treatment response focus on psychotherapy. In one study, RSFC between right insula and right middle temporal gyrus predicted response to behavioral activation treatment in medication-free patients with unipolar depression (112). A similar study showed that successful cognitive behavioral therapy increased RSFC between right insula and left supragenual ACC in adolescents with unipolar depression (113).

One study of transdiagnostic cognitive behavioral therapy attempted to use baseline RSFC to predict improvement in emotional regulation rather than clinical outcomes. Several interesting results were generated by this study. At baseline, neuroticism was negatively correlated with RSFC between right dorsal anterior insula and inferior parietal lobule, and perception of impaired affective control was positively correlated with RSFC between ventral anterior insula and bilateral dorsal/pregenual ACC. Greater improvements in emotional regulation were predicted by decreased RSFC between right dorsal anterior insula and right ventrolateral prefrontal cortex as well as by increased RSFC between bilateral dorsal anterior insula and bilateral amygdala (114).

Hippocampus

Hippocampus plays critical roles in memory, cognition, and regulation of stress in healthy controls. It appears to be implicated in several RSFC analyses as a downstream node rather than the primary seed itself. One study showed that the increase in RSFC of right hippocampus after electroconvulsive therapy correlated with clinical improvement in elderly patients with varying degrees of unipolar depression (115).

Brainstem Nuclei

Aminergic nuclei have been hypothesized to play a role in mood disorders. One study found that patients treated with selective serotonin reuptake inhibitors had increased RSFC between dorsal raphe nucleus and precuneus, angular gyrus, and bilateral cerebellum, increased RSFC between locus coeruleus and occipital lobe, left precentral gyrus, and

parahippocampal gyrus, and increased RSFC between ventral tegmental area and precuneus, left inferior parietal lobule, and bilateral middle/inferior temporal gyrus relative to patients treated with a serotonin norepinephrine reuptake inhibitor. By contrast, patients treated with a serotonin norepinephrine reuptake inhibitor had increased RSFC between dorsal raphe nucleus and right DLPFC, ventrolateral prefrontal cortex, and bilateral superior temporal cortex, increased RSFC between locus coeruleus and bilateral DLPFC, ventromedial prefrontal cortex, inferior temporal gyrus, and bilateral cerebellum, and increased RSFC between ventral tegmental area and left insula and bilateral cerebellum relative to the group treated with selective serotonin reuptake inhibitors (116).

In a separate study of young adults treated with selective serotonin reuptake inhibitors for unipolar depression, RSFC between ventral tegmental area and cuneus-occipital areas correlated with symptom improvement (117).

Independent Component Analysis

Independent component analysis (ICA) is a statistical method used to discover hidden factors (components, sources, or features) in a set of measurements or observed data such that the factors are maximally independent. The main advantage of ICA is that it provides a data-driven means by which to measure whole-brain connectivity with all components considered.

Despite its strengths, ICA has a number of disadvantages and limitations. First, the process of identifying components and selecting methods with which to run ICA (e.g., dual regression) is subjective and variable. Second, inter-session reliability of component strength has not been fully established, which limits the degree to which ICA can reliably measure longitudinal treatment effects. Third, the functional attribution of each ICA component is indirectly assumed based on the brain regions included the analysis. Moreover, the function of those brain regions has been extrapolated from healthy controls in tasks that may or may not have translational significance. For example, the salience network is not consistently implicated across tasks that claim to test salience. As such, the functional significance of ICA components may change over time and between studies.

Several strategies have been proposed to address these imitations. One strategy involves examining the correlation between the time series extracted from each ICA component. This analysis would presumably measure connectivity between putative networks in a way that parallels seed-based analysis (118, 119). Unfortunately, the neurophysiological significance of the correlation between ICA components and the stability of this correlation within and between scanning sessions remain unclear, making it challenging to use these measures as treatment biomarkers. A different strategy involves reporting hypotheses and results in the form of ICA components even when the study used seed-based analyses, or vice versa (80, 120, 121).

ICA of RSFC has revealed several components comprised of correlated brain regions. These brain networks are named after their putative function in healthy controls, which provides some speculative basis for psychopathology (122, 123). In this review, ICA component terminology will only be used for studies in which an actual ICA analysis was conducted.

Default Mode Network

The default mode network is primarily comprised of the medial prefrontal cortex, ACC, posterior cingulate cortex, and angular gyrus. This intrinsic organizational structure shows high connectivity during wakeful rest and low connectivity during most goal-directed tasks, although there are some exceptions to these generalizations (123–125).

The default mode network has been implicated in clinical response across various treatment modalities, including psychotherapy, medications, and neuromodulation. In one study, responders to cognitive behavioral therapy or cognitive processing therapy had significantly higher increases in default mode network RSFC than non-responders (126).

Medication studies have shown varying results. In one study, an intravenous infusion of citalopram was correlated with positive RSFC between default mode network and left precuneus and negative RSFC between default mode network and amygdala in a group of patients with major depressive disorder. Interestingly, healthy controls who received the same infusion also had a positive correlation between default mode network and amygdala relative to healthy controls receiving a placebo infusion (127). In different studies, baseline RSFC of default mode network with orbitofrontal cortex was negatively correlated with improvement after 12 weeks of duloxetine (128).

Neuromodulation studies show results that may be consistent but are difficult to contextualize. In one TMS study, baseline RSFC between default mode network and ventromedial prefrontal cortex and ACC was more than 80% effective at discriminating responders from non-responders (119). In an ECT study, RSFC of default mode network to DLPFC was normalized after ECT response in late-life depression, and this increase in RSFC differentiated remitters from non-remitters (129).

Salience Network

The salience network, which is primarily comprised of insula and dorsal/pregenual ACC, is responsible for triaging stimuli and integrating multimodal information in healthy controls. As such, it is widely involved in communication, socialization, and self-monitoring (130). The salience network has most recently been implicated in TMS response. One study showed that baseline RSFC in the salience network was positively correlated with TMS treatment response (119). This result was replicated in a different study specifically examining early treatment response to TMS (131).

Inter-Network Connectivity

There are relatively few ICA studies of how inter-network connectivity may correlate with or predict treatment response in mood disorders. One study showed that higher baseline RSFC within the default mode network and between the default mode network and the central executive network predicted response to sertraline monotherapy in a relatively large sample of patients with unipolar depression (121).

Effective Connectivity

Effective connectivity is a means to infer causal or directional influences between brain network nodes (132). There were not many ICA studies examining treatment response in mood disorders. In one study, baseline fronto-insular effective connectivity was positively correlated with early response to TMS (131).

Graph Theory Analysis

The application of graph theory to neuroimaging has yielded unique insights into network-wide properties rather than the strength of connectivity from a specific seed region (133). In this approach, a connection or adjacency matrix is used to summarize the nodes (brain regions) and edges (connections) of a brain network. A number of measures can be used to assess the matrix, including centrality (e.g., pageRank centrality, subgraph Centrality) assortativity (e.g., resilience), segregation (clustering coefficient, transitivity), and integration (e.g., diffusion efficiency).

The main advantage of graph theory is that it can provide a single variable for a network. As such, changes in that metric can be used to assess how an intervention affects the network. Despite this advantage, there are several limitations to consider First, the availability of numerous metrics can lead to numerous statistical analyses. Second, the stability of these metrics over time has not been established. Third, the significance of these metrics to putative function or clinical symptoms is unclear.

In this section, graph theory metrics are discussed in the context of treatment response in mood disorders.

Centrality

There are only a few studies examining a whole brain centrality measure in the context of mood disorder treatment. One study used eigenvector centrality to identify network nodes that are densely connected and sensitive to serotonergic medications. In this study of late-life depression, patients who remitted with venlafaxine showed significant RSFC increases between right precentral gyrus in the central executive network and significant decreases between right inferior frontal gyrus, supramarginal gyrus, and default mode network. Moreover, remitters showed significantly greater eigenvector centrality in bilateral inferior frontal gyrus and medial frontal gyrus than non-remitters (134). Using a slightly different metric, a study in patients with bipolar disorder showed that lithium treatment normalized mania-related connectome indices, reflected in part by significantly decreased right amygdala clustering coefficient (135).

One study assessed several metrics, including a centrality metric, to assess the effects of TMS on unipolar or bipolar depression. In this study, successful adjunctive TMS to dorsomedial prefrontal cortex resulted in significant increases in betweenness centrality in the stimulation target as well as in right amygdala, ventral striatum, and temporal pole. The authors noted that responders and non-responders showed opposing patterns of connectivity lateralization, and that patients with preserved hedonic function may be more responsive to dorsomedial TMS (136).

Assortativity

The study mentioned above that captured decreased right amygdala clustering coefficient with lithium treatment in bipolar disorder also assessed assortativity, which can be thought of as the degree to which a network node connects to similar nodes in a complex network. This study found that successful lithium treatment increased assortativity in a mood regulation network (135).

Global Brain Connectivity

One study leveraged the rapid-acting antidepressant effects of ketamine infusions to assess functional dysconnectivity changes in patients with major depression.

Responders to ketamine showed significant increases in global brain connectivity with global signal regression in lateral prefrontal cortex, caudate, and insula. The authors suggested that ketamine normalizes the dysconnectivity between these regions and the rest of the brain in major depressive disorder (137).

Connection Density

Connection density can be thought of as the number of observed connections relative to the number of possible connections in a graph or network. A few studies have examined this metric in the context of mood disorders treatment. The study of patients with bipolar disorder taking lithium that assessed clustering coefficient and assortativity also examined connection density. In this study, patients with bipolar disorder showed decreased mean connectivity in a network of which the largest percentage of differential links were with left posterior superior frontal gyrus, a midbrain region consisting of the red nucleus, substantia nigra, and ventral tegmental area, and right amygdala. Moreover, decreases in mania ratings were correlated with the decreases in in mean connectivity of this network (135).

A different study used network density and other measures to test the hypothesis that patients with dysthymic disorder have greater RSFC within the default mode network. At baseline, patients with dysthymic disorder showed higher default mode network RSFC than healthy participants, with specific elevations noted between posterior cingulate cortex and medial prefrontal cortex, bilateral lateral parietal lobes, and precuneus. After 10 weeks of duloxetine, patients with dysthymic disorder showed significantly reduced connectivity in many of these same connections. This "normalizing" effect was most prominent between posterior cingulate cortex, right lateral parietal cortex, and right inferior temporal gyrus (138).

Other Methods

Aside from seed-based analysis, ICA, and graph theory analysis, several other methods have been developed to examine functional connectivity. A few examples that will not be covered here include regional homogeneity analysis (59, 139) and four-dimensional (spatiotemporal) consistency of local neural activities (FOCA) (140). These methods are not frequently used in treatment studies, and their functional significance and stability over time have not yet been established.

This section will briefly review coherence metrics, fractional amplitude of low-frequency fluctuation (fALFF), and machine learning.

Coherence Metrics

One study of patients with treatment-resistant depression receiving bilateral electroconvulsive therapy created maps of network coherence in each patient by using the mean time series of the default mode network (defined by ICA) as a regressor for each voxel within the default mode network. Maps from responders were compared to maps from non-responders and healthy controls using permutation testing. Patients with depression showed significantly decreased network coherence in precuneus and angular gyrus relative to healthy controls, and this difference normalized in electroconvulsive therapy responders but not non-responders. The authors interpreted this finding as preliminary evidence that electroconvulsive therapy reconnects a part of the default mode network to the broader network (141).

Fractional Amplitude of Low-Frequency Fluctuation

One of the primary goals of fALFF is to quantify the local, low frequency signals that often gets averaged across larger regions and frequency bands (59). This quantification is done by conducting a Fourier transformation on the BOLD signal and measuring power in ranges below 0.01 Hz. Several studies have employed fALFF to examine treatment effects of psychotherapy, medication, and neuromodulation for mood disorders.

A psychotherapy study used fALFF and other analyses to probe how cognitive remediation therapy changes intrinsic neural activity in patients with major depression. At baseline, patients with depression had reduced functional network strength in bilateral prefrontal systems. Intrinsic neural activity increased in right inferior frontal gyrus after cognitive remediation therapy, and activity changes in several areas including left inferior parietal lobule, left insula, left precuneus, and right caudate were associated with cognitive improvement (142).

A medication study used functional connectivity, effective connectivity, and fALFF to argue that major depression is associated with abnormal pulvinar oscillations and abnormal causal interactions between pulvinar and several nodes of default mode and posterior insular networks. They also show provide data that duloxetine can ameliorate this pulvinar pathophysiology (143).

A neuromodulation study used seed-based analysis and fALFF to probe the neurobiological substrates of electroconvulsive therapy response in unipolar or bipolar depression. At baseline, BOLD signal fluctuations (fALFF) in subcallosal cingulate cortex were significantly higher in patients with depression than they were in healthy controls. Successful electroconvulsive therapy significantly decreased these signal fluctuations (fALFF). Also, baseline signal fluctuation (fALFF) abnormalities predicted treatment response (144).

Machine Learning

Machine learning is a broad term that generally refers to the process of finding patterns in large, high dimensional datasets by training a computational model to predict unseen data. There are several ways to use machine learning to study treatment effects on brain networks. One study used a method called alternating decision trees to build models that accurately predicted late-life depression diagnosis and antidepressant treatment response with ∼87 and 89% accuracy, respectively. Amongst other measures, these models included structural and functional connectivity. Lower RSFC of dorsal default mode network was specifically associated with positive treatment response (145).

A neuromodulation study leveraged RSFC and machine learning techniques to explore biomarkers of individual response to transcranial magnetic stimulation for depression. At baseline, patients with depression had low signal in caudate, prefrontal cortex, and thalamus. RSFC in default mode and affective networks was associated with treatment response. Using these findings, the authors successfully trained support vector machines to predict individual treatment response with 85–95% accuracy (146).

Machine learning has also been used to assess and predict individual response to electroconvulsive therapy. In one study, RSFC and multivariate pattern analysis identified a network centered in dorsomedial prefrontal cortex (including DLPFC, orbitofrontal cortex, and posterior cingulate cortex) that was 85% sensitive and 85% specific for individual response. A different network centered in the ACC (including DLPFC, sensorimotor cortex, parahippocampal gyrus, and midbrain) showed 80% sensitivity and 75% specific for individual response (147). A different study largely corroborated these results. In this study, a radial support vector machine was trained using arterial spin labeling and BOLD signal RSFC before electroconvulsive therapy for depression. The model predicted non-responders and responders with 74 and 64% accuracy, respectively, using connectivity strength among frontoparietal networks (including DLPFC), motor and temporal networks (near electroconvulsive therapy electrodes), and rostral/subgenual ACC (148).

DISCUSSION

Summary

There are several reviews of task-based and RSFC biomarkers of mood disorders (46–55), but few evaluate evidence across diagnoses, models, and treatment modalities. In this review, we examined biomarker data categorized by analytic technique: (1) reference region (seed-based) analysis, (2) ICA, (3) graph theory analysis, and (4) other methods. This review supports our *a priori* hypothesis that there is no single mood disorder RSFC treatment biomarker validated across diagnoses, models, treatment modalities, and independent datasets.

Reference Region (Seed-Based) Analysis: ACC, DLPFC, and Amygdala

Reference region (seed-based) analyses appear to be the most commonly used technique to assess RSFC biomarkers of treatment response in mood disorders. In some ways, it is the simplest and most direct way of discovering brain networks functionally connected to an *a priori* ROI (58, 149). Within this disproportionately large sample, ACC emerged as the region with

the most consistent evidence across studies. This emergence has face validity given the well-established role of ACC as a nexus of cognitive (dorsal) and affective (ventral) processing (150–153). There are several lines of convergent evidence that Brodmann area 25 within the ACC plays a particularly critical role in mood regulation, driven in part by invasive and non-invasive neuromodulation. In TMS studies, stimulating DLPFC regions more functionally connected to Brodmann area 25 appears to enhance response (73, 154). This technique is emerging as the preferred targeting method in clinical trials (155, 156). Brodmann area 25 is also the most frequent target for deep brain stimulation, a controversial intervention for refractory depression with intriguing but mixed results (81–87). ACC also has unique cytoarchitecture (e.g., spindle cells or von Economo neurons) that could theoretically explain its role in mood disorders, but current imaging modalities have limited capacity to investigate this premise (157).

The other region that appears to emerge most consistently from reference region analyses is amygdala, which has structural and functional connections to prefrontal cortex, anterior cingulate, and other regions implicated in mood regulation. The role of amygdala has been identified previously (158, 159), but the current review emphasis its importance across diverse treatment modalities and contextualizes its role as both primary seed region and downstream network node for other seed regions. Interestingly, relatively few neuromodulation studies have focused on amygdala as a primary seed region.

Independent Component Analysis: Default Mode Network

ICA appears to be the next most frequently used analysis technique for treatment biomarkers in mood disorders. This multivariate analysis avoids some of the biases and restrictions inherent to univariate reference region analysis (58, 160). Default mode network emerged as the construct with the most evidence across studies, which again implicates ACC as a critical node. It is difficult to evaluate default mode network as a biomarker of treatment response because it is so broadly implicated across tasks, non-tasks, and patient populations. Whereas, some studies report on connectivity exclusively within the default mode network, others reported on connectivity differences between individual nodes of the network and other network nodes that are not considered part of the network. There is also the possibility of the entire network correlating with another identified network, but this possibility raises several statistical and methodological challenges. It is also important to note that default mode network lacks specificity for mood disorders, further raising questions about how best to characterize it as a biomarker (161, 162).

Machine Learning Analysis: Too Few Studies

There was no single region or network that emerged from graph theory analyses or other analyses, although these studies generally invoked individual network nodes identified with other analyses. A few machine learning studies were compelling in generating models with high predictive sensitivity and specificity, but these studies were in limited sample sizes and were not tested in independent datasets. They also typically lack causal evidence in the form of brain lesions or brain stimulation.

A Speculative Integration of Results

Despite the lack of consistent biomarker across diagnoses, models, treatment modalities, and independent datasets, the current results generally corroborate existing literature on the brain circuitry implicated in mood disorders. The DLPFC and ACC are frequently shown to be critical hubs in a network that mediates depressive symptoms. DLPFC lesions and blood flow changes correlated with depression motivated the earliest TMS studies (163, 164), and lesions with functional connectivity to this region are associated with depression (165). One of the main regions connected to DLPFC is Broadman area 25 (24) of the anterior cingulate cortex. Several studies have shown that activity in this region correlates with depressive symptoms (91, 166). From an explanatory model perspective, BA25 shows structural and functional connectivity to regions that could theoretically mediate depressive symptoms beyond low mood or sadness. Connections to medial and dorsolateral prefrontal cortex, orbitofrontal cortex, anterior, and posterior cingulate, amygdala, and hippocampus could mediate affective and executive symptoms, and connections to insula, hypothalamus, and monoaminergic brainstem nuclei could mediate neurovegetative symptoms (167). This theoretical model would encompass many of the regions and networks identified in this review.

One of several topics that needs to be further explored is the convergence and divergence between unipolar depression, bipolar depression, and mania. Presumably there is a region or network of regions that serves as a central regulator of mood, maintaining the balance between depression and mania as opposite ends of a mood spectrum, but more information is needed. There has been some recent progress in understanding mania as a state, although symptom specificity remains a challenge. For example, lesions associated with mania show a specific connectivity pattern that includes right orbitofrontal cortex, right inferior temporal gyrus, and right frontal pole (168). Future studies will continue to refine understanding of how mood is regulated throughout structurally or functionally connected brain networks.

Limitations

There are several limitations that should be considered when interpreting the present results. First, the studies summarized in this review were heterogeneous in terms of patient populations, imaging acquisition quality or duration, analytic methods, and inter-scan reliability. It is beyond the scope of this review to outline these differences in fine detail, but nevertheless it is important to acknowledge that direct comparisons of potential biomarkers between heterogeneous studies should be done with caution. Second, the frequency with which a node or network is mentioned does not necessarily imply replication. For example, ACC could appear to be the region with the most evidence because the greatest number of studies chose it as a reference region or studied it in the context of the default mode network in

ICA studies. The inverse problem applies to graph metric studies, which were fewer in number and thus difficult to contextualize. Third, this review does not address RSFC signal-to-noise or inter-scan reliability. RSFC is a dynamic measure, and studies could be using short scans that do not capture enough data (169). The dynamic nature of RSFC is particularly problematic for treatment effect studies that reply on repeated scans over time. There are ways to address these issues at the individual experiment level (170), but it is difficult to assess them in a review. Fourth, it is difficult to assess study design and implementation, particularly in terms of blind integrity and appropriate use of control participants. There are other limitations of this review, to say nothing of the construct validity of "mood disorders" as a category, but those previously discussed are some of the most basic ones to consider when interpreting the present results.

In order to evaluate candidate biomarkers, classification analyses should be run to assess the sensitivity and specificity in distinguishing responders from non-responders. This analysis should subsequently be validated in an independent dataset. A few studies have taken this approach, but the field at large is far from a validated RSFC biomarker of treatment response in mood disorders (118, 145, 146). It may also be the case that a single biomarker is unlikely to be successful because mood disorder are dynamic, heterogeneous, and multifactorial (171–173).

Future Directions

Several strategies have been proposed to advance the study of candidate RSFC biomarkers and neuroimaging more broadly (174). One strategy focuses on individual- rather than group-level analyses, which could theoretically advance precision medicine in psychiatry as outlined by RDoC (5). This approach likely requires robust and repeated sampling from individuals over time, presenting both logistical and statistical challenges (174, 175). A seemingly opposite approach is to invest in larger samples that presumably enhance the power of data-driven analyses. This approach is evidenced by mega-analyses, mega-analyses, and a multitude of multi-site clinical projects such as Establishing Moderators and Biosignatures of Antidepressant Response in Clinical Care (EMBARC), International Study to Predict Optimized Treatment in Depression (iSPOT-D), The Predictors of Remission in Depression and Individual and Combined Treatment (PReDICT), Response to Lithium Network (R-LiNK), and others (176, 177). Larger datasets may increase statistical power, but they also potentially compound noise and variables.

A third approach involves transdiagnostic studies, which presumably avoids the assumptions of searching for neurobiological correlates of symptom clusters that lack biological validity. Examples of this approach typically focus on the "p factor" of general psychopathology, with the long-term strategy of potentially reverse engineering clinical constructs based on brain networks rather than symptom clusters (178–180). This long-term strategy has a number of challenges and is particularly difficult to implement with respect to treatment response.

A fourth strategy is to study focal brain lesions or brain stimulation to assess causality in networks that are potential biomarkers (181, 182). Distinguishing correlation from causation is challenging in traditional neuroimaging studies because network changes may be a cause of, an effect of, or an adaptation to a mood disorder or its treatment (21, 181). A computational technique called network mapping leverages the statistical power of the human connectome (183) to map atrophy coordinates, lesions, or stimulation sites to whole-brain networks rather than single brain regions (21). This technique has been used to identify new neuromodulation treatment targets, and to optimize existing neuromodulation treatment targets for neuropsychiatric conditions (154, 184). Network mapping is powerful, but it also has several limitations as a retrospective meta-analytic technique using normative connectome data to examine symptoms caused by lesions. It is also challenging to assess causality with lesions because brain disorders, like most disorders in medicine, have biopsychosocial aspects to them.

CONCLUSIONS

Many disparate findings have been reported for RSFC biomarkers of treatment response in mood disorders. These findings are complicated by small sample sizes, potential biases, and study heterogeneity. As such, no single biomarker has been identified or validated across diagnoses, models, or treatment modalities. Despite these current limitations, there are several future direction that could facilitate the identification of treatment response biomarkers in mood disorders.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.565136/full#supplementary-material

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Reduced Plasma Dopamine-β-Hydroxylase Activity Is Associated With the Severity of Bipolar Disorder: A Pilot Study

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Dopamine-β-hydroxylase (DβH) is an enzyme converting dopamine to norepinephrine, a key neurotransmitter in mood disorders, such as major depressive disorder (MDD) and bipolar disorder (BD). Due to overlapping symptomology of unipolar and bipolar depression, the present study attempted to explorer if the plasma DβH activity could discriminate the depressive episodes of BD from MDD. The aim of this study was to compare the plasma DβH activity among MDD patients (n = 104), BD patients (n = 101), and healthy controls (n = 160). Clinical characteristics and cognitive function were assessed using the Young Mania Rating Scale (YMRS), Hamilton Depression Scale (HAM-D), Hamilton Anxiety Scale (HAM-A), Patient Health Questionnaire-9 (PHQ-9), and Repeatable Battery for the Assessment of Neuropsychological Status (RBANS). Our data showed a lower plasma DβH activity in patients with BD, not MDD, than that in controls. For the BD patients, the plasma DβH activities were negatively correlated with HAM-D scores and HAM-A scores. However, there was no significant correlation between plasma DβH activity and severity of depressive symptoms in MDD patients. No significant correlation between DβH activities and cognitive assessments neither in BD nor in MDD patients. The present study provides evidence that BD is associated with decreased circulating DβH activity.

Keywords: dopamine-β-hydroxylase, bipolar disorder, major depressive disorder, mood disorder, cognitive function

INTRODUCTION

Bipolar disorder (BD) is a complex and chronic psychiatric disease, with a prevalence to range from 0.5 to 5% in community-based samples (1, 2). BD is a disabling disease due to its early onset, severity and chronic nature although relatively rare (3). The important character of BD is the alternative episodes of mania and depression. However, major depressive disorder (MDD) and BD at depressive phase are similar in clinical manifestations, their overlapping symptomology makes difficult to differentiate BD at depressive phase from MDD (4). More importantly, the use of antidepressants in BD patients have limited efficacy even might increase the possible switch to

manic episodes (5). Therefore, it is important to find biomarker to differentiate BD at depressive phase from MDD, thereby to improve the therapeutic effect on mood disorders.

Unfortunately, the underlying mechanisms of BD and MDD have not yet been fully elucidated, while the monoaminergic theory has been regarded as the main cause of both MDD and BD (6–8). According to the monoamine hypothesis, monoamine neurotransmitters, including serotonin (5-HT), dopamine (DA), and norepinephrine (NE), have always been the key aspects in mood disorders. Although previous genetic, biological and pharmacological studies confirmed the important role of 5-HT in MDD (7, 9), accumulating evidence suggested the contribution of dysregulated DA and NE transmission on both MDD and BD (10–12). DA and NE play an important role not only in cognitive function, but also in emotion regulation (13, 14). In general, depression was associated with reduced activity in the dopaminergic signaling system (15), whereas mania was associated with increased dopaminergic signaling (16). For example, depressive symptoms have been linked with hypodopaminergic transmission in the reward system (17), and dopaminergic stimulant agents augment the efficacy of antidepressants (18). On the other hand, hyperdopaminergic mice showed mania-like behaviors (19), and changed levels of 3-methoxy-4-hydroxyphenylglycol (MHPG, metabolites of NE) and homovanillic acid (HVA, metabolites of DA) were found in BD patients compared to controls (10, 20). Thus, the different changes of DA and NE transmission may contribute to explore the mechanism of MDD and BD.

Dopamine β-hydroxylase (DβH) is the key regulatory enzyme required to synthesize NE from DA (21), and it is also important to maintain the brain DA/NE balance. DβH is located in both central (catecholamine vesicles) and peripheral systems (sympathetic nerves and adrenal medulla) (22, 23). Since DβH can be released from catecholamine vesicles, the protein can be determined in the plasma or serum (21). Previous studies with mice deficient in DβH demonstrated the role of DβH in mood disorders. The birth rate of DBH gene knockout mice was much lower than wild-type mice, and the surviving mice almost died in the first week of life (24), suggesting the important role of DβH in development and survival. Considering the important effect of NE and DA in cognition, several evidences indicated that DβH might play a role in the cognitive defects in mental disorders (25–29), although DβH-deficient patients failed to display neurocognitive impairment (30, 31).

Abundant evidence has shown that low serum/plasma DβH activity might be a risk factor for mental illness (21, 25, 27, 32). Furthermore, the genetic, biological and pharmacological studies indicated the important role of DβH in BD and MDD. The *DBH* gene variants were proved to join in predicting individual differences in social and affective processing (33). Ates et al. indicated the mutated *DBH* gene increased the risk of BD (34). In addition, the serum DβH activity was significant decreased in BD patients in depressive state compared with MDD patients (35), while it was lower in MDD patients than that in healthy controls (36–40). Interestingly, compared with drug-naive BD patients, serum DβH activity was higher in lithium-treated BD patients (41). However, other studies showed the contrary results (42, 43). Animal studies indicated stress increased expression of DβH mRNA and protein in mood-related areas in the brain (44, 45). The contrary studies suggested the complex role of DβH in patients with mood disorders, even between MDD patients and BD patients in depressive state.

Due to overlapping symptomology of unipolar and bipolar depression, the present study attempted to explorer if the plasma DβH activity could discriminate the depressive episodes of BD from MDD. The aim of this study was to investigate whether plasma DβH activity differs between: (i) BD patients and healthy controls (HCs), (ii) MDD patients and HCs, (iii) BD patients and MDD patients. The plasma DβH activity was tested in individuals with three groups: BD patients, MDD patients and HCs. Furthermore, the associations of DβH activity and phenotypes of patients were also assessed in this study.

MATERIALS AND METHODS

Subjects

Subjects were recruited from Beijing Anding Hospital (China) from September 2014 to September 2016. The ethics committee of Beijing Anding Hospital approved the research. All of the individuals provided written informed consent to participate in the present study after fully explaining the purpose and procedure. Male and female patients aged 16 to 60 years who met the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for MDD, or BD were recruited. All patients were screened using the Structured Clinical Interview for DSM-IV Axis I disorders-Patient Edition (SCID) by experienced psychiatrists. In the present study, all the patients with MDD were undergone depressive episodes, while the BD patients also with a current depressive episode were recruited.

The inclusion criteria of all patients were as follows: (1) aged 16 to 60 years; (2) formal education $\geq$9 years; (3) total scores of Young Mania Rating Scale (YMRS) $\leq$ 6. The exclusion criteria of all patients were as follows: (1) comorbidity with other psychiatric disorders, such as schizophrenia; (2) electric convulsive therapy in recent 3 months; (3) were or had a history of substance dependence; (4) severe suicidal tendencies; (5) severe physical diseases, such as neurological diseases, cardiovascular disease, hepatic or renal diseases; (6) current pregnancy or breastfeeding.

The present study recruited gender-matched HC individuals with no history of psychosis or cognitive impairment. HCs underwent a psychiatric interview by experienced clinicians using the SCID to exclude psychiatric disorders. HCs were excluded when encounter the following situations: (1) had any lifetime DSM-IV psychiatric disorder; (2) had severe physical diseases, such as neurological diseases, cardiovascular disease, hepatic or renal diseases; (3) had a family history of psychiatric diseases; (4) were or had a history of substance dependence; (5) were current pregnancy or breastfeeding.

Clinical Assessments

Clinical assessments were administrated by experienced psychiatrists, who were blinded to participants. The patients were assessed with several clinical scales, including the Young

Mania Rating Scale (YMRS) (46), Hamilton Depression Scale (HAM-D) (47), Hamilton Anxiety Scale (HAM-A) (47), and Patient Health Questionnaire-9 (PHQ-9) (48). In addition, the cognitive function of each participant was assessed with Wechsler Adult Intelligence Scale (brief form), Stroop's color-word test (49), and Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (50). Five aspects of the RBANS were evaluated, including attention, speech, visual span, immediate memory, and delayed memory.

Plasma DβH Activity Assay

Peripheral blood from each individual was collected in this study. Plasma was harvested and stored at −80 °C until they were used for the detection of DβH activity. The DβH activity was detected with the method reported from our laboratory (25), which was based on the enzymatic conversion of tyramine (substrate) to octopamine by DβH. Briefly, the reaction mixture (100 μL) was composed of 1 M CH3COONa buffer (pH = 5.0), 0.2 M sodium fumarate, 0.2 M ascorbic acid, 1,500 U catalase, 0.2 M tyramine hydrochloride, 0.02 M pargyline, 0.2 M N-ethylmaleimide, pure water and plasma (5 μL). After being incubated at 37°C for 1 h, the reaction was stopped by adding 20% HClO4 (20 μL). The supernatant was transferred to high performance liquid chromatography (HPLC) system after centrifugation (2,000 rpm) for 10 min. Octopamine concentration was measured by column-switching, reverse phase HPLC system (U3000, Thermo Fisher Scientific, Waltham, MA, USA), with electrochemical detection. Electrodes 1 and 2 of the cell were set at +700 and −320 mV, respectively. Synephrine was used as internal standard. Octopamine and synephrine were separated by a 5 μm particle size reversed-phase SB-C18 analytical column (2.1 × 150 mm) obtained from Agilent Technologies (Santa Clara, CA, USA). The mobile phase was composed of 50.9 mM potassium acetate, 14.3 mM citric acid, 1.38 mM 1-octanesulfonic acid, and 9.5% methanol (v/v). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical Analysis

The data were analyzed using SPSS with version 20.0 (SPSS Inc., Chicago, IL, USA). Demographic and clinical characteristics were compared among three groups using χ^2-tests or one-way analysis of variance (ANOVA). Consideration of the significant difference in age, the covariance analysis was used to compare the continuous variables, including plasma DβH activities among groups with age as a covariate. On the other hand, the multivariate logistic regression analysis was used to evaluate the associations between discontinuous variable, including sex, family history, current state of illness, and mood disorders susceptibility with adjustment for age. Two-way ANOVA was used to evaluate the interaction effects between groups and gender, or compare the plasma DβH activity in patients with different episodes. The comparison of plasma DβH activity in patients with different kinds of drugs was also used with two-way ANOVA. In order to minimize the bias of demographic data on the analysis, the partial correlation was used to analyze the association of DβH activity and clinical and cognitive assessments, with age, education, duration of illness and first onset age as covariates. The level of significance was set at $p < 0.05$. However, the corrected p-value was set at 0.0167 considering the multiple testing of cognitive functions of the patients in the partial correlation analysis.

RESULTS

Demographic and Clinical Characteristics

A total of 389 individuals were initially screened for this study, and 24 (14 in MDD group, six in BD group, and four in control group) were excluded due to the declining to participate, not drawing blood or not meeting the inclusion criteria. Ultimately, the study population comprised of 104 MDD patients, 101 BD patients and 160 HCs. Although more than 90% patients completed all the clinical and cognitive assessments, only 116 HCs completed all the clinical assessments. The comparison of demographics and clinical characters among these three groups is shown in **Table 1**. Notably, there was a significant difference in age among groups ($F = 6.795$, $p = 0.001$). Significant differences in the YMRS, HAM-D, and HAM-A total scores were found among three groups. More concretely, the HAM-D, HAM-A, and PHQ-9 scores were significantly increased in MDD or BD patients compared to HCs. However, the YMRS scores were higher in BD patients than MDD patients or HCs. Furthermore, these differences still exist after covariance analysis controlling for age (**Table 1**). In addition, according to the assessments of intelligence quotient (IQ), RBANS, and Stroop's tests, the MDD or BD patients showed significant deficits in cognitive function.

Plasma DβH Activity

The average plasma DβH activity in each group was shown in **Figure 1** (the value was 17.31 ± 11.85 in HCs, 15.77 ± 11.19 in MDD patients, 13.49 ± 7.56 in BD patients). Covariance analysis (controlling for age) showed a significant decrease in plasma DβH activity in BD patients compared to HCs ($p = 0.005$). However, there was no significant difference in DβH activity between MDD patients and HCs ($p = 0.634$). It should be noted that no significant difference in plasma DβH activity was found between BD and MDD patients ($p = 0.245$).

In addition, we also analyzed the sex difference in plasma DβH activity among three groups. No significant difference in plasma DβH activity was found between males and females in these three groups (**Figure 1B,** $F = 0.830$, $p = 0.363$). **Figures 1C,D** showed there was no significant association in plasma DβH activities with age in each group (all $p > 0.05$).

In order to exclude confounding factors, the patients were divided into first-episode and multi-episode patients (**Figure 2**). Only 10% patients ($n = 10$) were in first-episode in BD, while this ratio was 38% ($n = 40$) in MDD. Though there was no significant difference in plasma DβH activity between first-episode and multi-episode patients, the multi-episode patients showed a decrease trend compared with first-episode patients in both MDD (14.45 ± 8.96 and 17.87 ± 13.91, respectively) and BD (13.32 ± 7.35 and 15.10 ± 9.66, respectively).

TABLE 1 | Demographics and other characteristics in three groups.

	HC	MDD	BD	F	*p*	$p_{Covariance}$
General characteristics						
n	160	104	101			
Gender (male/female)	80/80	54/50	62/39	3.413[a]	0.182	0.121[c]
Age (years)	30.42 ± 9.21	34.77 ± 11.13	30.48 ± 10.40	6.795	0.001	NA
Education (years)	13.77 ± 3.17	13.06 ± 3.07	13.09 ± 3.13	1.826	0.163	0.200
Duration of Illness (months)	NA	67.75 ± 69.83	86.90 ± 73.72	3.647	0.058	0.002
First onset age	NA	29.59 ± 11.25	23.49 ± 9.25	17.933	<0.001	0.002
Family history (yes)	NA	26	32	1.101[a]	0.294	0.238[c]
Type of first episode (depression/mania)	NA	NA	75/26			
Current state (remission/episodes)	NA	48/56	58/43	2.84[a]	0.096	0.067[c]
Medication (treated/untreated)						
Mood stabilizers	NA	3/101	71/30			
Antipsychotics	NA	20/84	63/38			
Antidepressants	NA	79/25	27/74			
Symptom assessment						
YMRS	1.13 ± 2.13	0.88 ± 1.84	3.27 ± 6.02	11.358	<0.001	<0.001
HAM-D	0.40 ± 1.13	11.34 ± 8.95	8.92 ± 9.06	53.776	<0.001	<0.001
HAM-A	0.42 ± 1.09	10.82 ± 9.46	7.94 ± 8.40	47.699	<0.001	<0.001
PHQ-9	3.36 ± 6.02	10.39 ± 7.55	7.57 ± 7.38	17.634	<0.001	<0.001
Cognitive assessment						
IQ	108.42 ± 17.46	100.69 ± 16.32	98.74 ± 13.11	9.715	<0.001	<0.001
RBANS						
Attention	108.16 ± 14.54	102.05 ± 18.57	101.53 ± 14.11	4.878	0.008	0.001
Speech	98.14 ± 18.65	90.42 ± 14.56	85.37 ± 14.63	14.857	<0.001	<0.001
Visual span	100.65 ± 17.76	96.98 ± 17.09	98.18 ± 15.70	1.127	0.325	0.229
Immediate memory	91.14 ± 18.06	83.42 ± 20.09	80.22 ± 17.09	8.431	<0.001	<0.001
Delayed memory	93.85 ± 13.24	85.59 ± 19.15	83.58 ± 15.16	10.383	<0.001	<0.001
Total scores	97.27 ± 15.54	88.92 ± 17.64	85.82 ± 11.34	14.523	<0.001	<0.001
Stroop						
Single word time	15.49 ± 5.02	17.24 ± 6.12	17.76 ± 5.42	4.139	0.017	0.001
Monochromatic time	20.17 ± 6.28	24.03 ± 9.81	26.74 ± 11.12	11.298	<0.001	<0.001
Double words time	19.20 ± 8.24	21.30 ± 10.09	23.55 ± 10.61	4.558	0.011	<0.001
Double color time	36.64 ± 12.11	41.70 ± 15.13	44.79 ± 16.56	7.043	0.001	<0.001

Values represent mean ± S.D.

[a] means χ^2 test. [c] means multivariate logistic regression analysis.

HC, Healthy controls; MDD, Major depression disorder; BD, Bipolar disorder; YMRS, Young Mania Rating Scale; HAM-D, Hamilton Depression Scale; HAM-A, Hamilton Anxiety Scale; PHQ-9, Patient Health Questionnaire-9; IQ, Intelligence quotient; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status.

Associations of Plasma DβH Activity and Clinical Variables

No significant associations were found between DβH activities and clinical assessments or cognitive function in MDD patients (**Table 2**, all $p > 0.05$). However, significant negative correlations were found between DβH activities and HAM-D scores ($r = -0.234$, $p = 0.021$), or HAM-A scores ($r = -0.201$, $p = 0.041$) in BD patients (**Table 3**, **Figure 3**). Nevertheless, plasma DβH activities showed no significant correlation with cognitive assessments in BD patients (all $p > 0.0167$).

Discussion

In this study, we enrolled 365 subjects, including 104 MDD patients, 101 BD patients, and 160 HCs, to complete the plasma DβH activity assay and clinical assessments. Five findings were obtained in our study: (1) Patients with BD, not MDD, showed a significant decrease in plasma DβH activities compared with HCs; (2) No significant differences in plasma DβH activities were found between the BD and MDD patients; (3) Significant negative correlations were found between DβH activity and mood-related assessments in BD patients; (4) There was no correlation between DβH activity and cognitive function in BD; (5) In contrast to BD patients, no correlations were found between DβH activity and clinical symptoms or cognition in MDD patients.

In the present study, we found that the plasma DβH activity was significantly lower in BD patients; this was in line with the previous studies in BD (37, 41). It is interesting to note that

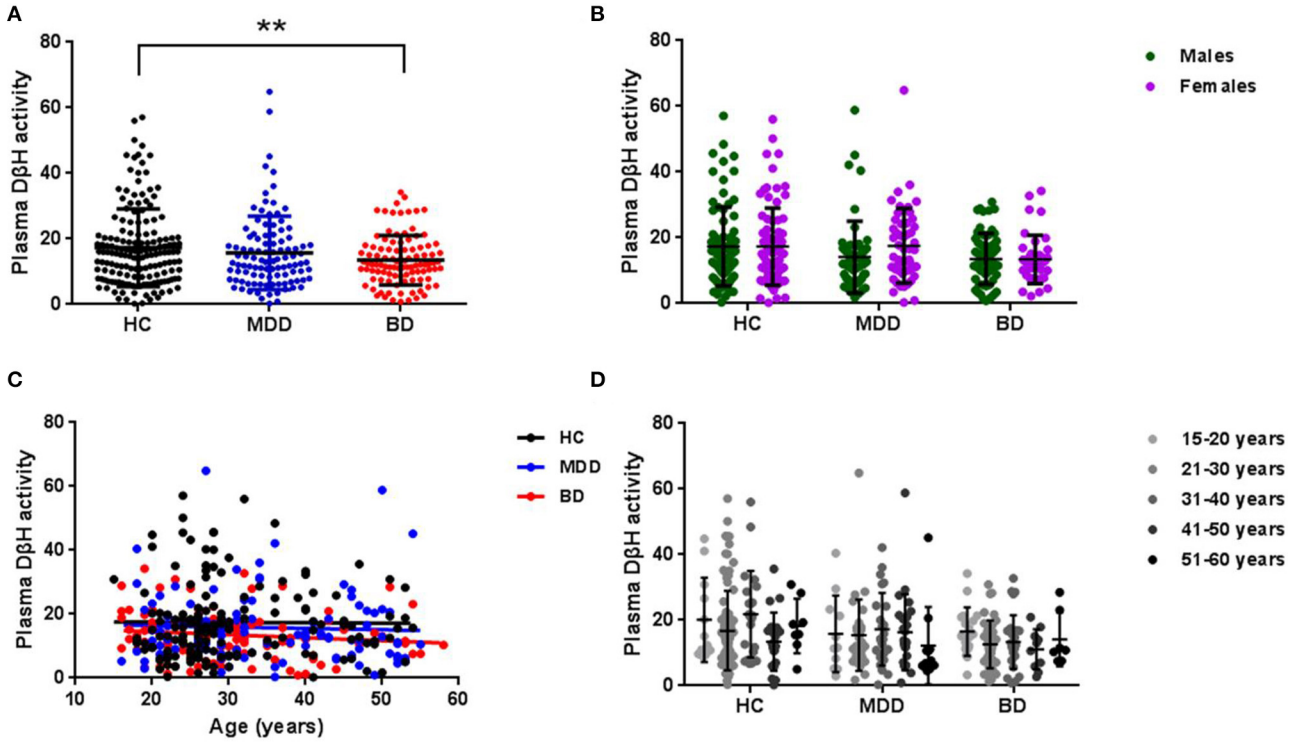

FIGURE 1 | Plasma DβH activity in patients with MDD or BD, and healthy controls (HC). **(A)** Compared the plasma DβH activity in HC ($n = 160$), MDD patients ($n = 104$), and BD patients ($n = 101$). **(B)** Showed the sex difference of DβH activity among HC ($n = 80$ in males; $n = 80$ in females), MDD patients ($n = 54$ in males; $n = 50$ in females), and BD patients ($n = 62$ in males; $n = 39$ in females). **(C,D)** Showed the age-dependent changes of DβH activity in each group. Data was presented as mean ± S.D. $^{**}p < 0.01$.

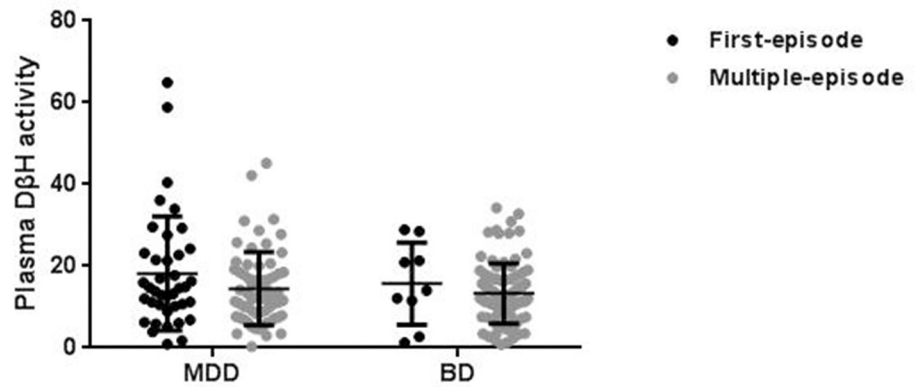

FIGURE 2 | Plasma DβH activity in patients with different episodes, including first-episode (FE) and multi-episode (ME) in MDD and BD. There was no significant difference in DβH activity in patients with different episodes in neither MDD ($n = 40$ in FE; $n = 64$ in ME) nor BD ($n = 10$ in FE; $n = 91$ in ME) groups. Data was presented as mean ± S.D.

we also found no significant difference in plasma DβH activity between BD and MDD patients. This may be due to the similarity of biological mechanisms and phenotypes between BD and MDD (30, 33). In addition, the DβH product, NE also showed similar changes in BD and MDD (20, 51–53). For example, NE was used as a stress factor to induced depression in several studies (54, 55), while stress was also a major risk factor of BD (56). Interestingly, there is report showed CSF NE concentration increased while DA levels decreased in rats after stress (57). The decrease of DβH activity may be one of the reasons for the alteration in NE/DA imbalance. The studies of animal models of depression or mania proved the changes in NE system (1, 3, 58–61). Furthermore, the

TABLE 2 | Associations between plasma DβH activity and clinical or cognitive assessments in patients with MDD.

	N	r	*p*
Symptom assessment			
HAM-D	104	−0.059	0.559
HAM-A	104	−0.165	0.101
PHQ-9	54	−0.114	0.261
Cognitive assessment			
IQ	98	−0.083	0.427
RBANS	97	−0.066	0.531
Stroop			
Single Word Time	93	0.151	0.159
Monochromatic Time	92	−0.085	0.429
Double Words Time	92	0.024	0.822
Double Color Time	91	−0.059	0.588

MDD, Major depression disorder; HAM-D, Hamilton Depression Scale; HAM-A, Hamilton Anxiety Scale; PHQ-9, Patient Health Questionnaire-9; IQ, Intelligence quotient; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status.

TABLE 3 | Associations between plasma DβH activity and clinical or cognitive assessments in patients with BD.

	N	r	*p*
Symptom assessment			
HAM-D	101	−0.234	0.021
HAM-A	101	−0.201	0.041
PHQ-9	69	−0.105	0.304
Cognitive assessment			
IQ	97	0.203	0.051
RBANS	97	0.103	0.326
Stroop			
Single Word Time	94	−0.109	0.308
Monochromatic Time	95	−0.17	0.107
Double Words Time	95	−0.053	0.618
Double Color Time	95	−0.023	0.831

BD, Bipolar disorder; HAM-D, Hamilton Depression Scale; HAM-A, Hamilton Anxiety Scale; PHQ-9, Patient Health Questionnaire-9; IQ, Intelligence quotient; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status.

NE metabolism disruption also might be caused by the reduced DβH in BD. The metabolite of NE, MHPG in the circulating system was found higher in BD patients than controls (4, 35), but contrary results were also reported in several studies (10, 20). Interestingly, neuromodulation treatment on mood disorders was also associated with up-regulation of NE system or DβH expression (45, 62, 63). The inconstant results in plasma DβH activity and NE levels in MDD or BD indicated the complex role of DβH in mood disorders. One of the possible reasons for the contrary results might be the difference in detected tissues, such as the blood or the CSF; another reason might be different episodes with the BD patients, such as the manic or the remission state. In addition, the antidepressants, especially NE reuptake inhibitors improved the depressive symptoms directly through regulation of NE levels (64, 65). Together, the present study showed a decrease of plasma DβH activity in BD patients, which may result in the reduced levels of NE to induced the symptoms of patients. Regrettably, plasma levels of NE were not detected in our study.

The main factor affecting DβH activity is heredity. Numerous studies indicate that *DBH* gene is a major quantitative trait locus that regulates blood and CSF DβH activity (25, 66). Previous studies have reported several single nucleotide polymorphisms (SNPs) which correlate with plasma DβH activity (21, 66–68). For example, a previous study reported −1021C>T (rs1611115) accounted for 35–52% of the variation in DβH activity in African American, European American and Japanese (69); while our previous study showed the ratio was 12.6% in Chinese (25). However, few studies explored the association between *DBH* gene polymorphisms and mood disorders. For example, one study showed that the 1603C>T polymorphism of the *DBH* gene is associated with susceptibility to BD in a Turkish population (34). Zhou et al. reported that *DBH* 5′- Ins/Del polymorphism might be associated with susceptibility to MDD in a Chinese population (70). These results indicated the regulated role of *DBH* gene mutation in DβH activity and its association with mood disorders (71).

Though evidence suggested low NE concentration was associated with MDD and BD, several studies showed that NE levels were different in patients between MDD and BD. It is thought that high levels of NE resulted in mania, while low levels of NE led to depression (72). Wiste et al. compared the tyrosine hydroxylase (TH, the key enzyme of DA synthesis) immunoreactive cells in locus coeruleus (LC) among different subjects (73), and found that the TH immunoreactive cells in LC in BD patients were about half of those in controls or MDD patients, suggesting the lower NE transmission in BD. In addition, neuronal damage in LC also emerged in BD, not MDD (74). However, no significant difference in plasma DβH activity was found in our study. This result should be further confirmed due to several confusion factors in the present study. First, only 10% patients ($n = 10$) were in first-episode in BD, while this ratio was 38% ($n = 40$) in MDD in our study. Compared with first-episode patients, multi-episode patients showed decrease trend in both BD and MDD, suggesting the difference in DβH activity might relate to the duration of disease. Second, several studies indicated the influence of antidepressants and mood stabilizers on DβH activity (41, 75–77). In our study, the patients were treated with different psychotropics, including antidepressants (escitalopram, duloxetine), antipsychotics (olanzapine, aripiprazole and quetiapine), and mood stabilizers (lithium and valproic acid). For example, MDD patients were treated with different drugs (76% antidepressants, 19% antipsychotics and 3% mood stabilizers), meanwhile, BD patients were also treated with different kinds of drugs (70% mood stabilizers, 62% antipsychotics, and 27% antidepressants). However, our study did not show the significant difference in patients treated with different kinds of psychotropics (**Supplementary Figure 1**, $p > 0.05$). Previous studies indicated that the antidepressive

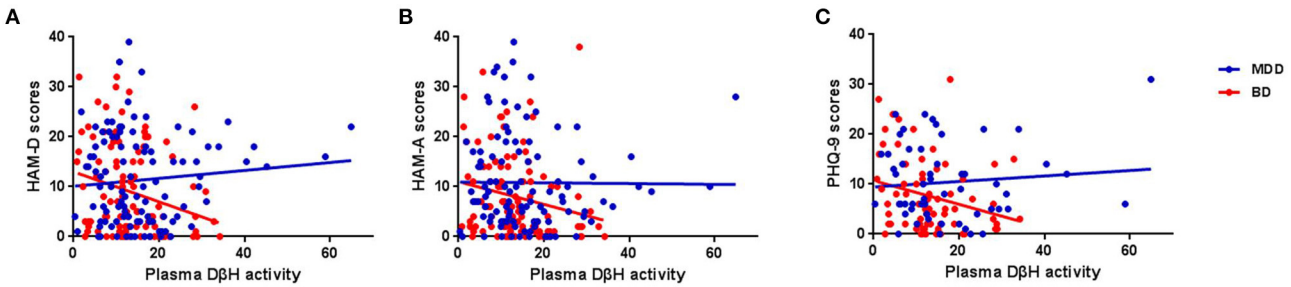

FIGURE 3 | Associations between plasma DβH activity and clinical assessments in MDD and BD patients. **(A–C)** Showed the associations between plasma DβH activity and Hamilton Depression Scale (HAM-D) scores, Hamilton Anxiety Scale (HAM-A) scores, and Patient Health Questionnaire-9 (PHQ-9) scores, respectively. Significant negative correlations were found between DβH activities and HAM-D scores ($r = -0.234$, $p = 0.021$), or HAM-A scores ($r = -0.201$, $p = 0.041$) in BD patients. However, no significant correlation was found between plasma DβH activity and clinical assessments in MDD patients (all $p > 0.05$).

effect of mood stabilizers and antidepressants might be partly mediated by DβH and NE system (75, 78). Moreover, the DβH activity was normalized during antidepressant therapy or mood stabilizers treatment (40, 41, 79). However, our present study showed similar plasma DβH activities in patients with different drug treatment groups, suggesting the similar regulation effect of different kinds of drugs. On the other hand, this inconsistency might also relate to the relative small sample size of patients and different medication duration (2–85 months).

To our knowledge, we firstly reported that plasma DβH activity was associated with anxiety and depressive symptoms in BD. Previous studies showed low DβH activity in CSF and serum in patients with mood disorders (37), and lower plasma DβH activity in untreated patients with BD was found than that in controls and lithium-treated patients (41). However, no reports have shown a clear correlation between plasma/serum DβH activity and severity of mood disorders. We found significant negative correlations between plasma DβH activity and anxious and depressive symptoms in BD. In other words, lower DβH activity is associated with more severe anxious or depressive symptoms in BD. This confirmed the important role of plasma DβH activity and monoamine neurotransmitter system in BD. However, no significant correlations were found between plasma DβH activity and mania severity (YMRS scores) in our study. This might relate to the current state of the BD patients when recruited in the present study. About 57% BD patients recruited in our study were in the remission stage, and the others were in the depressive stage (**Table 1**). It was a great pity that there was no BD patients in the manic state, while the YMRS scores were 3.27 (SD = 6.02). Further study should be conducted in the BD patients with manic state.

However, several limitations should be noted in this study. First, this study focused on the DβH activity in MDD and BD. However, other factors might also affect the dopaminergic transmission, such as polymorphisms of dopaminergic related genes as an endophenotype of MDD. Second, the three study groups, including patients with MDD or BD and HCs, were not fully age-matched. These variables were corrected in the analysis. Third, a majority of patients were receiving antipsychotics and antidepressants, which have confounding effect to explore the role of DβH in mood disorders. Finally, only 10% patients were in first-episode in BD, while the ratio was 38% in MDD.

Taken together, this is a pilot study and shows a reduction of plasma DβH activity as well as hypoactivity of the noradrenergic system in patients with BD. The plasma DβH activity is here proposed as a measure to evaluate the severity of BD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of Beijing Anding Hospital approved the research. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CW, ZS, and QB obtained funding for this study. ZS and QB designed the research. ZS, QB, ZM, FL, WL, FH, and XM performed the experiments and statistical analysis. ZS, QB, YH, and CP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.566091/full#supplementary-material

Supplementary Figure 1 | Differences of plasma DβH activity in patients treated with different kinds of psychotropics. Data was presented as mean ± S.D.

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SYSTEMATIC REVIEW
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Augmenting Clinical Interventions in Psychiatric Disorders: Systematic Review and Update on Nutrition

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There is a strong relationship between a healthy diet and mental well-being. Several foods and food compounds are known to modulate biomarkers and molecular mechanisms involved in the aetiogenesis of several mental disorders, and this can be useful in containing the disease progression, including its prophylaxis. This is an updated systematic review of the literature to justify the inclusion and recognition of nutrition in the management of psychiatric illnesses. Such foods and their compounds include dietary flavanols from fruits and vegetables, notable antioxidant and anti-inflammatory agents, probiotics (fermented foods) known to protect good gut bacteria, foods rich in polyunsaturated fatty acids (e.g., Omega-3), and avoiding diets high in saturated fats and refined sugars among others. While the exact mechanism(s) of mitigation of many nutritional interventions are yet to be fully understood, the evidence-based approach warrants the inclusion and co-recognition of nutrition in the management of psychiatric illnesses. For the greater public health benefit, there is a need for policy advocacy aimed at bridging the knowledge gap and encouraging the integration of nutritional intervention with contemporary therapies in clinical settings, as deficiencies of certain nutrients make therapy difficult even with appropriate medication.

Keywords: psychiatry, mental disorder, microbiome, biomarker, probiotics, nutrition, food

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INTRODUCTION

Mental disorders are widespread and impact significantly on health (1). In 2016, mental and addictive disorders affected more than 1 billion people globally and contributed 7% of the global burden of diseases (2). However, mental disorders manifest differently; according to WHO, they are generally characterized by a combination of abnormal thoughts, perceptions, emotions, behavior, and relationships with others (1). Current treatment involves the use of drugs such as antidepressants, antipsychotics, sedative-hypnotics, anxiolytics, stimulants, and mood stabilizers, along with psychotherapy (talk therapy). Electroconvulsive therapy (ECT) involving the application of electrical currents to the brain is used in some disorders that are unresponsive to other treatments.

"Nutritional psychiatry" pivots on the impact of nutrition (food) on the state of mind and mood. This presents an opportunity to augment clinical interventions as well as to mitigate the adverse effects of medications used in the treatment of psychiatric disorders (3, 4). Accumulating literature suggests a significant relationship between poor diet and the exacerbation of mood disorders, such as anxiety, depression, and other neuropsychiatric conditions (5). The likelihood of a healthy diet to produce beneficial effects on mental health among clinical and non-clinical subjects deserves more attention (6), and dietary interventions need to be refined and scaled up for maximum benefit in the management of mental disorders (7–9).

This review aims at updating the concept of "Nutritional Psychiatry" by (i) highlighting the various biomarkers and molecular mechanisms that form the hallmark of various mental disorders; (ii) examining foods and food compounds that can ameliorate the mechanistic derangement as evidence for the possibility of incorporating dietary interventions in the clinical management of psychiatric disorders.

METHODOLOGY

Multiple online searches were carried out in the databases of Medline, Pubmed, Scopus and Google Scholar in May 2020 using terms like "Nutritional psychiatry," "food and mental health," "diets in psychiatry," "nutrition and mental disorders," "food and food compounds and mental health," "biomarkers of psychiatric disorders," and "mechanisms of mental disorders." Sourced works of literature were screened, and full texts were obtained. Inclusion and exclusion criteria determined the suitability of the literature used in this review. In particular, studies were included if focusing on a whole food, supplements, or compounds (isolated from food) targeting psychiatric disorders. Articles were excluded when (a) not relevant, i.e., the nutritional source was meant to mitigate illnesses different from mental disorders, (b) unavailable in English, and (c) unavailable in full-text.

RESULTS AND DISCUSSION

Search Results

One hundred and eighty-two (182) studies were found in the initial search. After a screening of both titles and abstracts, 59 articles were excluded; in particular, 37 articles were not relevant, 16 full texts were unavailable, 3 were unavailable in English, 3 were duplicates. Further review of the full texts of the remaining 123 articles with strict application of the inclusion and exclusion criteria resulted in the exclusion of 26 articles, thus leaving 97 studies that were included in this review (**Figure 1**).

There are several types of diagnosable mental disorders that are known to cause significant alterations in behavioral, thoughts, emotional and functional disabilities. They include but are not limited to the following: depression (1), bipolar disorder (10), schizophrenia (11), dementia, autism spectrum disorder, generalized anxiety disorder (12, 13), attention-deficit/hyperactivity disorder (14), obsessive-compulsive disorder (15, 16), post-traumatic stress disorder (17) and eating disorders such as anorexia nervosa and bulimia nervosa (18).

MOLECULAR MECHANISMS AND BIOMARKERS OF PSYCHIATRIC DISORDERS

The term biomarker can be referred to as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathologic processes, or biological responses to a therapeutic intervention (19). It can be a gene, a group of genes, proteins, or other biomolecules (20). Due to the complexity of psychiatric disorders, biomarkers cannot be limited to molecular biology in psychiatry. Advances in neuroimaging methods have modernized the understanding of the bio-clinical substrata of many psychiatric disorders (21, 22). Clinical uses of biomarkers in psychiatry involve measuring them before the intervention and with the goal of predicting drug response, diagnosis, therapeutic failure, prognosis, pharmacotoxicity, and classification within diagnostic categories (23–26). They include inflammatory biomarkers such as high levels of cytokines and C-reactive protein (CRP), changes in serum molecules involved in pro-inflammatory and oxidative stress response, including hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis (27). Elevated levels of pro-inflammatory cytokines have been observed in patients with depression (28–31), schizophrenia (32, 33), and eating disorders (34).

Protein biomarkers involve the expression of proteins in the brains such as growth differentiation factor-15, hemopexin, hepsin, matrix metalloproteinase-7, retinol-binding protein-4, and trans-thyretin, which have been reported as biomarkers to distinguish patients with bipolar disorder from those without the disorder (35); up-regulation of microRNA utilized as a biomarker for diagnosis of patients with schizophrenia (36); increased cerebrospinal fluid levels of β-amyloid, tau, and phosphor-tau for Alzheimer's disease (37).

Disturbances in central and peripheral Neurotransmitters biomarkers are also indicators of mental disorders such as major depressive disorders. These neurotransmitters include dopamine, glutamate, γ-aminobutyric acid (GABA), and serotonin (38). Neurotrophic biomarkers such as expression of the brain-derived neurotrophic factor, BDNF in cognitive impairments in individuals with mental disorders is of utmost research interest (39). Electrophysiological biomarkers used in psychiatry include imbalances in resting heart rate (RHR), heart rate variability (HRV), respiration rate (RR), skin temperature (ST), skin conductance (SC) (40, 41), event-related potentials (ERP) and visual evoked potentials (42). ERP measures the electrical activity of the cerebral surface that represents a distinct phase of cortical processing. It is made up of two components, namely P300 positivity and N200 negativity (43). It has been reported that P300 activity may serve as a useful biomarker of attention and as a screen for combination-drug therapy in investigations of anti-Alzheimer drugs (44). In addition, several neuroimaging techniques like Magnetic Resonance Imaging (MRI), Positron Emission Tomography scan (PET scan), Single Positron Emission Tomography scan (SPECT scan), Magnetic [Resonance Spectroscopy (MRS), Functional Magnetic Resonance Imaging (fMRI), and Diffuse Tensor Imaging (DTI)

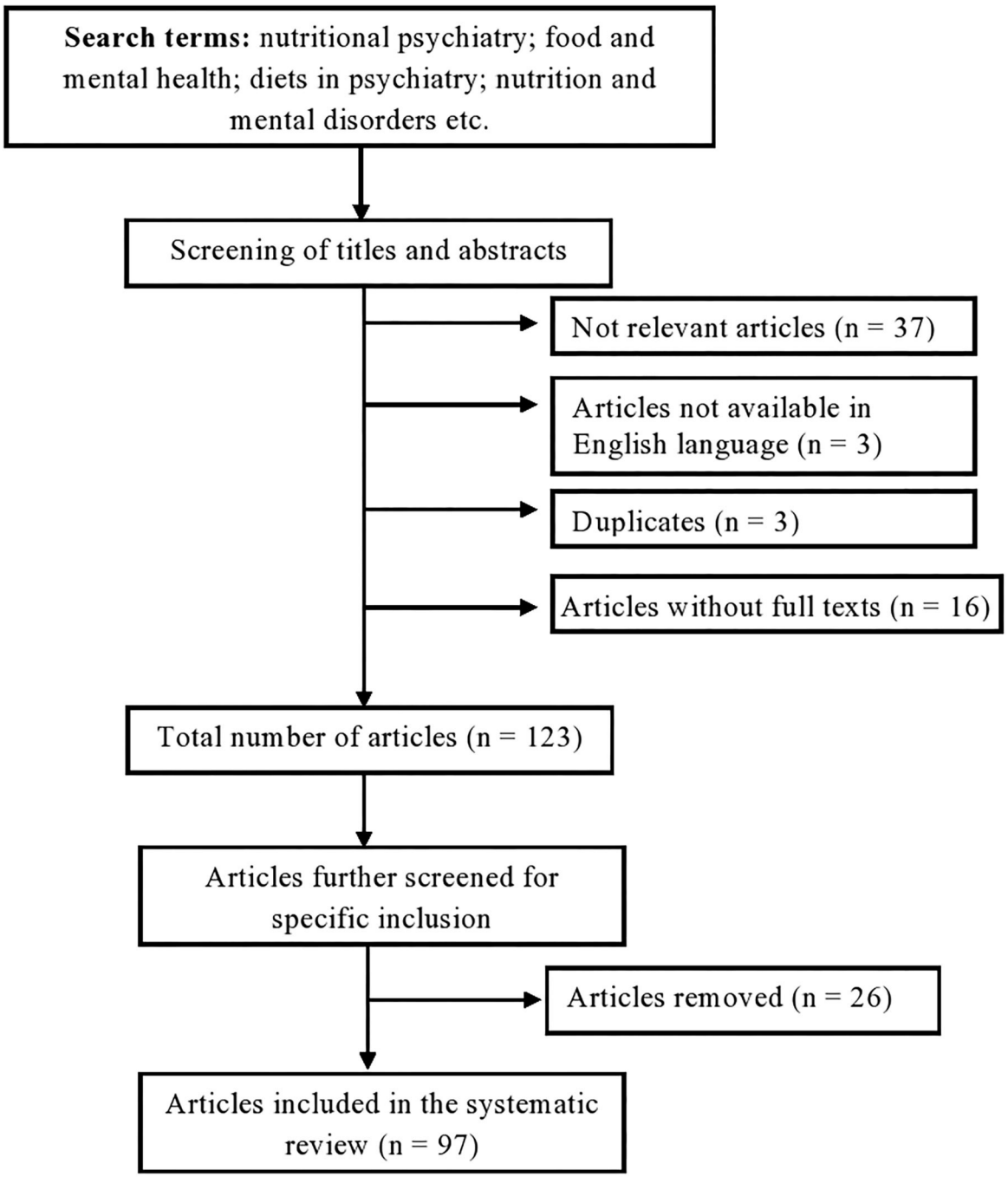

FIGURE 1 | Flow diagram of search selection PRISMA.

are currently employed to find biomarkers for mental illness (45) and to clearly elucidate the neural basis of the psychiatric disorder (40)]. The gut microbiota could control functional pathways in the brain and, therefore, useful as both biomarkers and potential drug targets in mental disorders (46). The gut microbiome has been demonstrated to play an essential role in the development and function of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the stress response and is involved in a range of psychiatric disorders, especially depression and anxiety disorders (47, 48). The use of antibiotics, Western diets, and excessive-stress lifestyles culminate in gut bacterial imbalances, known as dysbiosis, in addition to low diversity. Bacteria have the ability to produce GABA, tryptophan, 5-HT, and several neurotransmitters and monoamines MOA. Pathophysiology of mental disorders

has also been linked to bacterial translocation via increased gut permeability (49). Anxiety, stress, and depression can increase gut barrier permeability, resulting in a 'leaky gut,' which allows bacteria to seep into circulation, leading to the inflammatory response (50–52).

A schematic illustration of some biomarkers in psychiatric disorders is shown in **Figure 2**.

FOODS AND FOOD COMPOUNDS THAT AFFECT PSYCHIATRIC BIOMARKERS

A strong relationship between a healthy diet and mental well-being is often reported by people. Elation, mental health, and well-being have reportedly been increased by the increase in the consumption of fresh fruits and vegetables (53–56). Dietary flavanols, namely kaempferol, isorhamnetin, and myricetin (i.e., components of many fruits, vegetables, and tea), have been linked to a significantly lower risk of development of Alzheimer's disease (57). Many flavonoid classes, including flavonols, are anti-inflammatory and antioxidants.

Some common fruits like citrus (e.g., lime, lemon, orange, tangerine, grape), guava, cashew, mango, pawpaw, pineapple, avocado, banana, African star apple, sweetsop/ sugar apple, breadfruit, soursop, African bush mango, passion fruit, apple, dates (58) and vegetables (e.g., tomatoes, okra, eggplant, cucumber, beets, garlic, onion, and ginger) found in sub-Saharan Africa may be of nutritional psychiatry relevance (58). These fruits and vegetables have high levels of micronutrients such as zinc, magnesium, selenium, iron, and vitamins (59, 60). These micronutrients may modulate the risk of mental disorder, such as depression, via effects on the production and activity of monoamine neurotransmitters like serotonin, alterations to the HPA system, glutamatergic signaling, or inflammatory and oxidative stress (61, 62). These plant-based foods contain antioxidant phytochemicals, such as polyphenols, vitamin C, and flavonoids, i.e., substances whose antidepressant-like or anxiolytic effects have been reported (63, 64).

Several foods/food compounds are known to affect some psychiatric disorders in different ways. They include several phytochemicals like flavonoids, probiotics, omega-3 fatty acids, vitamins, myoinositol, Curcumin, plant parts like valerian root, milk thistle, and green tea (**Table 1**). These nutrients may affect mental disorders via several mechanisms such as the production and activity of monoamine neurotransmitters, neurotransmission, modulation of hippocampal neurogenesis, hypothalamic-pituitary-adrenal (HPA) system, anti-inflammatory and antioxidant effects, augmenting the production of brain-derived neurotrophic factor, BDNF or the protection of gut bacteria, among others. These bio-molecular mechanisms of dietary interventions in some mental disorders are summarized in **Figure 3**.

High doses of naturally occurring cocoa flavanols have been reported to reverse memory decline associated with age (68). Flavanols seem to selectively improve the function of the dentate gyrus, i.e., a region within the hippocampus that is associated with aging and age-related memory decline (68). The hippocampus is a region of the brain associated with memory, learning, and mood. The extent of neurogenesis in the hippocampus is directly related to cognition and mood. Modulation of hippocampal neurogenesis by diet is a possible mechanism by which nutrition affects brain function, plasticity, and mental health (69).

Hesperidin, i.e., a citrus-derived flavonoid, has been reported to have neuroprotective effects, particularly against depression,

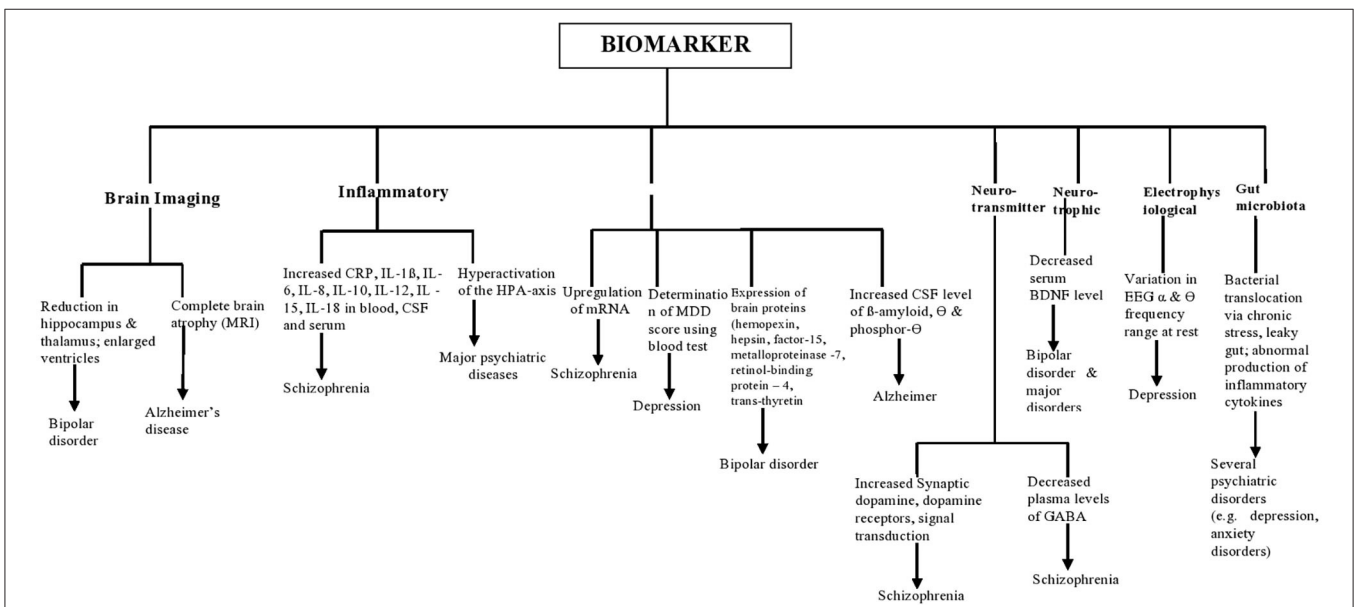

FIGURE 2 | Schematic illustration of biomarkers in some psychiatric disorders.

TABLE 1 | Some] foods/food compounds that affect psychiatric disorders.

Food or food compounds	Comment	References
Dietary flavanols (kampferol, isorhamnetin, myricetin)	They are components of many fruits, vegetables and tea. They lower risk of development of Alzheimer's disease	(57)
Hesperidin (citrus-derived flavonoid)	In depression: (i) Modulation of serotonergic 5-HT1A receptors and kappa opioid receptors in the hippocampus (ii) Anti-inflammatory and antioxidant effects.	(65–67)
Cocoa flavanols	Reverses age-related memory decline through modulation of hippocampal neurogenesis	(68, 69)
Mediterranean diet (whole grains, sea food, poultry, legumes, beans nuts, fresh fruits, leafy vegetables, healthy fats, and less red meat)	Protection against depression linked to enhanced production of brain-derived neurotrophic factor (BDNF)	(3, 70)
Probiotics (e.g., fermented foods such as yogurt active cultures)	(i) They protect gut bacteria; reduce cortisol stress via HPA; regulate hippocampal neurogenesis. (ii) Traditional African fermented foods contain live organisms capable of producing health-promoting compound and can act as probiotic strains.	(3, 40, 71) (72, 73)
Omega-3 fatty acids (from fish, seafood, grass-fed beef)	Effective in the treatment of ADHD, PTSD, major depressive disorder, bipolar depression. They affect neurotransmission, neurogenesis, gene expression and have antioxidants and anti-inflammatory properties.	(74–76)
Myoinositol	• An endogenous isomer of glucose; also present in nuts, grains, beans and fruits. Effective in treatment of OCD • Mechanism of action may involve modulating the reuptake of serotorin and increasing 5-HT2 receptor density.	(77–79)
Silymarin	• A flavonoid from the plant, Milk thistle. It has anti-inflammatory, antioxidant, antidepressant effects. • Increases, serotonin in the cortex and acts as a monoamine oxidase inhibitor	(80, 81)
Milk thistle	It has similar effects with fluoxetine in therapy of OCD but without severe adverse effects.	(82)
Valerian root (Valeriana officialis L)	Contains (i) aleuronic acid that is associated with modulation of GABA receptors (ii) Valepotriates, reported to be effective in treatment of psychotic symptoms of severe anxiety.	(83, 84)
St. John's Wort (*Hypericum perforatum*)	• It contains flavonoid. Its activity involves monoamine reuptake inhibition, neuroendocrine modulation, increased sensitization and binding to receptors (e.g., 5-HT). • It is equivalent to antidepressant in the treatment of depression	(85, 86)
Vitamins	Vitamin D: deficiency may affect OCD etiology by affecting the pathway of serotonin and catecholamine synthesis. It does this through regulation of the enzymes, tyrosine hydroxylase and tryptophan hydroxylase in addition to its neuroprotective effects. Vitamin B12: Deficiency causes depression, mania, psychosis Vitamin B9 (Folic Acid): deficiency result *in utero*- neurodevelopmental defects and is linked with depression in adults. Vitamin B3 (niacin): Deficiency causes pellagra with resultant dementia	(87–92)
Curcumin	A polyphenol obtained from tumeric plant. It reduces symptoms of depression.	(93, 94)
Epigallocatechin gallate (EGCG)	A polyphenol found in Green tea. It alleviates symptoms of stress and depression	(93)

learning, and memory deficits (95–97). Possible mechanisms of its antidepressant-like effects are regulation of serotonergic 5-HT1A receptors (65) and kappa opioid receptors in the hippocampus (66). Hesperidin has both anti-inflammatory and antioxidant effects (67). In a model of aluminum chloride-induced neuroinflammation in the hippocampus, the anti-inflammatory properties of hesperidin involve a reduction in the levels of pro-inflammatory mediators like tumor necrosis factor α (TNF-α) (98). Hesperidin has also been shown to protect the hippocampus by reducing levels of nitrate/nitrite while increasing levels of BDNF in the mouse (99). Its free radical scavenging and antioxidant abilities tend to ameliorate the shortfalls in the activity of glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase. In experimental models of stroke, irradiation, and LPS-induced endotoxicity, these antioxidant enzymes are down-regulated in the brain (100–102). The Mediterranean diet involves eating whole grains, seafood, and poultry (at least twice a week) and consuming legumes, beans, fresh fruit, leafy green vegetables, nuts (almonds, walnuts), cruciferous vegetables (cauliflower, broccoli), healthy fats (olive and canola oil), and a limited amount of red meat (3). According to a recent study, a Mediterranean diet and avoiding inflammation-producing foods may protect against depression (103). The mechanisms involved may be linked to enhanced production of BDNF, and therefore important functions such as neuroplasticity, neuronal survival, as well as growth and differentiation of new neurons and synapses (70). Low serum BDNF levels have been found in a number of psychiatric disorders like schizophrenia, major depressive disorder, PTSD, and Alzheimer's dementia (104). Diet can regulate or dysregulate

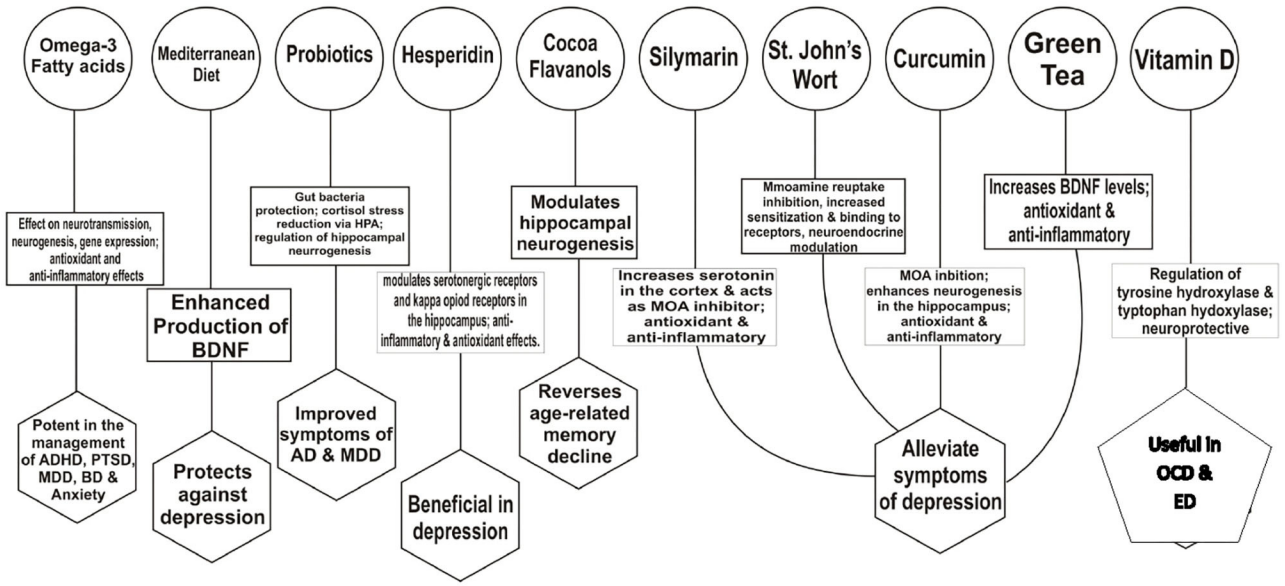

FIGURE 3 | Bio-molecular mechanisms of dietary interventions in some mental disorders. HPA, hypothalamic-pituitary-adrenal axis; OCD, Obsessive-Compulsive Disorder; MOA, Monoamine oxidase; BD, Bipolar disorder; BDNF, brain-derived neurotrophic factor; MDD, Major Depressive Disorder; ADHD, Attention deficit hyperactivity disorder; PTSD, Post traumatic stress disorder; AD, Alzheimer dementia; ED, Eating disorders.

the gut microbiome. Healthy gut microbiota is central in the regulation of serotonin metabolism because at least 90% of serotonin receptors are located in the gut (105). Alteration in the balance between "good" and "bad" bacteria may result in several diseases, including mood and cognitive disorders. Probiotic-rich foods (e.g., fermented foods such as yogurt with active cultures) are known to protect good gut bacteria (3). Fermented foods contain strains of Lactobacillus as well as yeasts and are vital because they contain both probiotic microbiota and microbial metabolites (59). Prebiotics, in their turns, include non-digestible fiber, which stimulates the growth of probiotics (70). Many prebiotics and probiotics reduce cortisol stress in healthy subjects (71, 106, 107). Several studies suggest that both individuals with clinically diagnosed cases and healthy individuals experiencing some anxiety and mood disorders benefit from the consumption of probiotics (108, 109). Several fermented foods are traditionally used in different parts of Africa' (110). These include fermented non-alcoholic cereals (mainly from sorghum, millet, and maize), starchy root crops (mainly from cassava), animal proteins (mainly dairy products), vegetable proteins (from legumes and oilseeds), and alcoholic beverages (e.g., from cereals, sap, honey, fruits) (111). These traditional African probiotics contain live microorganisms capable of producing health-promoting compounds like antimicrobials and essential nutrients or molecules with antioxidant activity (72, 73). The western diet, known for its content of ultra-processed foods, has been reported to change microbiome (gut environment), leading to reduced Lactobacilli (112), gut inflammation, and possibly contribute to disorders (113). Mediterranean diet reduces the numbers of inflammatory/pathogenic bacteria like Escherichia coli and increases important commensal bacteria such as Bifidobacteria (114), Clostridium cluster XVIa, and Faecalibacterium prausnitzii (115). Vegetarian diets have been reported to alter the microbial composition and reduce inflammation of the gut (116, 117). Pathogenesis of psychosis has been linked to anomalies in glucose tolerance, insulin resistance, mitochondrial dysfunction, and energy metabolism disturbances. These could be potential mechanisms for the effect of a ketogenic diet. This diet, high in fat, and low in carbohydrate, utilize ketone bodies as the fuel source for the brain, instead of glucose (118, 119). A report from investigators with the Nutrition Network of the European College of Neuropsychopharmacology (ECNP) postulates that a ketogenic diet may decrease seizures in children with epilepsy (5, 120).

Some diets have potentially harmful effects on the brain. A diet high in saturated fats and refined sugars has a powerful negative impact on brain proteins (neutrophins). Neutrophins are very important in depression: they protect the brain against oxidative stress and promote the growth of new brain cells (121). Del-Ponte and co-workers reported recently that food high in refined sugar and saturated fat might cause an increased risk for hyperactivity (ADHD) compared to fruits and vegetables (122). Eliminating the underlying suspected trigger foods may work as secondary prevention of food-induced ADHD: the "few-foods approach" is a diagnostic protocol allowing to determine whether or not individually composed few food diets (one food per week is added to the diet) are a trigger of ADHD. If the behavioral problems do not recur, the food can be included in the diet without restriction (123, 124).

Foods that contain aspartame, a food additive, are forbidden for people with phenylketonuria (a birth defect that causes the amino acid phenylalanine to build up in the body) as this

can result in brain damage, intellectual disabilities, behavioral symptoms, or seizures (125).

Several studies have corroborated the fact that deficiency of some vitamins and other essential nutrients lead to cognitive impairments (126, 127). Vitamin D plays an important role in immunity modulation, inflammatory response, and antioxidant processes, as well as in normal brain development and functioning, neurotransmission, neuroprotection, proliferation, and differentiation (80, 128, 129). Vitamin D deficiency can be associated with numerous neuropsychiatric diseases, including autism, major depressive disorder, schizophrenia, and Obsessive-Compulsive Disorder (OCD) (130, 131). Vitamin D deficiency may contribute to OCD etiology by (i) affecting the pathway of serotonin and catecholamines synthesis, (ii) regulation of the levels of the enzymes tyrosine hydroxylase and tryptophan hydroxylase, (iii) deprived neuroprotective effect (87, 88). In adults aged 65 years and above, higher vitamin D serum levels were associated with better attention and working memory performance (132). Vitamin D has also been reported to support the nervous system and brain functions such as impulsive behaviors, known to be of importance in the prognosis and treatment of patients with Eating Disorders (133). Vitamin B12 deficiency causes depression, lethargy, poor memory, fatigue, mania, and psychosis (89), while vitamin B3 (niacin) deficiency causes pellagra with resultant dementia (90). Deficiency of vitamin B1 (thiamine) causes beriberi and numbness as CNS symptoms, while vitamin B9 (folic acid) deficiency results in *in utero* neurodevelopmental defects and is linked with depression in adults (91, 92).

Foods rich in polyunsaturated fatty acids, PUFAs (e.g., Omega-3s), and polyphenols have also been reported to have beneficial effects in neuroinflammation, cognitive performance, mood, and stress reactivity (134–137). Omega-3 fatty acids are effective in the treatment of attention-deficit/ADHD, major depressive disorder, bipolar depression, and post-traumatic stress disorder, or PTSD (74, 75). Omega-3 fatty acids are found in fish, seafood, and grass-fed beef (70). Omega-3 fatty acids are an integral part of neuronal cell membranes and affect several physiological mechanisms in the central nervous system. They affect neurotransmission, gene expression, neurogenesis, neuronal survival and also have antioxidants and anti-inflammatory properties (76). A balance between omega-6 and omega-3 fatty acids seems to be relevant in some mental disorders, as high omega-6 to an omega-3 fatty acid ratio in the blood has been associated with major depressive disorder and ADHD (56, 70).

Myoinositol (MI), an endogenous isomer of glucose also present in nuts, grains, beans, and fruits, is used in the treatment of mental disorders. It is essential for the synthesis of membrane phospholipids and for the intracellular secondary messenger cycle (77). Although some studies found no evidence for the efficacy of myoinositol in OCD treatment, others have reported the effectiveness of myoinositol supplementation in the treatment of OCD (78, 138). Available clinical evidence suggests that MI may potentially be effective as monotherapy in OCD (80). The suggested mechanisms of action involve modulation of the reuptake of serotonin and an increase in 5-HT2 receptor density (79). Silymarin, a flavonoid derived from the plant Milk thistle (Silybummarianum), has been reported to have anti-inflammatory, antioxidant, immune modulator, sedative, and antidepressant effects (80). It increases serotonin in the cortex and acts as a monoamine oxidase inhibitor (81). The effect of milk thistle and fluoxetine are alike in the treatment of OCD, and their positive effect starts in the 5th week without severe adverse effects (82). Valerian root (obtained from the plant Valeriana officinalis L contains aleuronic acid associated with the modulation of GABA receptors) (83) and valepotriates (effective in the treatment of the psychotic symptoms of severe anxiety) (84). St John's Wort (*Hypericum perforatum*), which is of plant origin, has been reported to be equivalent to an antidepressant in the treatment of depression (85, 86). It contains flavonoids, and its neurobiological activity involves monoamine reuptake inhibition, neuroendocrine modulation, increased sensitization, and binding to receptors (e.g., 5-HT) (85). Although some herbal medicines may provide a synergistic effect with conventional drugs, there should be some precautions in the use of some herbal supplements and some pharmaceuticals, for example, St John's Wort with SSRIs due to potential adverse serotonin syndrome (80). Polyphenols are natural compounds present in plant-based foods. They have unique properties and are capable of combatting oxidative stress as well as stimulate the activation of molecules that aid in synaptic plasticity, thereby enhancing cognitive function (93). Notable examples of polyphenols include Epigallocatechin gallate (EGCG) from green tea and Curcumin from turmeric. Apart from their antioxidant and anti-inflammatory properties, their mechanisms of action involve increased expression of BDNF, which enhances the reversal of neuronal atrophy and behavioral deficits (139). Curcumin has been reported to mitigate symptoms of depression by enhancing neurogenesis in the hippocampus and frontal cortex (94). It also inhibits the action of monoamine oxidase enzymes, thus preventing the breakdown of monoaminergic neurotransmitters, thereby increasing serotonin and dopamine levels (140). Epigallocatechin gallate from green tea has been reported to alleviate symptoms of stress and depression (93).

CONCLUSION

The field of nutritional psychiatry though still new, is currently undergoing intensive research, resulting in several positive research findings. As with many other diseases, several foods and food compounds are known to modulate biomarkers and molecular mechanisms involved in the aetiogenesis of several mental disorders, and this can be useful in containing the disease progression, including its prophylaxis. While the exact mechanism(s) of mitigation of many nutritional interventions are yet to be fully understood, the evidence-based approach warrants the inclusion and co-recognition of nutrition in the management of psychiatric illnesses. For the greater public health benefit, there is a need to advocate for policies aimed at bridging the knowledge gap and encourage the utilization and integration of nutrition in addition to contemporary therapies in clinical settings, as deficiencies of certain nutrients make therapy difficult

even with the right medication. This is especially advantageous in developing, resource-challenged nations laden with inadequate healthcare funding for mental disorders, despite the condition being rife in the region and given the fact that these food substances are affordable and readily available in these nations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SO conducted the search, data extraction, and drafting of manuscript. CO and OO conceptualization, reviewed the draft manuscript, and certified final manuscript. CF reviewed the draft manuscript. All authors contributed to the article and approved the submitted version.

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Development and Internal Validation of a Novel Model to Identify Inflammatory Biomarkers of a Response to Escitalopram in Patients With Major Depressive Disorder

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Objective: The aim of our study was to identify immune- and inflammation-related factors with clinical utility to predict the clinical efficacy of treatment for depression.

Study Design: This was a follow-up study. Participants who met the entry criteria were administered with escitalopram (5–10 mg/day) as an initial treatment. Self-evaluation and observer valuations were arranged at the end of weeks 0, 4, 8, and 12, with blood samples collected at baseline and during weeks 2 and 12. Multivariable logistic regression analysis was then carried out by incorporating three cytokines selected by the Least Absolute Shrinkage and Selection Operator (LASSO) regression model. Internal validation was estimated using the bootstrap method with 1,000 repetitions.

Results: A total of 85 patients with Major Depressive Disorder (MDD), including 62 responders and 23 non-responders, were analyzed. Monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and lipocalin-2 were selected by the LASSO regression model. The area under the curve (AUC) from the logistic model was 0.811 and was confirmed as 0.7887 following bootstrapping validation.

Conclusions: We established and validated a good prediction model to facilitate the individualized prediction of escitalopram treatment for MDD and created a personalized approach to treatment for patients with depression.

Keywords: inflammatory biomarkers, major depressive disorder, escitalopram, predictive model, followed up study

INTRODUCTION

Major depressive disorder (MDD) is a mental disorder, diagnosed based on self-reported symptoms and observable signs, which causes significant distress and/or functional impairment (1, 2). Effective treatments for MDD are much needed, since MDD is associated with a high cost for care as well as high morbidity and mortality (3–5). Approximately one third of all patients with depression fail to respond to conventional anti-depressant therapies (6), contributing to the global burden of the disease. Due to the current, only partially effective trial-and-error approaches are adopted for treatment selection in MDD. Predictive biomarkers that guide selection of treatment could be particularly valuable. Biomarkers could have multiple uses in psychiatry, including disease

diagnosis and prediction of a therapeutic response (7). The incorporation of biomarkers into treatment of MDD could help improve efficacy of treatment and accelerate remission.

Much evidence exists regarding interactions between the brain and the immune system. Dysregulation of the immune system or inappropriate immune responses have been reported in various psychiatric disorders, particularly MDD. An aberrant inflammatory profile has been widely described for MDD and is believed to participate in the biological mechanisms involved in disease onset and response to treatment (8).

Accumulating data suggest that inflammation plays an important role in the pathophysiology of depression, and monitoring the therapeutic efficacy of drugs used to treat depression using immune parameters may identify unique patient populations (9, 10). The link between increased inflammation and depression was first reported in the early 1990s (11, 12) and led to the formulation of the macrophage hypothesis of depression (also known as the cytokine hypothesis of depression) (13, 14). Meta-analyses of the literature concluded that peripheral blood IL-1β, IL-6, TNF, and C-reactive protein (CRP) are the most reliable inflammatory biomarkers in patients with depression (10). Other factors that are associated with inflammation, such as adipokines and vascular endothelial factors, have also been shown to be involved in the pathophysiology of depression (15–17).

Regarding the potential clinical applications of the association between inflammation and depression, data indicated that inflammatory biomarkers can identify depressed patients who are less likely to respond to conventional anti-depressant treatment. Several studies have shown that anti-depressant treatments, mainly selective serotonin reuptake inhibitors (SSRIs), were associated with decreased levels of inflammatory markers (10). Different types of anti-depressant therapy may have diverse outcomes regarding changes in inflammatory cytokines (18–23). Therefore, specific associations between anti-depressant treatment and altered cytokine levels remain to be fully defined.

Immunity involves a complex interplay of multiple factors, so focusing on single inflammatory markers is likely to be inadequate. Few, if any, studies have assessed all immune- and inflammation-related factors, and whether there are specific aspects of the inflammatory response that are relevant to depression is unknown. Thus, the approach taken here, combining diverse measures of inflammation, may prove highly relevant from a clinical point of view. The least absolute shrinkage and selection operator (LASSO) method, which is a popular method for regression with high dimensional variables (i.e., genomics and proteomics) (24), was used to select the most useful predictive features from the primary data set. This is a form of regression analysis that includes a penalty for the magnitude of the regression coefficients to prevent overfitting (25). Consequently, this method is always selected to account for a large number of potentially correlated predictors (26).

The aim of our study was to identify immune- and inflammation-related factors with clinical utility for prediction of clinical efficacy in treatment of depression. We measured levels of a variety of inflammation-related markers, including cytokines, chemokines, lipocalin, vascular endothelial factor, and acute-phase reactants in plasma from clinical participants. We included as many factors as possible to identify the optimal panel of baseline inflammation-related factors that predict the anti-depressant efficacy of escitalopram. We sought immune- and inflammation-related biomarkers in depression in relation to treatment response, with the hypothesis that the anti-depressant effect of escitalopram could be predicted by baseline inflammation-related factors.

MATERIALS AND METHODS

Patients and Study Setting

Patients with MDD were recruited from the outpatient department at of Beijing Anding Hospital, Capital Medical University. A total of 85 participants were analyzed in this study. The inclusion criteria for the study were as follows: (1) age between 18 and 65 years; (2) diagnosis of MDD by a psychiatrist using the Structured Clinical Interview for DSM-IV criteria;(3) a severity rating on the 17-item Hamilton Depression Rating Scale (HAMD-17) of $\geq$14 and a total score on the 16-item Quick Inventory of Depressive Symptoms–Self-Report (QIDS-SR16) that was $\geq$11.

The exclusion criteria were: (1) history of any clinically significant disease or laboratory abnormalities that were not stabilized or were anticipated to require treatment during the study; (2) a positive pregnancy test or breast feeding; (3) significant risk of suicide, as evidenced by scoring 3 or 4 for HAMD-17 item 3 and risk of self-harm behaviors established by the investigator; (4) alcohol or substance abuse.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Human Research Ethics Committees. All participants were free to withdraw at any time during the study. All participants signed a informed consent. Only after obtaining written informed consent from participants were study-related procedures or assessments completed.

Study Design

Participants meeting entry criteria were administered escitalopram (5–10 mg/day) as the initial treatment (patients could reduce the dose if side effects could not be tolerated). The maximum dose of escitalopram used in the acute phase was 20 mg/day. Patients were treated by their psychiatrists at each outpatient visit and completed self-evaluation (QIDS-SR 16, FIBSER) and received observer valuations (HAMD-17) from clinicians at the end of weeks 0, 4, 8, and 12. Blood samples were collected at baseline and weeks 2 and 12 in the acute phase.

During the 14 days prior to enrolment, 10 patients with MDD were treated with escitalopram for no more than 7 days; the remaining 75 patients were not treated.

Drug/Therapy Combination

Antipsychotics, other anti-depressants, and mood stabilizers were prohibited during the study. Use of non-benzodiazepines such as zolpidem (≤10 mg/day), zopiclone (≤7.5 mg/day), and zaleplon (≤10 mg/day) was permitted for patients with severe insomnia. Benzodiazepines such as lorazepam were permitted in patients with significant symptoms of anxiety, except for the 8 h prior to assessment. Electroconvulsive therapy, transcranial magnetic stimulation, phototherapy, electro-acupuncture, biofeedback, and vagal nerve stimulation were also prohibited. Any systematic psychotherapies (psychoanalysis, cognitive comprehension, desensitization therapy, hypnosis therapy, Morita therapy) were prohibited, but general supportive psychotherapy was allowed.

Psychometric Assessment and Plasma Inflammatory Marker Measurements

Psychometric Assessment

Before each infusion, depression severity was rated using the Chinese version of the HAMD-17. According to HAMD-17 scores, responders were defined as having a 50% or greater reduction in the HAMD-17 total scores from baseline to week 12. The development of hypomanic symptoms was assessed using the Young Mania Rating Scale (YMRS). The HAMD-17 and YMRS scales were determined at baseline and weeks 4, 8, and 12. Inter-rater reliability (kappa values for categorical measures) was >0.8 for all measurements.

Plasma Inflammatory Marker Measurements

Peripheral blood samples were obtained by venipuncture from patients at baseline. Samples were collected into EDTA tubes and centrifuged at 2,500 rpm for 10 min at room temperature. The plasma was immediately removed, aliquoted, and stored at −80°C prior to cytokine measurements. The levels of 33 cytokines were assessed using four types of MILLIPLEX TM MAP Plex Kits (catalog number: HCYTOMAG-60K, HCVD2MAG-67K, HNDG2MAG-36K, CVD6MAG-67K; MERCK Millipore Corporation, Billerica, MA, USA) on the Luminex 200 platform (Luminex, Austin, USA) according to the manufacturer's instructions. All samples were run simultaneously for each panel and all assays were performed in duplicate. Duplicate samples from each patient were measured within one assay. All assays were carried out using a single lot number of reagents and consumables by a single operator, who was blinded to the sample sources. Data were collected using the Luminex PONENT v3.1 software and concentrations of the markers were determined using Milliplex Analyst v5.1 software.

Statistical Analysis

Cytokines with ≥30% of missing data (values outside the ranges of detection) were excluded (27, 28). For the remaining cytokines, values below the lower detection limit (LDL) were assigned a value of half the LDL, while those above the upper detection limit (UDL) were assigned a UDL value (29–31).

Eight patients were lost to follow-up at the 12-week visit and their responses were imputed by the HAMD-17 at the 8-week visit. Continuously coded variables were reported as the mean(sd) and analyzed by *t*-test or Wilcoxon rank sum test. Categorical variables were reported as frequencies and proportions and analyzed by chi-square test. In all statistical analyses, missing data comprised <1% and were handled with the multiple imputation procedure using the R package "MICE" under the assumption that data were missing at random. Outcome information was included in the imputation model to avoid attenuation of estimated effects in later analyses (32). A formal statistical test on these variables would have to consider the scale of the experiment with type I error due to multiple comparisons. Although *P*-values are reported for these data, all information from these variables is descriptive in nature.

To build a predictive model for response using demographic and cytokine data, we used the R package "glmnet" (33) to perform the LASSO logistic regression algorithm (24, 34). This allowed us to select variables that were most predictive of a response, among all of the 26 candidate features in the data set (22 detectable cytokines, age, gender, BMI, and baseline HAMD-17 score).

A multivariable logistic regression analysis was then refitted by incorporating three cytokines (monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and lipocalin-2) selected by the LASSO regression model. We assessed associations between the predictors and the outcome from resulting models using odds ratios (OR) with 95% confidence interval (CI) and *P*-value. Discrimination of the predicting model was assessed using the area under the curve (AUC) of the receiver operating characteristic and Harrell's concordance index (C-index). Calibration of the predicting model was assessed with a calibration curve and the goodness-of-fit of the model was assessed using the Hosmer-Lemeshow test (35) - $P > 0.05$ supported the goodness-of fit.

We estimated the optimism for all measures by internal validation using the bootstrap method (with 1,000 repetitions) with the relatively corrected C-index (36). Decision curve analysis (DCA) was then applied to determine the clinical relevance of the predictive model by calculating the net benefits at different threshold probabilities in the cohort (37).

All analyses were performed using R 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) and SAS (version 9.4; SAS Institute, Cary, NC).

RESULTS

Patient Characteristics and Cytokine Levels

A total of 85 patients with MDD were recruited to the study (32 males, 53 females; mean age 28.95 ± 7.56 years [range 18.7–56.0]). All patients were divided into response and non-response groups (62 responders, 23 non-responders). The final dose of medicine during the follow up was higher in the non-response group (17.83 ± 3.64 mg/day) than those in the response group (15.48 ± 3.92 mg/day). All demographic and disease data

TABLE 1 | Demographic and clinical characteristics.

Variables	MDD patients			T/χ2	*p*-value
	Non-response *n* (%)	Response *n* (%)	All *n* (%)		
Participants	23	62	85		
Gender[a]				0.11	0.740
Female	15(65.22)	38(61.29)	53(62.35)		
Male	8(34.78)	24(38.71)	32(37.65)		
Education[a]				3.32	0.190
Lower than Undergraduate	9(39.13)	13(20.97)	22 (25.88)		
Graduate	6(26.09)	16(25.81)	22 (25.88)		
Undergraduate	8(34.78)	33(53.23)	41 (48.24)		
Family history[a]				0.66	0.417
NO	19(82.61)	46(74.19)	65(76.47)		
YES	4(17.39)	16(25.81)	20(23.53)		
First episode[a]				0.00	0.963
NO	11(47.83)	30(48.39)	41(48.24)		
YES	12(52.17)	32(51.61)	44(51.76)		
	Mean (SD)	**Mean (SD)**	**Mean (SD)**		
Age (years)[c]	27.65(7.42)	29.43(7.62)	28.95 (7.56)	−1.41	0.159
Body mass index[b]	22.17(2.92)	22.87(3.63)	22.68(3.45)	−0.84	0.405
Onset age (years)[b]	23.57(6.89)	25.65(7.25)	25.08 (7.18)	−1.19	0.237
Duration of illness (years)[c]	3.04(4.12)	2.85(4.04)	2.91 (4.04)	0.23	0.817
Duration of current episode (years)[c]	0.35(0.78)	0.42(1.30)	0.40 (1.18)	0.21	0.832
Clinical assessments					
Baseline HAMD-17 scores[b]	19.87(4.25)	20.87(4.27)	20.60 (4.26)	−0.96	0.339
Endpoint HAMD-17 scores[c]	15.35(5.02)	4.46(3.28)	7.63 (6.28)	6.74	<0.0001
Baseline QIDS-SR scores	16.04(3.71)	14.90(3.23)	15.21 (3.38)	1.34	0.181
Endpoint QIDS-SR scores[b]	11.87(4.53)	4.88(2.78)	6.91 (4.63)	6.90	<0.0001

[a]*Chi-square;* [b]*Independent sample t-test;* [c]*Wilcoxon rank sum test.*
Body mass index is calculated as weight in kilograms divided by height in meters squared.

in the two groups are summarized in **Table 1**. No differences in age, sex, BMI, onset age, illness duration, or baseline HAMD-17 scores were significant between responders and non-responders. **Table 2** summarizes the levels of 22 detectable cytokines from the two groups in treatment cohort patients undergoing MDD.

Feature Selection

From the demographic (age, gender, and BMI), disease (baseline HAMD-17 score), and cytokine data, 26 features were reduced to three potential predictors on the basis of the 85 patients in the cohort (∼7:1 ratio; **Figures 1A,B**). MCP-1, VCAM-1, andlipocalin-2, had non-zero coefficients in the LASSO regression model (**Table 2**).

Model Development

The results of the multivariate logistic regression analysis for MCP-1, VCAM-1, and lipocalin-2 are presented in **Table 3**. A model incorporating the above independent predictors was developed and presented as a ROC curve (**Figure 2**) and nomogram (**Figure 3**). Analysis revealed that MCP-1 (OR, 1.0129; 95% CI, 1.0027–1.025), VCAM-1 (OR, 1.0082; 95% CI, 1.0031–1.014), and lipocalin-2 (OR, 0.9837; 95% CI, 0.9612–0.9972) were independently associated with treatment response. The AUC from the model was 0.811, the cut-off value for prediction score at the optimum point was 0.688, the sensitivity was 82.6% and specificity was 80.6%. The nomogram displays the multi-variant analysis effect of predictors on the risk of response at endpoint.

Model Performance and Clinical Utility

The shape of the curve on the calibration plots indicates that the model is well-calibrated (**Figure 4A**). A Hosmer and Lemeshow statistical test on the observed data for the model supported the goodness-of-fit of the model ($\chi 2 = 13.377$, $p = 0.063$). The C-index for the prediction nomogram was 0.811 (95% CI: 0.702–0.920) for the cohort and was confirmed as 0.7887 through bootstrapping validation, which suggested that the model had good discriminatory ability. In the response predicting model, apparent performance showed a good prediction capability. **Figure 4B** illustrates the decision curve analysis for the response predicting model. The decision curve showed that the model is useful between a threshold probability of 1 and 91%, and using

TABLE 2 | Inflammatory cytokine levels in treatment response and non-response groups.

Cytokines	Total participants	Non-responders (n = 23) Mean (SD)	Responders (n = 62) Mean (SD)	p-value
Log of CRP	3.75(0.55)	3.68(0.52)	3.78(0.56)	0.458[a]
Sqrt of G-CSF	9.16(2.60)	8.76(2.97)	9.31(2.47)	0.390[a]
Log of Eotaxin	1.83(0.17)	1.83(0.15)	1.83(0.18)	0.993[a]
FGF2(pg/ml)	59.22(33.28)	64.34(32.31)	57.32(33.69)	0.391[a]
Log of GM-CSF	0.62(0.31)	0.62(0.31)	0.61(0.31)	0.931[a]
Log of IFNγ	0.72(0.35)	0.65(0.36)	0.75(0.35)	0.268[a]
Log of IL-1Ra	1.19(0.78)	1.39(0.76)	1.11(0.78)	0.138[a]
Log of IL-12	0.47(0.30)	0.41(0.30)	0.49(0.30)	0.280[b]
Log of IL-17	0.46(0.32)	0.40(0.33)	0.48(0.32)	0.329[a]
Log of IL-7	0.61(0.38)	0.60(0.41)	0.61(0.37)	0.980[a]
Log of IP-10	2.43(0.18)	2.38(0.17)	2.45(0.18)	0.126[b]
Lipocalin-2(ng/ml)	80.06(63.24)	105.10(116.96)	70.78(15.79)	0.530[b]
MCP-1(pg/ml)	205.99(72.00)	182.88(51.23)	214.56(76.92)	0.060[b]
Log of MCP-1β	1.25(0.41)	1.24(0.49)	1.25(0.38)	0.660[b]
Log of PDGF	3.56(0.59)	3.64(0.54)	3.53(0.61)	0.451[a]
RANTES(pg/ml)	2142.78(1097.67)	1952.99(1216.42)	2213.18(1052.10)	0.335[a]
Log of SAP	4.95(0.20)	4.90(0.17)	4.97(0.21)	0.147[a]
sCD14(ng/ml)	2396.68(585.69)	2315.97(642.28)	2426.62(565.88)	0.442[a]
sICAM-1(ng/ml)	165.57(388.68)	124.53(129.86)	180.79(448.42)	0.752[b]
TNFα(pg/ml)	9.88(6.19)	8.51(3.48)	10.39(6.89)	0.101[a]
VCAM-1(ng/ml)	661.08(140.80)	600.13(114.41)	683.70(143.73)	0.013[b]
VEGF(pg/ml)	47.86(44.73)	50.98(41.22)	46.71(46.23)	0.533[b]

[a]*Independent sample t-test;* [b]*Wilcoxon rank sum test.*

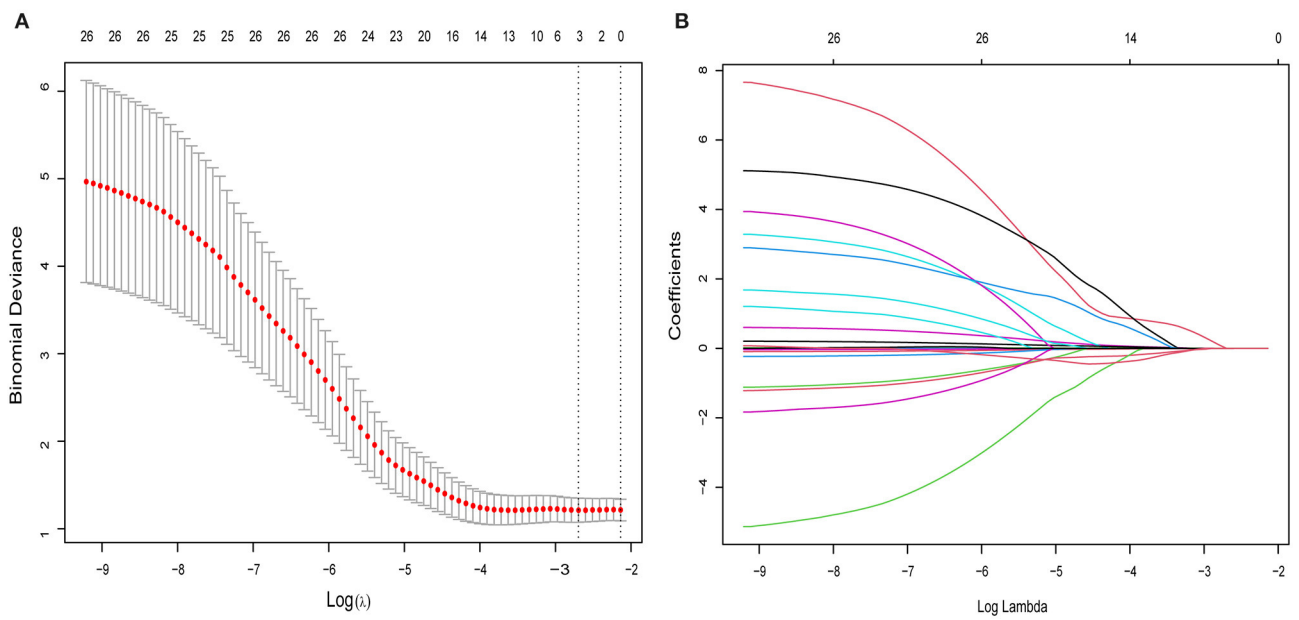

FIGURE 1 | Selection of cytokines, demographic, and clinical features using the LASSO binary logistic regression model. **(A)** Optimal parameter (lambda) selection in the LASSO model using 10-fold cross-validation via minimum criteria. The partial likelihood deviance (binomial deviance) curve was plotted vs. log (lambda). Dotted vertical lines were drawn at the optimal values by using the minimum criteria and 1 SE of the minimum criteria (the 1-SE criteria). **(B)** LASSO coefficient profiles of the 26 features. A coefficient profile plot was produced against the log (lambda) sequence. A vertical line was drawn at the value selected using 5-fold cross-validation, where optimal lambda resulted in five features with non-zero coefficients. LASSO, least absolute shrinkage and selection operator.

TABLE 3 | Prediction factors for response to treatment.

Variables	Prediction model		
	β	Odds ratio(95%CI)	*P*
MCP-1	0.0129	1.0129(1.0027,1.0258)	0.02575
VCAM-1	0.0081	1.0082(1.0031,1.0142)	0.00342
Lipocalin-2	−0.0164	0.9837(0.9612,0.9972)	0.04445

β, *regression coefficient; CI, confidence interval.*

this response predicting model to predict response adds more benefit to the scheme.

DISCUSSION

Utilization of investigative approaches coupled with multiplex immunoassay panels enables the assessment of a broad range of immune- and inflammation-related markers. In this report, we used commercially available multiplex kits to quantify a broad spectrum of inflammatory markers at baseline, in a cohort of MDD participants. Comparison of baseline factors between the 12-week treatment-responding group and the treatment-non-responding group of MDD patients revealed significant differences. Using a novel and appropriate statistical approach that simultaneously modeled dozens of sociodemographic, clinical, and inflammatory variables, we showed that inflammation-related markers at baseline can predict anti-depressant efficacy in patients with MDD. Our report describes the application of a machine learning approach to define potential inflammation-related predictors of response to the selective serotonin receptor inhibitor anti-depressant (SSRI) escitalopram, the most prescribed therapeutic drug for the treatment of depression (38), through combined LASSO and logistical regression. Three key variables (i.e., MCP-1, VCAM-1, and lipocalin-2) were identified and satisfactory performance was obtained using a parsimonious prediction model, with accuracy of 0.811.

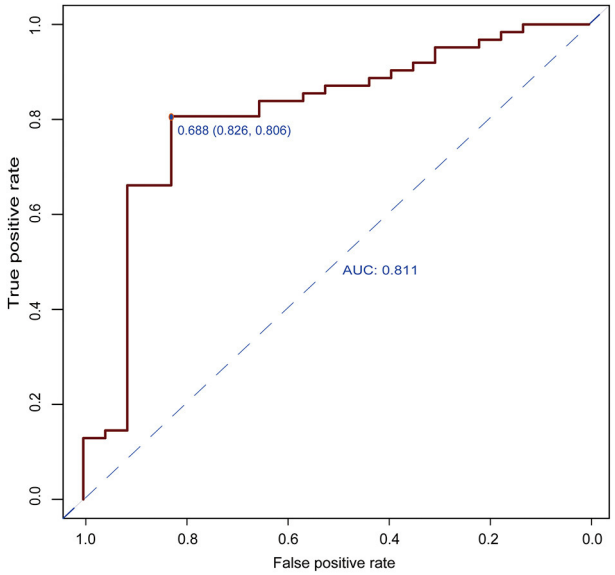

FIGURE 2 | Receiver operating characteristic (ROC) curves of the predictive model. The area under the ROC curve (AUC) from the model was 0.811, the cut-off value for the prediction score at the optimum point was 0.688, the sensitivity was 82.6%, and the specificity was 80.6%.

MCP-1

Chemokines are divided into two major families (CC and CXC) depending upon the presence or absence of an amino acid between the first two cysteines at the amino-terminal (39). Chemokines direct the cell trafficking needed to initiate T-cell-mediated immune responses and inflammation. MCP-1 is a member of the CC chemokine family and signals predominantly via the G protein-coupled CCR2 receptor. Some data point toward MCP-1 being an important mediator of the neuro inflammatory processes that take place in several neurological disorders, including autoimmune disease, obesity, and atherosclerosis. MCP-1 can affect cellular interaction, neuro modulation, and synaptic transmission, all of which are known to be altered in depression (39). In a previous study, Flaishon et al. demonstrated that CCL2 at pM levels can exert global suppressive effects on T-cell trafficking into inflamed lymph nodes. Thus, this chemokine may have clinical application as a general anti-inflammatory agent (40). The anti-inflammatory or pro-inflammatory effects of MPC-1 may be related to its specific dose.

Relatively few studies have investigated the association between MCP-1 and depression and the results of these studies are not consistent (41–44). Some reported MCP-1 levels decreased or increased after anti-depressant treatment (45, 46). In our study, MCP-1 was higher in the responding group than that in the non-responding group. The precise mechanism responsible for this association is unclear, although the neuroprotective function of neuronal chemokines (47, 48), and their ability to enhance dopaminergic activity in the central nervous system, could be possible explanations.

VCAM-1

The expression of VCAM-1 on endothelial and other cells is induced by inflammatory stimuli and cytokines (49). Inflammation and endothelial damage are potential mechanisms that link depression with cardiovascular disease (50). A growing body of data suggest that endothelial dysfunction is associated with several clinical conditions with high cardiovascular risk, including depression (51). Symptoms of depression are related to adverse cardiovascular prognosis in patients with heart failure. Furthermore, endothelial activation and damage is characterized by increased plasma levels of soluble VCAM-1 and other factors considered to be surrogate markers of vascular disease (52).

Although the precise mechanisms involved remain unclear, some studies indicate that patients with severe depression

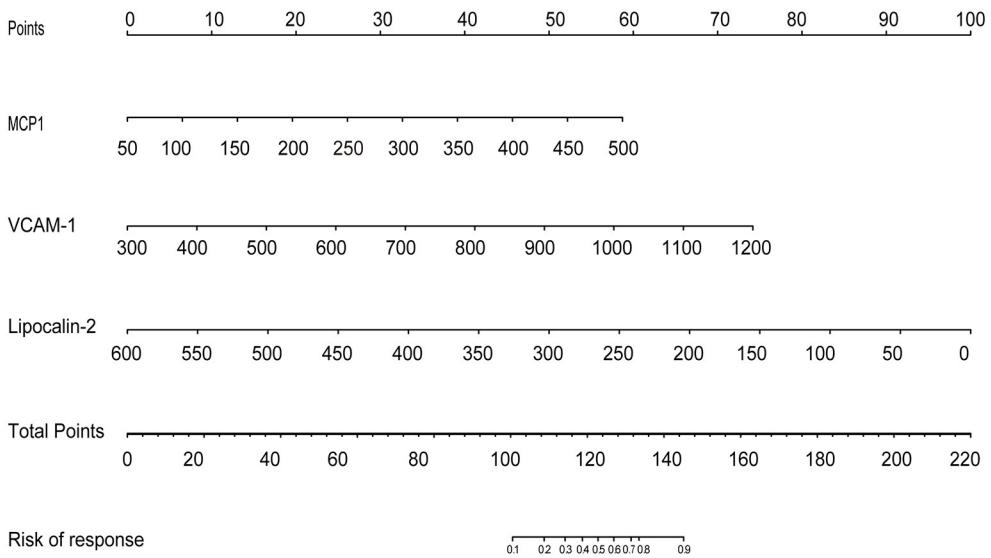

FIGURE 3 | The development of a predictive nomogram for MDD response. A response predicting nomogram was developed in the study cohort that included MCP1, VCAM1, and Lipocalin. First, it is necessary to locate the patient's MCP1, VCAM1, and Lipocalin level, on the corresponding axis. The score for each value is then assigned by drawing a line upwards to the line, and the sum of the three scores is plotted on the total points line. Next, a line should be drawn straight down to identify the patient's probability of achieving a response.

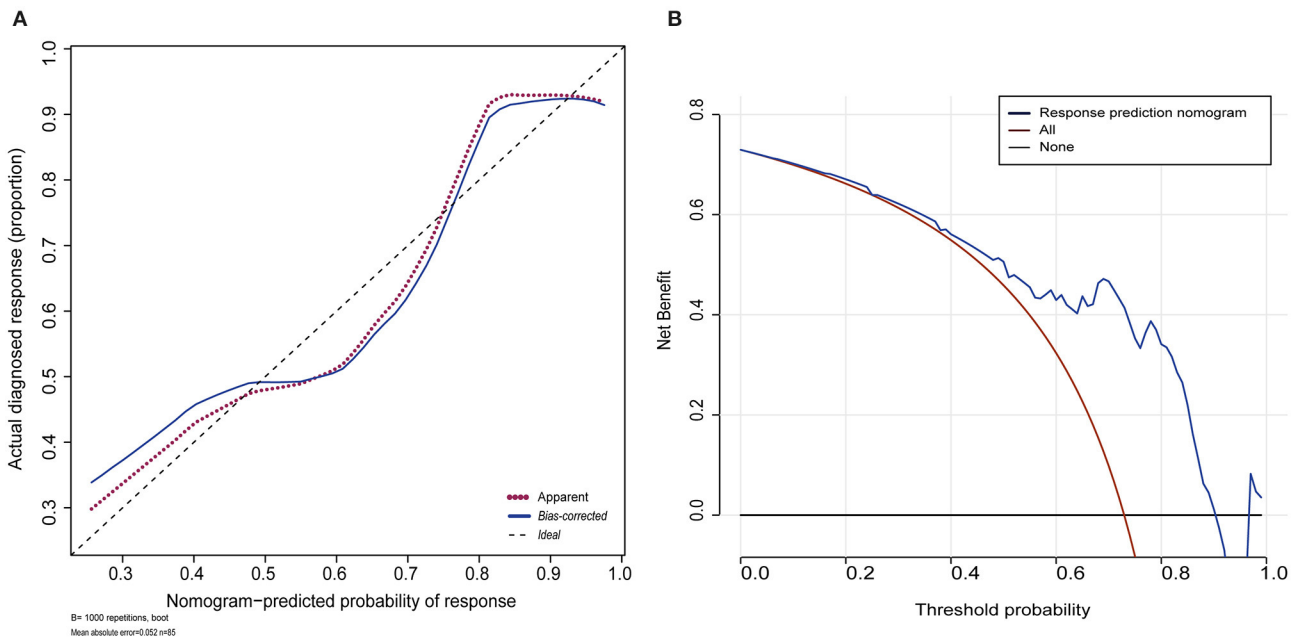

FIGURE 4 | Calibration plot for the nomogram and decision curve analysis of the logistic model. **(A)** Calibration plot for the nomogram. The dotted black line indicates the location of the ideal nomogram, in which the predicted and actual probabilities are identical. The dotted red line indicates the apparent accuracy of the nomogram without correction for overfitting. The blue solid line represents the bootstrap-corrected nomogram. **(B)** Decision curve analyses (DCA) demonstrating the net benefit associated with use of the response nomogram with regards to predicting a response to treatment. The *y*-axis represents the net benefit. The red line represents the treat-all-patients scheme. The black line represents the treat-none scheme. The blue line represents the predictive nomogram scheme. The decision curve shows that if the threshold probability is 1–91%, using this nomogram in the current study to predict response adds more benefit than an intervention-in-all-patients scheme or an intervention-in-none scheme.

treated with SSRIs have reduced cardiovascular risk compared to patients not receiving anti-depressant therapy (53, 54). Here, we demonstrated that VCAM-1 was significantly higher in the responding group than that in the non-responding group. Lopez-Vilchez et al. explored the potential modulating effect of anti-depressant treatment with escitalopram for 24 weeks. Their results show significant reductions in soluble VCAM-1 levels during treatment with escitalopram (55). Increased levels of soluble VCAM-1 have been reported in another study of severe depression (56), which supports the existence of endothelial damage and cardiovascular risk. However, despite this evidence, direct damaging effects on the endothelium of the humoral changes occurring in patients with depression remain poorly elucidated.

Lipocalin-2

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), is the product of the lcn2 gene and is a glycoprotein associated with a variety of inflammatory conditions (57–59). Elisabeth et al. reported that brain lipocalin-2 may be an important biomarker of neuro-inflammation (60). As an immune-related protein, lipocalin-2 is likely to perform a dual role in the nervous and immune systems, as has been attributed to other immune-related proteins. It has been demonstrated that increased circulating levels of lipocalin-2 are significantly associated with depression in patients with heart failure (17). To our knowledge, this is the first study to evaluate the moderator effect of baseline levels of lipocalin-2 on anti-depressant treatment outcomes. NGAL can lead to reduced hippocampal neuronal growth during stress (58), which links to the "neurotrophic hypothesis of depression" (59). NGAL is therefore an interesting inflammatory component and plays an important function in the pathophysiology of depression; the precise function of NGAL in brain homeostasis warrants further investigation.

Our study provides valuable evidence that MCP-1, VCAM-1, and lipocalin-2, are putative markers of MDD due to the significant differences between pharmacological therapy responders and non-responders. Although MCP-1, VCAM-1, and lipocalin-2, were shown to be correlated with the efficacy of escitalopram in this study and were also shown to be correlated with MDD in previous studies, the interaction of these factors with MDD or antidepressant treatment, need to be investigated further.

This was a predictive study conducted to demonstrate novel methodologic approaches, such as LASSO, to the identification of predictors of recovery from depression. LASSO, an increasingly common tool in genetic research (61, 62), minimizes false discovery, and increases the generalizability of the results (62). This method of choosing predictors not only surpasses univariable analysis in terms of outcome (63), it also enables the panel of selected features to be combined into an inflammatory signature. While previous research has similarly aimed to identify models that predict treatment outcome to anti-depressant medication in MDD (64, 65), few of them focused on the response to escitalopram.

When the model was tested on a randomly selected test dataset using the bootstrapping method, its discrimination was confirmed with an accuracy of 0.7887. Calibration plot and decision curve analysis also indicated good applicability and net benefit. Currently, nomograms are widely used as prognostic devices in medicine. Given the availability of treatments in clinical settings, this approach would optimally be used to assist clinical decision-making in conjunction with response prediction models for other treatments. Our findings also provide some insight into the pathways underlying the anti-depressant effects of escitalopram, since inflammatory molecules have been implicated as potential mechanisms (66, 67). The growing trend for machine learning will hopefully create high quality evidence for the understanding of depression and drive innovations in this field. The improvement of the performance of predictive models will help to personalize treatments with safety and efficiency (68).

There are several limitations to be considered when interpreting our results. Firstly, selection bias and inadequate representation of MDD patients may have occurred, since participants were mostly included on the basis of physician referral, which was not designed to develop or evaluate a clinical decision model. Secondly, risk factor analysis did not include all potential factors that could affect escitalopram efficacy. Thirdly, although the model was tested extensively with internal validation, further external validation and replication is required. Fourthly, due to the lack of healthy controls (HCs), this study was unable to determine how these markers change relative to HCs.

In conclusion, we have proposed and validated a relatively accurate prediction model to facilitate individualized prediction of escitalopram treatment in MDD and established a personalized approach for treating patients with depression. The relationship between immune and other biological systems is complex and multifaceted. Concurrent assessment of some of the parameters involved in the inflammatory response in depression might prove useful in furthering understanding of therapeutic mechanisms. In future studies, we plan to verify the sensitivity and effectiveness of the inflammatory factor panel in efficacy prediction, using larger cohorts of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research and Ethics Committee of Beijing Anding Hospital (2017–24). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JinZ: project administration and writing - original draft. JiaZ: methodology, data curation, formal analysis, and visualization. ZS: resources and investigation. LF: project

administration. XZ: methodology. JY: conceptualization and writing - review and editing. GW: conceptualization. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Mitochondria in Mood Disorders: From Physiology to Pathophysiology and to Treatment

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Mitochondria are cellular organelles involved in several biological processes, especially in energy production. Several studies have found a relationship between mitochondrial dysfunction and mood disorders, such as major depressive disorder and bipolar disorder. Impairments in energy production are found in these disorders together with higher levels of oxidative stress. Recently, many agents capable of enhancing antioxidant defenses or mitochondrial functioning have been studied for the treatment of mood disorders as adjuvant therapy to current pharmacological treatments. A better knowledge of mitochondrial physiology and pathophysiology might allow the identification of new therapeutic targets and the development and study of novel effective therapies to treat these specific mitochondrial impairments. This could be especially beneficial for treatment-resistant patients. In this article, we provide a focused narrative review of the currently available evidence supporting the involvement of mitochondrial dysfunction in mood disorders, the effects of current therapies on mitochondrial functions, and novel targeted therapies acting on mitochondrial pathways that might be useful for the treatment of mood disorders.

Keywords: mitochondrial dysfunction, mood disorders, bipolar disorder, major depressive disorder, novel therapies

INTRODUCTION

Mitochondria are cellular organelles known to be involved in diverse biological processes, such as adenosine triphosphate (ATP) production, metabolism of reactive oxygen species (ROS), calcium (Ca^{2+}) homeostasis, cell death and survival (1), as well as in synaptic plasticity. Mitochondria are abundant in neuronal dendrites and synaptic terminals. In the brain, which uses high amounts of ATP and does not have the ability to store it (2), their activity is crucial for the modulation of neuronal activity, short- and long-term neuronal plasticity, cellular resilience, and behavioral adaptations, mainly through actions on long-term potentiation (3–6). Moreover, several lines of evidence suggest roles for mitochondria in supporting the different bioenergetic requirements of highly proliferative neural stem cells and postmitotic neurons (7). In this respect, the adaptation of the energy supply to the energy demand and mitochondrial health is central to cellular homeostasis, and appropriate neuronal function (8–10).

Mitochondrial dysfunction is considered a multifactorial phenomenon since it may have multiple causes and affects numerous neurobiological processes, altering synapsis and enhancing apoptosis, which could play a role in the potentially progressive long-term course of some psychiatric disorders (1). Several studies have focused on the presence of impaired energy metabolism in patients with mood disorders, which indicates that mitochondrial dysfunction may play an important role in various aspects of these conditions (2). In summary, the "mitochondrial hypothesis" suggests that mood disorders are triggered, in part, by mitochondrial dysfunction, which can be intimately linked to a wide range of processes associated with treatment outcomes, disease progression, and severity (11, 12). Moreover, mitochondrial dysfunction could pre-dispose vulnerable individuals to these disorders and lastly, be an important target for current and novel potential therapies for mood disorders (2).

Every cell depends on energy production from mitochondria, with much higher demand in neurons, especially in gray matter, which has a high number of synapses and mitochondria (13–15). Besides energy production, mitochondria are sources of cellular growth substrates and play crucial roles in oxidative/nitrosative stress, cell resilience, and death pathways (3, 16, 17).

Mitochondria are the only organelles in the cell that contain their own DNA, called mitochondrial DNA (mtDNA), which contains 37 genes that encode 13 proteins, 22 tRNA, and 2 rRNAs. These genes encode 13 protein subunits of the electron transport chain (ETC). Genes from nuclear DNA (nDNA) code the rest of the mitochondrial proteins (15) and play a role in the regulation of mitochondrial function. In contrast to nDNA, mtDNA is vulnerable to DNA damage due to constant exposure to reactive oxygen species (ROS) and at times insufficient DNA repair mechanisms (18). Moreover, a number of proteomic studies have been conducted to decipher the mitochondrial proteome. Several mitochondrial databases that list the number of mitochondrial proteins are available nowadays (19).

Mitochondria contain two membranes, an outer and an inner membrane, an intermembrane space, and an intracellular matrix. The intracellular matrix contains several enzymes, which participate in the tricarboxylic acid (TCA) cycle and are responsible for the generation of NADH and $FADH_2$ (20). These redox cofactors are required for the generation of ATP through oxidative phosphorylation *via* the ETC, present within the folds on the inner mitochondrial membrane or cristae, as explained in **Figure 1** (21–23).

Given their diverse roles, mitochondria possess several mechanisms to maintain a healthy and functional mitochondrial pool (29), such as neutralizing ROS by antioxidant defenses, the unfolded protein response (UPR), mitochondrial dynamics, biogenesis, and mitophagy (30).

Apart from their involvement in cellular energy production, mitochondria also play an important role in regulating the process of apoptosis through both intrinsic and extrinsic pathways (31). In normal conditions, apoptosis removes those neurons and glia that are functionally compromised or unable to make neuronal connections (15). In the intrinsic mitochondrial-mediated pathway, stimuli such as high levels of intracellular cytoplasmic Ca^{2+} or ROS, as well as the activation of proapoptotic proteins (i.e., Bcl-2 family members) in the outer mitochondrial membrane (32), trigger a cascade of processes that activate caspases. This results in cleavage of several proteins, DNA fragmentation and cell death (33, 34). In the extrinsic pathway, activation of extracellular death receptors enhances processes that alter membrane permeability, resulting in leakage of proapoptotic factors and apoptosis (34).

Ca^{2+} homeostasis is another key process in which mitochondria are involved, with Ca^{2+} a principal secondary messenger that is involved in the regulation of neurotransmission and neuroplasticity in the brain (15). The mitochondrial outer membrane is permeable to Ca^{2+}, and the inner membrane contains Ca^{2+} uniporters for its inward movement, and Na^+/Ca^{2+} and Ca^{2+}/H^+ antiporters for its outward movement (35). Moreover, mitochondria form highly specialized signaling hubs with the ER through the mitochondria-associated membranes (MAMs), allowing the regulation of lipid synthesis and rapid transmission of Ca^{2+} signals between these organelles (36).

Mitochondrial Ca^{2+} concentrations increase when cytosolic Ca^{2+} levels are high and in case of high-energy demand, and decrease when cytosolic levels are low, or the ATP/ADP ratio is high. Ca^{2+} can modulate oxidative phosphorylation machinery by different mechanisms, including direct binding, enhancing post-transcriptional modification, and also by the activity of a Ca^{2+}-dependent binding protein. It also binds to complex IV and reduces ATP inhibition of this enzyme, enhancing ATP production even in situations of high ATP concentrations (24). ATP synthesis is also enhanced by stimulation of the aspartate-glutamate carriers (AGCs) and the ATP-Mg/Pi (i.e., Ca^{2+}-binding mitochondrial carrier protein, SCaMC-3) transporters on the inner mitochondrial membrane. Ca^{2+} also leads to increased NADH synthesis and higher production of pyruvate (15).

However, when Ca^{2+} levels are excessive in the intracellular space or mitochondria they induce stress and excitotoxicity, ATP production is reduced (37, 38), and Ca^{2+} is extruded

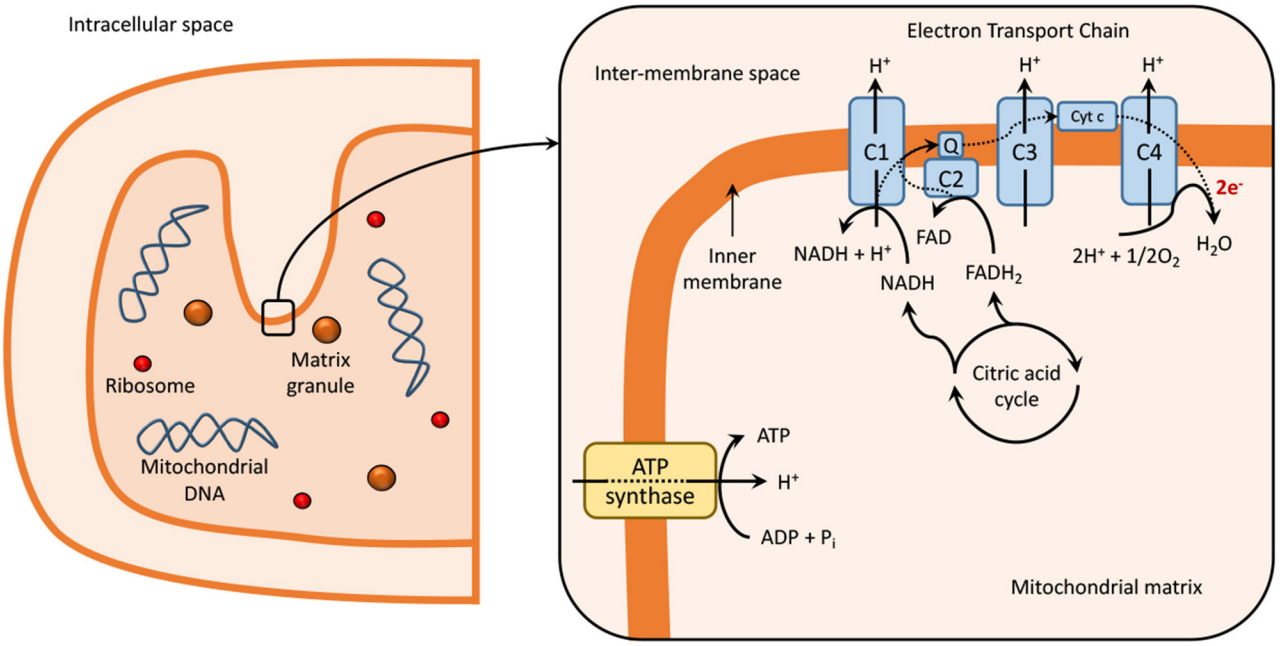

FIGURE 1 | Mitochondria at normal physiological conditions. Dashed lines represent electron transport in the electron transport chain reaction (ETC). The ETC is localized within the inner mitochondrial membrane or cristae of the mitochondria (21–23) and is composed of five multimeric protein complexes (I-IV and ATP-synthase or complex V) that are responsible for ATP production by oxidative phosphorylation. Complex I, or nicotinamide adenine dinucleotide (NAD+), and/or complex II (succinate dehydrogenase), begin the process of oxidative phosphorylation by catalyzing the transfer of electrons from NADH and $FADH_2$, respectively, to coenzyme Q (or ubiquinone). The transfer of electrons is serially conducted through complex III (ubiquinol: cytochrome c oxidoreductase), cytochrome c and complex IV (cytochrome c oxidase), to the terminal acceptor, generating an electrochemical proton gradient that enhances ATP production in complex V *via* oxidative phosphorylation (1, 24, 25). During this process, single-electrons can escape and produce a single-electron reduction of O_2, forming superoxides and other ROS (24, 26, 27). Impaired functioning of ETC can result in excessive ROS production, which leads to the damage of DNA, lipids, proteins, and other molecules in a process known as oxidative damage (1, 24, 27). The generation of ROS is also related to signaling physiological processes, such as synaptic plasticity and memory (28). C, complex; Cyt c, cytocrome c; e-, electron; ECT, electron transport chain; FAD, flavin adenine dinucleotide; Q, coenzyme Q; NAD, nicotinamide adenine dinucleotide.

through the Na^+/Ca^{2+}exchanger and the mitochondrial permeability transition pore (mPTP). Impairment in the control of mitochondrial membrane permeabilization, by mPTP, has been suggested to be responsible for the mitophagy of depolarized mitochondria, induction of apoptosis, and necrosis (15). Ca^{2+} homeostasis is regulated by different proteins, enzymes, and cellular signaling networks, which may be risk pathways for mood disorders when they are altered (15).

The maintenance of a healthy mitochondrial pool is critically regulated by the dynamics and turnover of the mitochondrial population (29). At the organelle level, mitochondrial quality is sustained through the synthesis of new mitochondria, fusion and fission, and the elimination of damaged mitochondria (30, 39). The balance between fusion and fission events shapes mitochondrial networks to meet metabolic demands (40, 41).

A considerable amount of literature has demonstrated that neuronal activity regulates mitochondria and synapses (42, 43). Neurons depend on oxidative metabolism to meet their high-energy needs (10). Thus, to match the actual local needs in neurons, mitochondria constantly move along microtubes networks, changing mitochondrial trafficking, distribution, anchoring, and membrane dynamics (44).

Mitochondria also regulate synaptic plasticity by transducing some of the effects of glutamate and BDNF. BDNF expression and signaling are promoted by some environmental factors, such as physical activity and cognitive stimulation (2, 45). On the other hand, studies have shown that BDNF enhances ATP synthesis and mitochondrial respiration through several mechanisms, including increases in glucose transport, upregulation of the mitochondrial biogenesis, and respiratory coupling efficiency (46, 47). Moreover, ATP is necessary for the mobilization of synaptic vesicles to the active sites of synapse in neurons. The ATPase complex, by producing cAMP, activates PKA kinase, which allows the mobilization of synaptic vesicles (48). When ATP production is reduced, as in mood disorders, neuronal transmission is consequently impaired (15, 49, 50).

Mitochondria also play a critical role in the neurogenesis, the process of neural stem cell proliferation and differentiation into new neurons. Numerous studies have shown that the mitochondrial genome and mitochondrial proteins are required for neuronal differentiation (51–53). Moreover, accumulating evidence has indicated that the development of a mature mitochondrial network in terms of mitochondrial function and structure is necessary during the differentiation of induced pluripotent stem cells (iPSCs) (54–56).

The aim of this article is to provide a focused narrative review of the currently available evidence supporting (1) the involvement of mitochondrial dysfunction in mood disorders, (2) the effects of current therapies on mitochondria, and (3) novel targeted therapies acting on mitochondrial pathways that might be useful for the treatment of mood disorders. To this end, a literature search was conducted to identify relevant original research articles, reviews including systematic reviews and meta-analyses containing evidence regarding the role of mitochondria in mood disorders, from MEDLINE, SCOPUS, EMBASE, ClinicalTrials, ISI Web of Science and Google Scholar. Based on these reports, we provide a critical overview of the current state of the role of mitochondria in mood disorders, ranging from physiology to pathophysiology, and therapeutic strategies, as well as perspectives on future directions.

Take-Home Message

Mitochondria are cellular organelles involved in a number of biological processes, with a key role in maintaining neuronal homeostasis. They are involved in energy production, metabolism of ROS, calcium homeostasis, apoptosis, synaptic plasticity and neurogenesis, modulating neuronal activity and preventing neuronal damage. In mood disorders, mitochondrial dysfunction leads to the impairment in cellular homeostasis with dysregulation in these mechanisms.

OVERVIEW AND DISCUSSION

Mitochondrial Dysfunction in Mood Disorders

As mentioned above, mitochondria are the main source for cellular energy but are also responsible for other processes that are crucial for cell functioning and survival, such as apoptosis and Ca^{2+} homeostasis (57). Impaired mitochondrial functioning may result from a number of causes, including altered expression of mitochondria-related genes, changes in the regulation of mitochondrial biogenesis, mitochondrial structural abnormalities, changes in oxidative phosphorylation and variations in metabolite levels (57). The above-discussed functions make mitochondria indispensable in several network processes, as well as they are associated with aging and a plethora of pathological conditions, such as Alzheimer's, Parkinson's, and Huntington's disease, amyotrophic lateral sclerosis, and psychiatric diseases (6, 8, 57–60). The hypothesis that mitochondrial dysfunction is associated with these conditions is supported by reports that have associated mitochondrial diseases with psychiatric symptoms, especially mood and cognition (1, 61, 62).

Mitochondrial Bioenergetics and Redox in Mood Disorders

The brain is an organ with the highest energy consumption, unique membrane lipid composition, and depends on mitochondrial oxidative phosphorylation, being unable to store glycogen. Since brain mitochondria produce high quantities of ATP but also ROS and RNS, this organ is vulnerable to oxidative damage, which occurs when the oxidative load exceeds antioxidant capacity (2).

Metabolic Changes

A number of studies using neuroimaging and post-mortem brain tissue from patients with BD have shown lower numbers of neuronal and glial cells and lower brain volume in prefrontal and limbic brain regions. Growing evidence suggests mitochondrial dysfunction is implicated in these changes through a reduction in oxidative bioenergetic generation and a shift to anaerobic glycolysis and consequently impaired neuroplasticity, phospholipid metabolism and Ca^{2+} homeostasis (15, 24). In addition, alterations in various regions of the brain in neurometabolites, including high-energy compounds, have been found in patients with mood disorders. In summary, it has been described that patients with mood disorders have lower levels of phosphocreatine (PCr), N-acetyl-aspartate (NAA), adenosine diphosphate (ADP), and ATP (63, 64). In patients with major depressive disorder (MDD), hypermetabolism could be a consequence of depression severity (65), whereas hypometabolism appears linked to less severe illness (66–69).

Moreover, studies have noted negative correlations between NAA/Creatine + PCr or NAA levels and illness duration in BD (70, 71), with an enzymatic reaction rate abnormality present in BD in the creatine kinase (CK) system, based on the decrease in the forward rate constant of the CK enzyme without alterations on ATP and PCr levels, as well as by downregulation of CK in post-mortem brains of BD patients (72, 73). This hypothesis is consistent with a previous study that showed that individuals with BD could maintain average brain concentrations of high-energy compounds at rest, but there is an underlying abnormality in the mechanism that generates new ATP, which can be uncovered when energy demand is increased (72). Apart from this, studies showing increased lactate and taurine levels and a reduced brain intracellular pH suggest that there is a shift from oxidative phosphorylation to glycolysis as a major source of energy generation in BD (74, 75). Elevated lactate is present, especially in manic phases, in the frontal cortex, caudate, cingulate, and anterior cingulate cortex, which could mean either an overall increase in ATP demand, or defective oxidative metabolism (76).

Mitochondrial Changes in the Electron Transport Chain

By drawing on the hypothesis of mitochondrial impairment on mood disorders, several studies in post-mortem brain, skeletal muscle or blood from patients with mood disorders have shown changes in the enzymatic activities linked to the TCA cycle and ETC, as well as impairment in mitochondrial oxygen consumption. Studies in post-mortem samples of patients with BD and MDD have shown that many mitochondria-related genes are downregulated compared with controls (77). For instance, some studies reported decreased expression of some of the complex I subunits in the cerebellum in bipolar and depressed patients compared with controls (57, 78–81). Not only is decreased expression present, but decreased activity is also reported in MDD and BD patients. A recent study confirmed

previous findings showing that the citrate synthase (CS), complex II, and complex IV activities were decreased, while the complex I activity and complex I/citrate synthase ratio were significantly increased in blood platelets of BD patients during a depressive episode. Supporting these findings, Valvassori et al. (82) in isolated mitochondria from peripheral blood mononuclear cells (PBMCs) showed a decrease only in complex II activity in bipolar depressed patients. In contrast, in MDD patients, physiological respiration, the maximal capacity of the electron transport system, and respiratory rate after complex I inhibition are decreased, as well as activity of complex II and CS (83). However, there are studies on mitochondria isolated from PBMCs and blood platelet showing no significant differences in ETC activity in MDD and BD patients (84–87).

Oxidative Damage

Based on the premise that mitochondria are the primary source of ROS, replicated studies documented alterations in multiple aspects of oxidative stress, including an increase in the production of ROS and a reduction of the antioxidant capacity, in MDD and BD patients. Compared to healthy controls, depressed patients show an increase in oxidative stress markers involved in lipid peroxidation (88–90) and a decrease in antioxidant markers (91), as well as lower brain ATP levels (92). Patients with BD have increased lipid peroxidation products in the cingulate cortex (93), and also increased markers of oxidative and nitrosative damage in the prefrontal cortex (1, 94, 95). A meta-analysis that assessed eight oxidative stress markers in patients with BD, including 971 patients with BD and 886 controls, reported an increase of markers of lipid peroxidation, DNA/RNA damage and nitric oxide in the group with BD (96).

Since oxidative damage is the result of the balance between oxidative products and the antioxidant defense, some studies in mood disorders have investigated this system, including superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), and glutathione peroxidase (GPx) (34, 97). Animal studies have shown that chronic stress is associated with lower brain concentrations of GSH, SOD and catalase (98–100). Studies in post-mortem brains of patients with BD have shown lower expression of SOD, microsomal GST, and GPx in frontal areas and lower expression of GPx in the hippocampus (101–103), and reduced activity of SOD and catalase in these patients (104, 105).

However, in some studies SOD activity appears increased in BD during the manic and depressive episodes (104, 106–108), whereas there are studies showing decreased SOD levels in manic patients (1, 109). Savas et al. (106) found increased SOD levels in euthymic bipolar patients (106), whereas others found decreased activity in the euthymic phase (104, 107). A study reported increased activity of GPx in euthymic bipolar patients (107) but not in depressed or manic patients, whereas another showed increased GPx levels in depressed bipolar patients compared to healthy controls (110). Other studies did not find any differences in GPx activity compared to a control group or across different mood states (95, 104). The same uncertain pattern is observed regarding catalase activity. Studies in chronic patients have shown decreased or unaltered catalase activity (105, 107). Contrary to these reports, BD depression at baseline presented a significant increase in catalase levels, with a lower SOD/CAT ratio (110), which was confirmed by previous findings (108). This may be explained by a compensatory mechanism in the early phases of BD, or heterogeneity in other data domains. Compared to controls, reduced GSH and glutathione S-transferase were increased among patients with late-stage BD (95).

Calcium-Dependent Functions

When ATP production is reduced, mitochondrial and cellular functions are impaired due to changes in mitochondrial membrane potential, reducing mitochondrial capability for Ca^{2+} uptake. Studies in brains from bipolar subjects have shown altered intracellular free Ca^{2+} levels in blood cells and olfactory neurons (111, 112). Bipolar patients evidence high cellular Ca^{2+} levels in all states, but especially in mania (113, 114), and also changes in the expression of genes involved in Ca^{2+} signaling, neuroactive ligand-receptor interaction, and protein kinase PKA/PKC signaling pathways. Moreover, the authors also found changes in the action potential system (115). Indeed, excess Ca^{2+} affects both neuronal excitability and signaling cascades regulating gene expression, leading to perturbation of multiple neuronal processes, such as dendrite development, synaptic plasticity, and excitatory/inhibitory balance (116).

Calcium/Calmodulin Dependent Protein Kinase Kinase 2 (CaMKK2), is the core component of the Ca^{2+}-calmodulin (Ca^{2+}-CaM) dependent signaling pathway in neurons (117). Through activation of AMP-activated protein kinase (AMPK) and the master mitochondrial regulator, PGC1α, tightly linked to the circadian clock (118), CaMKK2 regulates mitochondrial function and whole-body energy balance. Bipolar disorder is associated with mutations that affect the function or experssion of CaMKK2 (119). Decreased CaMKK2 function leads to decreased BDNF expression, a known biomarker of BD. Lastly, the activity of CAMKK2 is regulated at least in part by the multi-site phosphorylation of the catalytic domain termed the S3-node in a switchable bidirectional manner, a phenomenon critical for understanding the biphasic nature of the disorder (120, 121).

Studies have also reported that DISC1, a protein involved in mitochondrial dynamics and a putative risk factor for BD and MDD (122), interacts with the IP3R1 modulating endoplasmic reticulum-mitochondria Ca^{2+} transfer (123). One study by Dwivedi et al. (124) showed an increase in IP3R1 binding sites and protein levels in platelets of depressed patients. Moreover, Scaini et al. (125) found that BD patients had higher levels of VDAC and TSPO, suggesting that these could deregulate mitochondrial Ca^{2+} signaling and increase ROS production.

Mitochondrial Morphology

Other findings that support the hypothesis about the association between mitochondrial dysfunction and mood disorders are changes in mitochondrial morphology, distribution, and degradation. A study undertaken by Cataldo et al. (126) showed that prefrontal neurons from post-mortem brain samples obtained from patients with BD and peripheral cells from patients with BD contain a larger number of smaller-sized mitochondria. The same authors showed an abnormal pattern of clumping and marginalization in the intracellular distribution

of mitochondria in peripheral cells, as well as atypically shaped mitochondria (ring- or cup-shaped mitochondrial profiles), suggesting subtle changes in the critical network architecture of mitochondria in the cells (127). Moreover, Mertens et al. (115) showed that iPSC-derived hippocampal dentate gyrus-like neurons of patients with BD had smaller mitochondria than those from healthy controls. As previously described, the balance of fusion and fission modifies the overall morphology of the mitochondrial network (40, 127). Thus, the alterations in these processes observed by downregulation of the mitochondrial fusion-related proteins Mfn-2 and Opa-1 and an upregulation of the fission protein Fis-1 in PBMCs from BD patients (128) might explain the abnormal mitochondrial morphology and distribution findings in patients with BD.

Mitochondrial Degradation and Apoptosis

By drawing on the concept of mitochondrial quality control, Scaini et al. (125) have been able to show that BD patients presented a downregulation of mitophagy-related proteins, Parkin, p62/SQSTM1 and LC3A in PBMCs, followed by NLRP3-inflammasome activation. In summary, the imbalance in mitochondrial fission and fusion toward fission, followed by a decrease in the levels of mitophagy proteins and an increase in the caspase-3 protein levels (125, 128) could suggest that the number of damaged mitochondria exceeds the capacity of mitophagy, and apoptosis becomes the dominant pathway to minimize tissue damage in BD (129, 130). Indeed, evidence has shown that apoptotic genes, such as FAS, BAK and APAF-1 are upregulated in the hippocampus of patients with BD (103). Moreover, Bcl-2, an antiapoptotic protein, is downregulated in BD patients due to different polymorphisms, resulting in Ca^{2+} homeostasis dysregulation and increased glutamate levels. This is added to the endoplasmic reticulum (ER) stress response seen in all states of BD, mainly in mania (34, 129). Chronic mild stress was shown to reduce the expression of BAG-1, a gene that enhances the anti-apoptotic effects of Bcl-2. This causes the activation of caspases, BCL-2-associated X protein (BAX), and BCL-2 antagonist/killer (BAK) in the mitochondria, which leads to the alteration of membrane permeability and neuronal death (15).

PI3K and Akt are other proteins related to cell survival and proliferation. Their transcription is upregulated in mania, and this pathway is activated by oxidative stress and IL-6, and regulated by AMPK, suggesting that this pathway is active in bipolar mania (103). Akt promotes mitochondrial survival *via* different routes, such as inhibiting cytochrome c release into the cytosol, which is the final act of mitochondrial apoptosis (131). It also activates the ETC and promotes a shift to glycolytic energy generation in BD. PI3K activates mTOR, which stimulates oxidative phosphorylation (76). Moreover, GSK-3α and GSK-3β are activated in an environment of chronic oxidative stress, such as in BD, with greater activation in mania than in depression. Their inactivation has been correlated with measured clinical improvement (76).

GSK-3 promotes cellular apoptosis by the activation of Fas receptor, also promoted by TNF-α, but which also has a role in neuroprotection. In mania, TNF-α activates GSK-3 to promote neuronal survival, since GSK-3 upregulates NFκβ, and this inhibits TNF-α mediated apoptosis, may inhibit oxidative phosphorylation and promote aerobic glycolysis. TNF-α inhibits mitochondrial biogenesis, which is prevented when SIRT-1 activity is increased (76). Increased levels of NFκβ and SIRT-1 have been found in mania compared to bipolar depression and healthy controls (76). SIRT-1 levels are lower in bipolar depression than in euthymia, and TNF-α levels may be lower in depression than in mania (76). A dysregulated NFκβ system plus genetically influenced anti-apoptotic elements might enable the increased mitochondrial function in mania and the cyclical nature of BD (132). A recent study found an association between the downregulation of 20 genes related to the apoptosis pathway, TNF-α, TLR, and NFκβ signaling pathways and major depressive disorder (76). Moreover, NFkβ causes an increase in cytoplasmic CREB levels in BD patients, which is of interest as the activity of BDNF against ROS is mediated *via* CREB transcription, and BDNF levels are lower in mania than in depression and lower in BD patients compared to controls (133). Studies also demonstrated that CREB is involved in neurogenesis and is reduced in depression (134).

Inflammatory Changes

Chronic inflammation has been found to be present in all phases of BD (135), since it promotes a pro-inflammatory environment with an increase in cytokine levels, such as IL-1β, IL-6, and TNF-α, and increased nitric oxide in brain and plasma (2). These changes are higher in bipolar depression than in unipolar depression and highest in mania. A meta-analysis showed that patients with major depressive disorder had higher levels of plasma IL-6, TNF-α, and soluble interleukin-2 receptors (sIL-2R) (2).

The aforementioned evidence on mitochondrial bioenergetics pathophysiology in mood disorders is summarized in **Figure 2**, which represents the biphasic mitochondrial model in BD in depression (reduced mitochondrial biogenesis) and mania (increased mitochondrial biogenesis), and the derived biological processes in the mitochondria, including oxidative stress, inflammation, genetic damage, increased permeability, cytotoxicity, and apoptosis.

Other specific changes have been observed in mood disorders, which are mentioned in the next sections.

Genetic Changes

Genetic findings also support mitochondrial dysfunction in BD. Some studies have shown that subjects suffering from mitochondrial diseases frequently develop psychiatric symptoms, especially mood symptoms (134).

Increased expression of mitochondrial fission genes and a decreased expression of mitochondrial fusion genes have been associated with depressive behavior in mice (126, 136). Pathological isoforms of DISC1 lead to abnormal neuronal development and mood disorders (137). Genome-wide association studies (GWASs) studies have identified multiple *loci*, with a small effect, associated with BD risk, including CACNA1C, ANK3, ODZ4, SYNE1, and TRANK1 (34, 122, 138, 139). In addition, Kataoka et al. (140, 141) demonstrated the potential roles of *de novo* protein-altering mutations and calcium-related

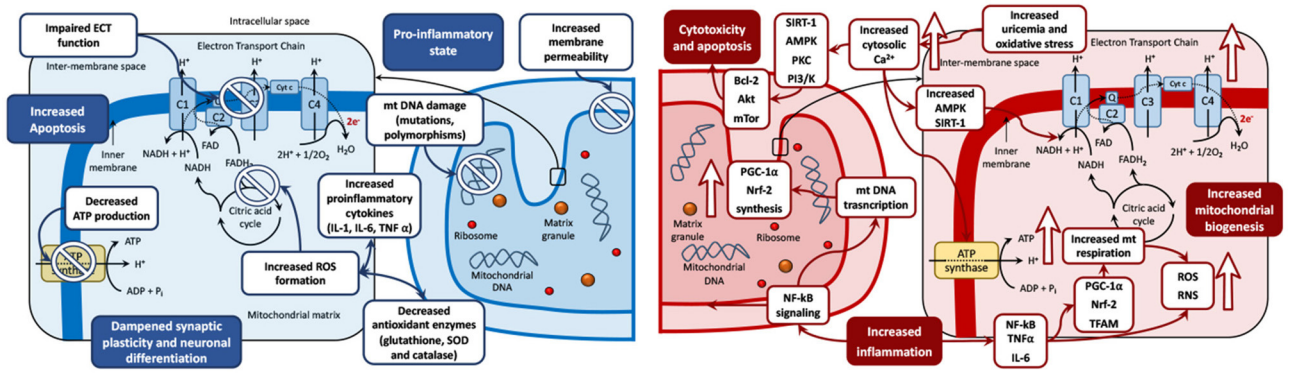

FIGURE 2 | The biphasic mitochondrial model in bipolar disorders. Left: Depression mitochondrial model—Decreased mitochondrial biogenesis. Changes during depressive phases include mitochondrial DNA damage (including mutations and polymorphisms), membrane permeability, and increased formation of ROS. These imbalances lead to a pro-inflammatory state, with increased levels of pro-inflammatory cytokines (IL-1, IL-6 and TNFα) and decreased antioxidant enzymes (glutathione, SOD and catalase). These disturbances can cause cytotoxicity, increased apoptosis, and dampened synaptic plasticity and neuronal differentiation. Antidepressant drugs have shown the capacity to restore mitochondrial disregulation by reestablishing the oxidant/antioxidant balance and counteract the negative effects of depression on the mitochondria. Right: Mania mitochondrial model—Increased mitochondrial biogenesis. Upward arrows symbolize an increase. Changes during manic phases include increased inflammation and elevated production of ROS and RNS, driven by increased activity of the NF-kB signaling pathway. NF-kB signaling stimulates mitochondrial biogenesis *via* the upregulation of PGC-1α, Nrf-2, and TFAM. PGC-1α and Nrf-2 stimulate mitochondrial respiration, which is a further source of oxidative stress *via* ROS and RNS production. Increased oxidative stress could induce an increase in the levels of cytosolic Ca^{2+} ions seen in mania compared to other phases of the disease. Elevated Ca^{2+} levels can stimulate oxidative phosphorylation and ATP production and may lead to the activation of AMPK and SIRT1, which may increase the activity of NAD+. In an environment of increasing oxidative stress, the activity of SIRT1, AMPK, PKC PI3/K are increased. This can foster mitochondrial survival leading to cytotoxicity and cell death *via* activation of proapoptotic pathway cascades (Bcl-2, Akt and mTor among others). Increased uric acid levels increase the uptake of Ca^{2+} ions by mitochondria, increase the mitochondrial membrane potential and therefore enhance ATP production. Antimanic drugs including mood stabilizers and antipsychotics may restore mitochondrial dysregulation by counteracting the mitochondrial imbalance leading to neurogenesis, neuroplasticity, and cell survival. ADP, adenosine diphosphate; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; C, complex; Cyt c, cytochrome c; e-, electron; ECT, electron transport chain; FAD, flavin adenine dinucleotide; IL, Interleukin; Q, coenzyme Q; mt DNA, mitochondrial DNA; mTOR, mechanistic target of rapamycin; NAD, nicotinamide adenine dinucleotide; NF, nuclear factor; Pi, inorganic phosphate; PI3/K, Inositol 1,4,5 triphosphate; PKC, Protein Kinase C; ROS, reactive oxygen species; RNS, reactive nitrogen species; TFAM, mitochondrial transcription factor A; TNFα, tumor necrosis factor-alpha.

genes in BD. Considering the relationship between *de novo* mutations and clinical phenotypes, the same authors observed significantly earlier disease onset among the BD probands with *de novo* protein-altering mutations when compared with non-carriers. Although no specific mutations in mtDNA have been associated with BD (142), some mtDNA haplogroups showed significantly lower cerebellar pH, which is also seen in the disorder. Moreover, a rare gene variant of mtDNA, 3644T>C, might be associated with BD, since patients showed a prevalence of 1.43% of the gene variant whereas the prevalence was 0.13% in healthy controls (34, 143). On the other hand, deletions of mtDNA were more commonly found in post-mortem cerebral cortex of patients with BD compared to controls (34, 144), and also in a patient who suffered from depression (145–147). However, other studies did not replicate these findings, which may be due to different methodologies and different brain regions studied (148).

Another study reported higher levels of circulating cell-free mtDNA in patients with MDD compared to healthy controls, while mtDNA content was not significantly different (149–151). Moreover, a recent study found a higher mtDNA copy number and a decreased DNA methylation status in the peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1α) promoter in patients with MDD, which leads to reduced expression of mitochondrial genes (2, 152). In contrast, Czarny et al. (153) showed that the cellular mtDNA copy number did not differ between healthy and depressed subjects, but it showed a lower capacity for degradation and a higher number of lesions compared to controls (154).

In BD, a meta-analysis for BD-mtDNA copy number studies with a low level of heterogeneity revealed a significant lower mtDNA copy number in patients (154). In contrast, another meta-analysis with a higher level of heterogeneity identified no significant differences between mtDNA copy numbers in BD patients. A recent study showed a decrease in mtDNA copy number and an epigenetic aging acceleration in post-mortem hippocampus from BD patients (155). Focusing on mood-specific states, Wang et al. (156) found that during the depressed and manic states, patients with BD had significantly lower mtDNA copy numbers (157), with the degree of DNA damage associated with the severity of manic and depressive symptoms (157).

Purinergic Dysfunction

The purinergic system appears dysregulated in patients with depression and BD (158). In oxidative stress, the activity of SIRT-1, AMPK, PKA, PKC, GSK, and inositol triphosphate are increased, as well as antiapoptotic proteins, such as Bcl-2, PI3K, mTOR, Akt, and uric acid. Their activation stimulates oxidative phosphorylation *via* different routes. As the mitochondrial function is increased, oxidative stress is higher and different

pathways are activated in order to mitigate the cytotoxic effects of oxidative stress without inducing apoptosis.

Uric acid levels seem to be increased in all phases of BD but are higher in mania than depression or euthymia, which reflects an increase in energy production (159). Increased uric acid levels allow a greater uptake of Ca^{2+} ions by mitochondria, increased mitochondrial membrane potential, and thus higher ATP production. Lowered levels of uric acid have been described as a risk factor for developing mood disorders. Uric acid acts as a scavenger of peroxynitrite, which has high mitotoxic activity (2). It has other neuroprotective effects, such as increasing AMPK activity, which regulates the function of the CLOCK:BMAL-1 complex and upregulates the activity of SIRT-1, leading also to adaptative responses to oxidative stress for mitochondrial survival and functioning.

Other studies have found that cAMP and PKA are upregulated in BD and regulate the rate of oxidative phosphorylation through the phosphorylation of proteins and enzymes involved in ATP synthesis, such as cytochrome c oxidase, enhancing mitochondrial protection. Cytochrome c oxidase, the terminal respiratory enzyme, key for ATP synthesis, is a metabolic marker for neuronal functional activity (160), with its alterations related to depressive symptoms. The cAMP response element-binding protein (CREB) stimulates cAMP-dependent transcription of ETC enzyme complexes and other proteins from mtDNA, thus stimulating oxidative phosphorylation. The activity of CREB, which enhances the upregulation of CK, key for neuroprotection and energy production, is altered in BD, leading to higher or lower levels of CK in mania and lower levels in mixed states (76).

Genetic variations in the purinergic system and in a number of genes involved in cAMP signaling have been found in BD, which highlights the role of cAMP/CREB on circadian clock genes and to maintain ATP production. Higher activity of antiapoptotic proteins, enzymes and signaling cascades has been observed in mania, which enhances mitochondrial activity (2, 76).

Circadian Clock Genes and Oxidative Phosphorylation

Oxidative stress can enhance changes in circadian clock systems, although chronic oxidative stress provokes pro-survival effects. High levels of ROS resets circadian clocks and induces a range of prosurvival responses and different expression of clock genes secondarily to a pro-inflammatory environment, such as activation of cAMP/CREB signaling. Polymorphisms in clock genes can modify cellular sensitivity to oxidative stress or genotoxic insults. Dysregulation of systems involved in oxidative stress and genetic changes in clock proteins could explain some of the observations in circadian systems in BD.

PKC and inositol triphosphate play a role in the pathogenesis of BD, being associated with and downstream of intracellular Ca^{2+} levels. In mania, elevated functioning of PKC has been found, which acts by stimulating and protecting mitochondria. Cytosolic Ca^{2+} activates ATP synthesis enhancing the activity of AMPK through different routes, such as increasing NAD+ and the activity of SIRT-1 (2).

A number of gene variations have shown increased susceptibility for developing more severe forms of BD. These genes control circadian NAD+ concentrations, which increase the activity of SIRT-1 and SIRT-3, and this stimulates oxidative phosphorylation. NAD+ and SIRT-1 directly activate ATP production and upregulate circadian genes, suggesting a pathway of influence in mood disorders (2).

Hypothalamic-Pituitary-Adrenal Axis

Depression is linked to hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis due to an impairment of the corticosteroid receptor-mediated feedback. This leads to increased secretion of corticotropin-releasing factor (CRF) in the hypothalamus and causes increased levels of glucocorticoids both in the brain and peripherally, being translated into increased mitochondrial activity (2).

In the mitochondria, glucocorticoids form a complex with the antiapoptotic protein Bcl-2 to inhibit the formation of Bax-containing pores on the mitochondrial outer membrane. They also reduce the release of Ca^{2+} and cytochrome c from the mitochondria, inhibiting apoptosis (134, 161). Nevertheless, a chronic increase in glucocorticoid levels can cause neuronal toxicity and respiratory chain dysfunction, excessive ROS generation, apoptosis, and cell death in skeletal muscle cells and hippocampus (134, 162). Studies in rats treated with lipopolysaccharide have found sex-specific alterations in glucocorticoid receptors, which could be explained by changes in inflammation-induced expression of genes involved in oxidative metabolism (15, 163, 164).

Glutamate and Dopamine in Mitochondrial Dysfunction

Glutamate

Glutamate is implicated in mood disorders. Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, causes a rapid antidepressant effect in patients with MDD (165, 166). This effect might be due to increased BDNF expression (134, 163), modulation by 5-HT receptors, and interactions with inflammatory processes (134, 164). Glutamate levels are higher in brains of patients with mania than other phases of illness, suggestive of dysfunction of the glutamatergic system. In depression, astrocyte density is reduced and, as a consequence, the ratio of glutamine to glutamate is not properly maintained by the glutamate recycling pathway (134, 167). Moreover, mitochondrial energy production is reduced in glutamatergic neurons in patients with MDD (168). High glutamate levels and consequently high intracellular Ca^{2+} levels promote apoptosis. On the other hand, activation of glutamate receptors also stimulates ATP production, and expression of p53, which can produce an increase of mitochondrial respiration, production of ROS and reduction of GSH (169).

Dopamine

Increased dopamine levels have been noted in mania. Some studies report lower dopamine transporter (DAT) binding in the striatum in unmedicated depressed or euthymic bipolar patients (2). Higher dopamine transmission and impaired DAT

function in mania could be explained by elevated oxidative and nitrosative stress, which is higher in mania than in other phases of illness (170). Excessive dopamine levels in mania can also cause damage to nuclear and mtDNA by chronic nitrosative and oxidative stress. However, this is repaired by high dopamine and uric acid levels, which act in a synergistic way to repair free radical-mediated damage (76). In this environment, dopamine can protect neurons against glutamate-induced excitotoxicity, stimulate oxidative phosphorylation, and activate p53, which induces anti-apoptotic activity and inactivates tyrosine hydroxylase, which is necessary for the synthesis of dopamine. Consequently, high dopamine and glutamate levels together with high uric acid levels may not have the expected detrimental effects. Moreover, pro-apoptotic signals may induce the expression of anti-apoptotic genes such as BCL-2, inhibiting the protein Bcl-2 toxicity and apoptosis and stimulating oxidative phosphorylation (76).

All this evidence suggests that changes in mitochondrial function in MDD and BD could be key elements in order to better understand the role of the currently used pharmacotherapy and also to develop novel therapies and new treatment strategies, which will be covered in the next section.

Take-Home Message

Mitochondrial dysfunction may result from different causes, being some of the alterations related with several network processes in which mitochondria are indispensable.

Some of the changes observed in mood disorders include alterations in mitochondrial neurometabolites and metabolic dysfunction, decreased expression and activity of the ETC complexes, increased oxidative damage, altered calcium homeostasis, and changes in mitochondrial morphology, distribution, and degradation. In addition, increased apoptosis, chronic inflammation, Increased expression of mitochondrial fission genes and other genetic changes, including polymorphisms in clock genes, have been observed in mood disorders. Increased mtDNA degradation, purinergic dysfunction, and hyperactivity of the HPA axis, with higher glucocorticoid levels, are other findings reported in mood disorders. Finally, increased glutamate and dopamine levels have been reported in manic episodes. Nevertheless, current evidence is scarce and further studies are needed to assess these changes in mood disorders.

EFFECTS OF PHARMACOTHERAPY ON MITOCHONDRIAL FUNCTIONS

Since mitochondrial dysfunction has been related to the pathophysiology of mood disorders, including factors such as increased oxidative stress, decreased ATP production, and dysregulation of Ca^{2+} homeostasis (2), numerous studies have focused on their role as possible drug targets for pharmacological treatments (171, 172). In this regard, conventional psychotropic drugs for mood disorders, including mood stabilizers, antidepressants, and antipsychotics, have demonstrated to have molecular mitochondrial properties, such as neuroprotection, enhancement of mitochondrial function or prevention of cellular apoptosis (173), illustrated in **Table 1**. In addition, novel interventions are being studied and developed to be used as adjunctive therapies for mood disorders, as noted in **Table 2**.

Mood-Stabilizing Drugs

Mood stabilizers are considered first-line drugs in BD to either treat mood episodes or to prevent future recurrences (214). Although the mechanism of action of mood-stabilizing drugs is

TABLE 1 | Effects of conventional pharmacotherapy on mitochondrial functions.

	Molecular mitochondrial properties	**Clinical properties**	
	Neuronal survival	**Inflammation and oxidative/nitrosative stress**	
Mood stabilizers			
Lithium (174–177)	Reduces apoptosis*	Prevents excessive mitochondrial calcium influx*	Mood-stabilizing properties in BD and antidepressant properties in MDD
	Enhanced neuroprotection and neurotrophism	Reduces oxidative stress*	
	Reduced cortical atrophy in BD	Antioxidant effect*	
Valproic acid (178, 179)	Reduces apoptosis*	Reduces oxidative stress in mitochondria*	Mood-stabilizing properties in BD
		Antioxidant effect*	
Antidepressants (99, 180, 181)	Reduce apoptosis*	Increase mitochondrial biogenesis*	Antidepressant properties in BD
	Enhanced neurotrophism	Reduce oxidative stress (mitochondrial and peripheral)*	Risk of manic switch
Antipsychotics (182–185)		Reduce oxidative stress in brain mitochondria*	Antimanic and mood-stabilizing properties in BD

*All data represents human clinical studies unless explicitly stated in table (*animal studies).*
BD, bipolar disorder; MDD, major depressive disorder.

TABLE 2 | Effects of novel therapies on mitochondrial function.

Novel therapies	Molecular mitochondrial properties		Clinical properties
	Neuronal survival	**Inflammation and oxidative/nitrosative stress**	
Pramipexole (186, 187)			Antidepressant efficacy in treatment-resistant BD
Nutraceuticals			
N-acetylcysteine (188–190)		Reduces oxidative stress (in brain and periphery)*	Improves depressive and reduces manic symptoms
Omega-3 fatty acids (191, 192)		Reduce oxidative stress	Better functioning in BD
		Increase antioxidants	Improve depressive symptoms
Alpha-lipoic acid (193–196)	Reduces apoptosis*	Reduces oxidative stress*	Reverses and prevents amphetamine-induced behavioral and neurochemical alterations*
	Enhanced neuroprotection*		
Acetyl-L-carnitine (194–196)	Reduces apoptosis*		Improvements in depressive disorders
	Enhanced neuroprotection*		
S-Adenosylmethionine (197–199)		Reduces oxidative stress*	Improvements if supplemented in depressive disorders
			Potential risk of manic switch in BD (one study)
Creatine monohydrate (200)			Improvements in depressive symptoms
			Potential risk of manic switch in BD (one study)
Leucine, isoleucine, and valine (201)			Reduction in manic severity (one study)
L-tryptophan (202)			Reduction of manic symptoms
			Potential risk of depressive switch in BD (one study)
Carnosine (203, 204)		Reduces oxidative stress*	Improvement of behavior, cognition, and overall well-being
Inositol (205, 206)			Improvements in depressive symptoms in BD
Coenzyme Q10 (207)		Reduces oxidative stress	Improvements in depressive symptoms and functioning in BD
Melatonin (208–210)		Increases BDNF and ERK1/2*	Improvements in depressive symptoms. Scarce effects proven in BD.
		Reduces peripheral oxidative stress*	
Vitamin C and E (211)			Improve severity in depression
Vitamin B3 (211)		Reduces oxidative stress*	Enhances social behavior*
Folic acid (212)		Reduces oxidative stress*	Reduction in manic symptoms
Ketogenic diet (213)			Reports on mood stabilization

*All data represents human clinical studies unless explicitly stated in table (*animal studies).*
BD, bipolar disorder; BDNF, brain-derived neurotrophic factor; ERK1/2, extracellular signal regulated kinases.

not clear, some studies suggest that mitochondrial dysfunction and oxidative stress may be therapeutic targets of these drugs.

Some studies have reported that mood stabilizers, apart from altering glutamatergic neurotransmission, decrease intracellular pH, increase expression of the anti-apoptotic gene BCL-2 (by blocking the inhibition produced by histones), regulate expression of other genes, reduce elevated intracellular Ca^{2+} and increase Ca^{2+} storage capacity in the ER, and also induce mitochondrial migration to synaptic terminals, modulating neurotransmission (174, 215–217). These findings in aggregate suggest that they may reduce the symptoms of mood disorders at least in part by augmenting mitochondrial activity (63, 214). Lithium and valproate inhibit glutamate-induced apoptosis and oxidative damage to lipid and protein in cerebral cortical cells (218, 219). They also inhibit cytochrome c release from mitochondria. This reduces oxidative stress by stabilizing the inner transmembrane potential of mitochondria and prevents caspase-2 and caspase-3 activation, and cell death (174). Chronic

treatment with lithium and valproate has also been shown to inhibit amphetamine-induced hyperactivity (24, 219). Lithium, valproate and carbamazepine are known to reduce inositol levels (220) and augment autophagy in cell cultures (221).

Other animal studies on the antioxidative properties of mood stabilizers have shown that chronic treatment with valproate or lithium is associated with increased ER stress proteins and related proteins, such as calreticulin (222), in cortical and PC12 cells (215, 223). These proteins are involved in antioxidative effects and mitochondrial functioning (216, 224). Moreover, lithium and valproate have other neuroprotective functions, such as regulation of the expression of GST isoenzymes in cerebral cortex, which is a group of detoxification enzymes that inhibit oxidative damage to lipid and protein in cerebral cortical cells, and GSH levels, accelerating conjugation processes (225, 226). However, with low levels of the rate-limiting synthesis enzyme (glutamate-cysteine ligase) and low levels of GSH, lithium and valproate neuroprotective effects are inhibited, which indicates that adequate GSH levels could be important for efficacy (227, 228). Other studies have shown increased oxidative damage to lipids in patients with BD, with higher lipid peroxidation ameliorated after mood-stabilizer treatment, which supports the previous findings (24, 229). Together this evidence indicates that mood stabilizers may reduce the symptoms of BD by enhancing mitochondrial activity (24).

Lithium

Apart from its neuroprotective functions mentioned above, lithium ameliorates BD associated cortical atrophy and maintains cortical thickness (219). Regarding Ca^{2+} regulation, lithium prevents excessive Ca^{2+} influx triggered by the N-methyl-D-aspartate receptor. In contrast, animal studies suggest that lithium can allow an increase of Ca^{2+} concentrations by desensitizing mitochondria against Ca^{2+}, preventing a further response leading to apoptosis. Lithium is also correlated with increased activity of complexes I, II and III, enhances the expression of the scavenger glutathione transferase (184), facilitates mitochondrial respiration, and has other antiapoptotic properties previously mentioned (177, 230).

In rat studies lithium has been shown to inhibit inflammatory signaling pathways related to toll-like receptor 4 (TLR4), which may reduce phosphorylation of NFkβ, reducing inflammatory gene expression and also levels of caspase-3, which might prevent neuronal apoptosis (175, 231). Other studies have shown that the c-Jun N-terminal kinase, which is known to mediate oxidative toxicity, is inhibited by lithium (184, 232). Apart from enhancing this antioxidant defense, lithium has also been reported to increase the activity of SOD (233), of GPx, and total antioxidant reactivity levels in the brain (234).

Lithium also inhibits GSK-3b, a phosphorylating kinase that inhibits the conversion of pyruvate into acetyl-CoA by pyruvate dehydrogenase and also activates BAX (235). Its mutations have been shown to alter lithium response in BD. The inhibition produced by lithium may enhance ATP production and inhibit apoptosis (235, 236). Moreover, in rat models where amphetamines were used, GSK-3b was shown to enhance dopamine activation, leading to the hypothesis that lithium may contribute to maintaining normal levels of dopamine (94).

GSK-3b and phosphoinositide signaling pathways regulate BDNF, which has a complex role in mood disorders. Nevertheless, it is thought to be a potential drug target, since neurotrophic effects of lithium have been related to the increase in hippocampal BDNF in the presence of a neurotoxic insult (235, 237). Phosphoinositol is increased in patients with BD in the central nervous system and is reduced by lithium (184), causing lower levels of myoinositol in this group of patients (238, 239). In contrast, few animal studies have shown contrary effects of lithium treatment, such as the lowering of complex II and IV activity and enhanced ROS formation (240), with reduced antioxidant levels (241). It has also been shown to enhance the activity of caspase-3, leading to apoptosis (242).

Valproic Acid

In rats, valproate has shown to lower amphetamine-induced citrate synthase and to inhibit succinate dehydrogenase, thought to be related to its mood-stabilizing effects (243). Valproate also protects mitochondria from ouabain-induced lipid peroxidation and superoxide formation (176, 244). It could act as a cytoprotective agent in the presence of cytotoxic factors, but alone could inhibit mitochondrial functions (245, 246).

Nevertheless, other studies have shown valproate lowers levels of some cofactors, such as creatine and CoA, involved in the uptake of long-chain fatty acids into mitochondria, which leads to reduced beta-oxidation (214). Valproate has also been shown to enhance ROS generation by inhibition of complex II, and to induce mPTP opening, with a reduction of membrane potential, leading to the release of cytochrome c and apoptosis. Valproate inhibits ATP synthesis when pyruvate is used as a substrate (214). In rat studies, valproate has shown to inhibit glutamate-driven oxidative phosphorylation (247, 248). In cases of impaired ETC structure, valproate inhibits complexes I and IV activity and SOD levels (179).

Other Mood Stabilizers

Although there is scant evidence regarding other mood stabilizers apart from lithium and valproate, changes found in animal studies with carbamazepine treatment include the reduction in mitochondrial respiration, ATP synthesis, and membrane potential, and also the inhibition of Ca^{2+}-induced swelling of liver cells (241, 249, 250). Lamotrigine has been shown to inhibit the effects of rotenone, a cytotoxic agent, and maintain mitochondrial membrane potential, preventing mPTP opening and increasing GSH levels (251). Its neuroprotective effects could be due to complex I inhibition (252).

Antidepressants

In animal models of depression (214, 253), antidepressants seem to increase mitochondrial biogenesis and enhance antioxidative capacity against oxidative stress (139, 184). For instance, venlafaxine increases expression of anti-apoptotic and antioxidant mitochondrial genes (254), and agomelatine may similarly scavenge free radicals (11, 255). As with electroconvulsive therapy, they reduce peripheral

inflammatory cytokines (33), which is supported by the reported antidepressant activity of celecoxib, a cyclooxygenase 2 (COX-2) inhibitor (256, 257). Antidepressants also increase autophagy and neural plasticity (134, 258). Since mitochondrial dysfunction has been linked to mental disorders (184), cytochrome c oxidase and apoptosis inhibition have been studied as potential new treatment approaches (259).

In patients treated with antidepressants, there is an increase in the levels of BDNF mRNA (260) and a reversion of the decrease in CREB levels seen in patients with depression (261), which could be mechanisms of action in mood disorders (262). Animal studies have also shown that some antidepressants inhibit complex I in brain mitochondria, reducing its metabolic function (263). A number of studies have shown that some antidepressants, including fluvoxamine, fluoxetine, sertraline, paroxetine, nortriptyline, and venlafaxine, alter ETC activity in mitochondria (241, 264). The reduction of ROS production could explain their beneficial effects (265–267).

Fluoxetine also promotes cytochrome c oxidase and glutamate dehydrogenase activity in presynaptic mitochondria of rat hippocampus (160, 268), and inhibits multiple other enzymes in mitochondria (11, 181). Increased cytochrome c oxidase activity in the female hippocampus by fluoxetine could improve outcomes in women (269–271). Apart from altering mitochondrial energy production, fluoxetine might affect the mitochondrial processes *via* the glucocorticoid receptor (GR) (33, 272).

Antidepressants are also involved in apoptosis, playing a complex role that depends on cell and brain structure type. One study reported that paroxetine, fluoxetine and clomipramine increased levels of apoptotic markers (cytochrome c and DNA fragments), but imipramine did not have any effect (24, 273). Desipramine induced apoptosis by activating the caspase pathway in glioma cells (274), while fluoxetine and amitriptyline protected PC12 cells from cell death (275). Nortriptyline inhibited neuronal cell death, protecting isolated mitochondria against programmed cell death, inhibiting the release of apoptotic mitochondrial factors and caspases (276). Fluoxetine has been shown to prevent stress-induced apoptosis in the hippocampus, but not in the prefrontal cortex (277, 278). In summary, different mitochondrial functions, such as ATP synthesis, generation of ROS, and cell death, are important targets of antidepressants.

Antipsychotics

Few studies have explored mitochondrial modulation by antipsychotics (279, 280). Olanzapine has shown to increase SOD activity and protect PC12 cells from oxidative damage by H_2O_2 (20, 281), and also to prevent the decrease in membrane potential and ROS overproduction induced by beta-amyloid peptide (282). In two mice studies, quetiapine increased mitochondrial ETC activity and reduced markers of oxidative stress in the prefrontal cortex, nucleus accumbens, amygdala, and hippocampus (24, 282–284). Scaini et al. [183, 185, 186) showed a significant decrease in all the functional parameters of mitochondrial oxygen consumption after treatment with clozapine and olanzapine in lymphoblastoid cell lines (LCLs) from healthy controls, and these effects were more prominent in cells treated with olanzapine. The same authors also demonstrated that the treatment with clozapine and olanzapine at high doses further decreased mRNA expression of Mfn-2 and Drp-1 in LCLs, supporting the notion that clozapine and olanzapine can potentiate mitochondrial dysfunction.

Novel Therapies

In the last years, a number of agents have been studied as potential therapeutic factors aimed to treat and improve the course of mood disorders, including factors involved in the glutamatergic pathway, insulin transduction pathway, melatoninergic system, purinergic system, endopeptidases, and also mitochondrial modulators (183). A number of the latter agents have been developed or studied with the aim of enhancing antioxidant defenses or mitochondrial functioning as adjuvant therapy to antidepressants (285).

Studies of Ca^{2+} channel blockers, such as diltiazem and verapamil, have been conducted as potential treatments for BD, but results in the literature are still controversial. It was hypothesized that their therapeutic effect may be due to the protection of neurons against the damage induced by excessive Ca^{2+} levels (2).

Pramipexole, a D2/D3 agonist approved for the treatment of Parkinson's disease and restless legs syndrome, upregulates Bcl-2 (286). It has also shown antidepressant efficacy in treatment-resistant bipolar patients (287), with a superior response rate compared to placebo and similar to SSRIs.

Some dietary supplements (or nutraceuticals) have been assessed as potential treatments in mood disorders (186, 187), since they may enhance mitochondrial function and brain energy metabolism and prevent ROS-induced damage. These include N-acetylcysteine (NAC), alpha-lipoic acid (ALA), acetyl-L-carnitine (ALCAR), S-adenosylmethionine (SAMe), coenzyme Q10 (CoQ10), creatine monohydrate (CM), and melatonin (201).

The molecular mitochondrial properties shown by novel therapies for mood disorders are summarized in **Table 2**.

Nutraceuticals

N-Acetylcysteine

N-acetylcysteine (NAC) is a GSH precursor, the major antioxidant agent in the brain (288) for preventing oxidative damage in the mitochondrial ETC (289). By increasing GSH levels, NAC may increase mitochondrial respiratory capacity and have neuroprotective functions by other mechanisms (285, 290). It can prevent oxidative damage to complex I (184), can enhance GST activity, and act directly against oxidant radicals (290, 291).

Some studies in BD have demonstrated that treatment with NAC can improve depressive symptoms, clinical response rates, symptom remission, quality of life and functioning (292, 293). Few clinical trials assessing the efficacy of NAC as adjunctive treatment in patients with BD have shown promising results (189, 281, 289, 294–298), with benefits in depressive symptoms of BD patients (63, 189, 285, 289), but not as maintenance treatment (189, 294). *Post-hoc* analyses suggested that NAC might be effective in later stages of BD (289) and also to reduce manic symptoms (281). However, there are recent negative trials, albeit smaller and of a shorter duration (298).

Clinical trials in depressive disorders also suggest the potential of NAC as adjunctive treatment in depression (299, 300). Although no differences in depressive symptoms were found in another clinical trial comparing NAC with placebo (189, 301), the NAC group showed a better response at the 16-week post-discontinuation endpoint. A meta-analysis including five studies assessing depressive symptoms with a follow-up of 12–24 weeks revealed significantly greater improvements in depressive symptoms and functionality with NAC compared to placebo (190).

Omega-3 Fatty Acids

Some studies have demonstrated modulatory effects of omega-3 fatty acids on mitochondria. Eicosapentaenoic acid (EPA; 20:5n-3) is a fatty acid that seems to protect against oxidative stress by replenishing oxidized lipids and increasing oxygen and glucose supply to the brain (301). Diets rich in omega-3 fatty acids have shown to upregulate cytochrome c oxidase, cytochrome b, and ATP synthases, leading to increased ATP formation (302, 303).

In a study of rodents with methylphenidate-induced mania, omega-3 fatty acids alone and in combination with lithium and aripiprazole reduced levels of SOD, CAT, and lipid peroxidation products (304). Stanley et al. (63, 305) demonstrated that docosahexaenoic acid (DHA; 22:6n-3) changes mitochondrial membrane phospholipid composition and mitochondrial function, protecting mitochondria against damage (306). A pilot study found significantly higher remission, greater improvements in depressive symptoms, and better global functioning in bipolar patients supplemented with omega-3 fatty acids, while no benefits in mania were found (306). Clinical improvements associated with omega-3 fatty acids intake are produced at least in part by modulation of BDNF levels (192). A study that included 10 different countries found a correlation between lower fish or seafood consumption with a higher prevalence of bipolar spectrum disorders (307, 308). Patients with BD have shown lower levels of erythrocyte DHA, ALA and EPA when compared to healthy controls (63, 309). Another study showed a trend toward lower levels of omega-3 fatty acids in relatives of patients with BD (191).

A systematic review of clinical trials assessing nutraceuticals showed positive and statistically significant results on depression in four out of nine studies (201), but none showed positive findings in mania. However, sample sizes were small, reducing the chance of positive results (192, 310). The previous evidence suggests that supplementation or increased consumption of omega-3 fatty acids may be beneficial in mood disorders, but additional studies are necessary to define their clinical efficacy more accurately.

Alpha-Lipoic Acid

Alpha-lipoic acid (ALA) is an antioxidant found in red meats, spinach, yeast, and other products (201). It facilitates glucose entrance into cells for ATP synthesis and recycling of endogenous antioxidants, such as CoQ10, vitamins C and E, and GSH (193). ALA has been demonstrated to reduce metabolic deficits, oxidative stress and apoptosis (by preventing glutamate-induced Ca^{2+} cellular influx) (311, 312), stimulate glucose uptake into cells, improve cognitive function and enhance neuroprotection (312, 313) and to stimulate mitochondrial biogenesis (195), but studies in mood disorders are lacking (312).

Acetyl-L-Carnitine

L-carnitine (ALCAR) is a compound obtained through the diet (314) that is biosynthesised from lysine and methionine. It enhances the entrance of fatty acids into the mitochondria for ROS scavenging and beta-oxidation, which leads to ATP and acyl-coenzyme A (acyl-CoA) production (315). Acyl-CoA enters the citric acid cycle (316, 317). Reported functions of ALCAR include neuroprotection, anti-apoptotic properties (315), inhibition of GABA production (316, 318), and enhancing of mitochondrial functioning (319). Animal studies of ALCAR show increased levels of ATP and PCr (195). Results regarding the reduction of oxidative stress are mixed, but coadministration with NAC or ALA has shown benefits (320). Moreover, supplementation with ALA and ALCAR may promote mitochondrial integrity in the hippocampus of aged rats (321). Some early clinical trials suggest the ALCAR has significantly greater efficacy than placebo as an augmentation treatment depressive disorders (63, 194). However, another study found no significant differences in depressive scores of ALCAR/ALA treatment compared to placebo (322–324).

S-Adenosylmethionine

S-Adenosylmethionine (SAMe) is formed from ATP and methionine and is needed for the synthesis of many neurotransmitters and for repairment and degradation of dysfunctional proteins. It is a precursor for GSH production, which plays an important role in reducing oxidative stress. It is also used for homocysteine synthesis, which in turn can regenerate SAMe (196).

Some studies suggest the efficacy of SAMe supplementation for depressive episodes as adjunctive therapy with a number of antidepressants SSRI, venlafaxine, or SNRIs (288). Nevertheless, studies with older antidepressants, including phenelzine, mianserin, and maprotiline, showed inconsistent results (197, 199, 325–327). One study in patients with BD showed SAMe might pose a risk for a manic switch (288), but randomized clinical trials in BD are still lacking (328). A recent large scale trial showed a numerical but not statistically significant benefit of SAMe in depression (198).

Creatine Monohydrate

Creatine is an antioxidant agent synthesized by the liver and kidneys which is also found in meat and fish (288). It can be obtained as a supplement in the form of creatine monohydrate (CM). Creatine is the precursor of PCr, a reservoir of inorganic phosphate, used for ATP synthesis by donating a phosphate to ADP (329). In the context of high-energy demand, PCr is rapidly converted to creatine to donate a high-energy phosphate to ADP to obtain ATP (330).

Creatine also attenuates the decreases in N-acetyl-aspartate (NAA), which acts as a marker of impaired mitochondrial function, and inhibits the activation of the mitochondrial permeability transition, suggesting neuroprotective effects

(331, 332). Other neuroprotective effects are intracellular Ca^{2+} buffering, extracellular glutamate reduction, and antioxidant effects (333–335). PCr and NAA concentrations are reduced in BD patients and this reduction correlates with clinical severity (336). Where PCr levels are diminished, CM supplementation may increase PCr and NAA production to promote neuroprotection (70, 337).

Despite limited evidence in mood disorders, CM has been associated with improvement in depressive symptoms in case studies (63). Benefits were seen in treatment-resistant depression in a small open-label study (338), which also suggested a risk of a manic switch after CM treatment in patients with BD (200). Before CM might be considered as an adjunctive treatment for the management of BD and depressive disorders, RCTs are necessary.

Other Aminoacids

In one study assessing the efficacy of leucine, isoleucine, and valine combination vs. placebo in 25 patients with BD, positive results were seen with significant reductions of the severity of mania within 6 h in the verum group, whose activity may be explained by competitive inhibition of phenylalanine and tyrosine, which are necessary for dopamine synthesis (288). L-tryptophan reduced manic symptoms in a study of 24 patients (339, 340). Moreover, a meta-analysis reported significantly reduced plasma tryptophan levels in patients with MDD (202). This aminoacid was shown to reduce depression scores in people with unipolar depression in methodologically limited studies, so further evidence is required in order to consider it as an adjunctive therapy (341).

Carnosine is a dipeptide made up of the amino acids beta-alanine and histidine that protects brain mitochondria and regulates the immune system (201, 342). It has also been studied for adjuvant treatment of depression (343, 344). So far, it has been demonstrated to reduce the effects of chronic stress in animal studies and to improve behavior, cognition, and overall well-being in human studies (204, 345).

Inositol

A pilot study using inositol, a glucose isomer, in 24 patients with BD found a significant reduction in depression scores after 3 weeks of treatment but not after 6 weeks (2, 203). Another 6-week study in 17 subjects with BD showed no significant reduction in depression or mania scores (206). Notwithstanding, both studies found a greater clinical response with inositol compared to controls, which suggests a potential benefit of this agent in BD (205).

Coenzyme Q10

Coenzyme Q10 is a component of the ETC complex involved in ATP synthesis (201). It acts as an antioxidant in mitochondria and lipid membranes (346). CoQ10 has been suggested to stabilize the mitochondrial membrane in the context of oxidative stress (63, 347). It also inhibits the activity of mPTP and increases complex I activity (348). One open-label placebo-controlled trial reported clinical improvement in depressive symptoms in older adults with bipolar depression using this supplement (63).

A randomized controlled trial comparing nutraceutical treatment (including ALC, CoQ10 and ALA, in addition to co-factors involved in mitochondrial function) with NAC and placebo in patients with depression did not show a significant difference between groups at the primary endpoint. However, the rate of change between baseline and week 20 post-discontinuation was significantly greater in the group previously treated with nutraceuticals compared with the placebo group on depression scores, and also on functioning. This suggests a delayed benefit of the combination or improvement of symptoms on withdrawal, which should be assessed in future studies (285). Thus, current evidence suggests that CoQ10 might be beneficial in mood disorders (288), but further clinical trials in mood disorders are necessary to confirm these early promising but non-definitive signals.

Melatonin

Melatonin is a hormone released in a circadian pattern by the pineal gland and other tissues in the body, including the brain. It has a number of functions and is an important antioxidant free radical scavenger (207, 288). Specifically, it stimulates the production of GSH (349) and increases the expression of genes related to antioxidative functions, such as glutathione peroxidase and SOD (210). Melatonin also seems to directly enhance mitochondrial function since it activates ETC complexes, increases mitochondrial membrane fluidity, and closes the mPTP. It also protects mtDNA against degradation, promotes the expression of mitochondrial genes coding for ETC complex subunits, and has neuroprotective properties (209). The beneficial effects of melatonin seem to be those related to ROS scavenging and actions linked to cytosolic proteins (209, 288).

Clinical studies in mood disorders do not show conclusive results. So far, melatonin has shown benefits improving depressive symptoms in patients with "winter depression" (209, 288) compared to placebo, whereas a controlled study in seasonal affective disorder did not show changes in atypical depressive symptoms (208), and a crossover study on patients with severe depression showed that patients taking melatonin had worsened dysphoria, sleeping patterns and weight gain (350).

Regarding evidence assessing melatonin for BD, one open-label study showed no significant effects on mood or sleep in rapid-cycling patients (351), whereas in another small open-label study it showed sleep-enhancing and antimanic effects in manic patients (352). As mentioned previously, agomelatine, an agonist of melatonin MT1 and MT2 receptors, has demonstrated preliminary evidence of efficacy in bipolar depression (353), but agomelatine has other actions on the serotonin system.

Vitamins and Minerals

Supplementation with vitamins C and E was shown to significantly improve severity in depression. One study where they were combined with monoaminergic antidepressants for 12 weeks showed they improved oxidative stress in subjects with MDD (354), but it was not a placebo-controlled design. Nicotinamide is a form of vitamin B_3 found in food, used as an antioxidative substance, and is also a precursor of NAD+. It is hypothesized to be effective for the treatment of mood disorders

(211), since it increases oxidative phosphorylation in the brain and enhances social behavior in high-anxiety rats (355). A small clinical trial evaluating magnesium as a potential adjunctive therapy for treating acute mania or rapid cycling BD showed a greater reduction of manic symptoms compared to controls (356), which might be due to the modulation of Ca^{2+} channel activity and its participation in neurotransmitter release (357). One study using folic acid for 17 BD participants showed no statistically significant differences on symptoms of depression compared to controls (201, 358), but in another where 88 patients with acute mania were initiated on valproate, folic acid at doses of 3 g showed a significant reduction of manic symptoms at week 3 compared to placebo (359). Thus, assessing folate levels and administering supplementation in patients with mood disorders could be beneficial for the clinical course of the acute episodes.

Ketogenic Diet

The effects of specific diets in mood disorders are still not clear despite evidence reporting that they can alter several biological processes. The exception is the Mediterranean diet, which has been associated with antioxidative properties and has shown antidepressant effects in a RCT (201, 360). One study assessing rats on a calorie-restricted diet showed that mitochondrial efficiency and oxidative damage in skeletal muscle were significantly increased in these rats, while antioxidant effects were significantly lowered in food-restricted rats that followed a high-fat diet. Thus, caloric restriction seems to predispose to higher mitochondrial efficiency and also to high-fat induced oxidative damage (361). Other studies have shown that the ketogenic diet (KD) upregulates mitochondrial antioxidant status and protects mtDNA from oxidant-induced damage (362). It has also shown effective anticonvulsant properties and has been suggested as a potential adjunctive therapy as a mood stabilizer.

The ketogenic diet consists of a low-carbohydrate diet that substantially changes the energetic source of the organism (213, 363), which switches from glucose to ketones bodies, obtained by breakdown of fatty acids. This causes alterations in neurotransmitter levels, hormones, and peptides (364), and an increase in oxidative phosphorylation and ATP synthesis (365, 366), increased GSH levels, reduced ROS production (367), reduced inflammatory levels and neuroprotection (368). A ketogenic diet seems to influence epigenetic changes involved in increased mitochondrial function and biogenesis (369), which might also be responsible for the increase of BDNF (370). The ketogenic diet stimulates the endogenous antioxidant system through the activation of nuclear factor erythroid-derived 2 (NF-E2)-related factor 2 (Nrf2), the major inducer of detoxification genes (371, 372), especially in the hippocampus (373). Despite the limited data regarding the ketogenic diet for the treatment of mood disorders, early reports support the hypothesis about its beneficial effects on mood stabilization (374).

Physical Exercise

Physical activity is directly related to increased mitochondrial biogenesis, increased mitochondrial content and oxygen utilization capabilities, and that aerobic exercise in the elderly ameliorates loss of skeletal muscle mitochondrial content (369). One study assessing the efficacy of fluoxetine and exercise in muscle cells of rats reported that physical activity increased cytochrome c oxidase activity compared with the group treated only with fluoxetine. Exercise increased citrate synthase activity in both fluoxetine and non-fluoxetine groups, and fluoxetine increased its activity only in the exercise group. On the other hand, exercise significantly decreased ROS levels in both fluoxetine and non-fluoxetine groups, with this reduction higher in the fluoxetine group (375). *Post-hoc* analysis of a trial of a mitochondrial combination therapy found the greatest benefits in those with the highest levels of physical activity (376). There is a meta-analytic level of evidence from RCT's that exercise has antidepressant effects. Hence, enhancing mitochondrial function through physical activity may provide a novel way to treat mood disorders (377).

Take-Home Message

Numerous studies have focused on the role of therapeutic agents targeting different mitochondrial functions that are altered in mood disorders. On one hand, mood stabilizers, antidepressants and antipsychotics have shown to promote neuroprotection, reduce oxidative stress and enhance mitochondrial function. On the other hand, novel interventions have been assessed as potential adjunctive therapies for mood disorders.

Some mitochondrial modulators have been developed or studied with the aim of enhancing antioxidant defenses or mitochondrial functioning as adjuvant therapies in mood disorders. Pramipexole has shown antidepressant effects by the upregulation of Bcl-2. Some dietary supplements or nutraceuticals have been found to enhance mitochondrial function and brain energy metabolism mainly by the reduction of oxidative stress. These include N-acetylcysteine (NAC), alpha-lipoic acid (ALA), acetyl-L-carnitine (ALCAR), S-adenosylmethionine (SAMe), coenzyme Q10 (CoQ10), creatine monohydrate (CM), and melatonin. Even though current evidence suggests they might be beneficial in mood disorders, further clinical trials are necessary to confirm these findings.

Melatonin has antioxidative functions and also enhances mitochondrial function. However, clinical studies in mood disorders have not shown positive results. Vitamin supplementation, ketogenic diet and physical exercise have also shown positive effects in mitochondrial function and mood disorders, with scarce evidence.

CONCLUSION

Mitochondria play a key role in different cellular functions, especially those related to energy production. A number of studies indicate the possible role of mitochondria in the pathophysiology of mood disorders, raising the possibility that the processes of energy generation and oxidative damage could be significant therapeutic targets for the treatment of BD with mood-stabilizing or other kinds of drugs as well as lifestyle approaches. A better knowledge of mitochondrial functioning could help identify impaired processes and specific treatment targets. This would increase the understanding of mechanisms of action of the drugs currently used and aid the development of

novel effective therapies to treat specific mitochondrial functions that might be used as the main therapy or as adjunctive treatment, especially for subjects that do not fully respond to conventional therapies. Research on changes in mitochondrial processes in patients with mood disorders might clarify how mitochondrial dysfunction can be considered a biological target. Further studies are needed to confirm that pharmacological treatments reduce or delay neuroprogressive changes in mood disorders, and to demonstrate the potential benefits of putative antioxidant substances.

AUTHOR CONTRIBUTIONS

All authors contributed in the preparation of the manuscript and gave approval for the final version.

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Efficacy of Sertraline Plus Placebo or Add-On Celecoxib in Major Depressive Disorder: Macrophage Migration Inhibitory Factor as a Promising Biomarker for Remission After Sertraline—Results From a Randomized Controlled Clinical Trial

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Introduction: Previous research delivers strong indications that inflammatory activation leads to treatment resistance in a subgroup of patients with Major Depressive Disorder (MDD). Thus, tailored interventions are needed. The present study aimed to find potential biomarkers that may enable patients to be stratified according to immune activation.

Methods: A phase IIa randomized placebo-controlled trial was performed to assess levels of inflammatory compounds in responders/remitters and non-responders/non-remitters to sertraline plus celecoxib ($n = 20$) and sertraline plus placebo ($n = 23$). Levels of macrophage migration inhibitory factor, neopterin, and tumor necrosis factor alpha were determined by enzyme-linked immunosorbent assay; response and remission were measured by reduction of the Montgomery Åsberg Depression Rating Scale score.

Results: Both treatment groups showed a significant decline in depression symptoms, but no difference was found between groups. A clear pattern emerged only for macrophage migration inhibitory factor: placebo remitters showed significantly lower baseline levels than non-remitters (a similar trend was seen in responders and non-responders) while celecoxib responders showed a trend for higher baseline levels than non-responders.

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Conclusion: Small subsample sizes are a notable limitation, wherefore results are preliminary. However, the present study provides novel insights by suggesting macrophage migration inhibitory factor as a promising biomarker for treatment choice.

The trial was registered in EU Clinical Trials Register (EU-CTR): https://www.clinicaltrialsregister.eu/ctr-search/trial/2009-011990-34/DE, EudraCT-No.: 2009-011990-34.

Keywords: inflammatory, Major depressive disorder, cytokine, response, biomarker, anti-inflammatory treatment

INTRODUCTION

In clinical practice, patients' response to antidepressant treatment often remains unsatisfactory. Around 20% up to 50% of depressed patients show non-response to at least two standard antidepressant drug trials (1, 2). Furthermore, remission rates across different antidepressant treatment options are at 28% after initial treatment attempt and remission rates further decrease with each treatment failure (3). Thus, identifying patients prone to treatment resistance is important to enable early use of alternative treatment options. Previous research has consistently shown that inflammatory activation plays a role in the pathophysiology of Major Depressive Disorder (MDD) and pro-inflammatory activation has been implicated in treatment resistance to standard antidepressant medication in several studies (4). Results indicate that low-grade inflammation is present in a subgroup of MDD patients (5, 6) characterized by higher levels of circulating pro-inflammatory compounds (7–9). Overall, higher levels of these compounds were associated with depression, though results of single study show some variety (10–13). In particular, compounds such as C-reactive protein (CRP), Interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) have been frequently described. Deficits in the T cell system and pro-inflammatory monocyte activation were also shown to be present in MDD patients (14–17).

Most studies investigate inflammatory markers from the periphery, while depressive symptoms result from dysregulations in the brain. Peripheral cytokines activate afferent nerves to the brain and can enter the brain themselves leading to further pro-inflammatory cytokine output by microglia in the brain (18–20). Besides the above-described parameters, markers of monocyte/macrophage activation (and endothelial function) have also been associated with MDD: Circulating levels of macrophage migration inhibitory factor (MIF) and neopterin were increased in depression as compared to healthy controls (21, 22). Monocytes are an important compound of the innate immune system because they are drivers of inflammation by releasing pro-inflammatory cytokines (23). Interestingly, multiple studies have confirmed a comorbidity between MDD and cardiovascular disease (24–26), and the link between MDD, low-grade inflammation, and cardiovascular events is highly suggested pointing to shared biological underpinnings (27). Thus, the use of anti-inflammatory drugs in MDD seems to be a reasonable approach to increase responsiveness. In patients with atherosclerosis, cyclooxygenase-(COX-)2, prostaglandin E receptors, and prostaglandin E synthase-1 were overexpressed in plaques and peripheral blood mononuclear cells [PBMCs; (28)]. Furthermore, stimulated macrophages exposed to high levels of oxidized low-density lipids (oxLDL) exhibit higher COX-2 (29). Thus, COX-2-inhibitors may be a promising approach because they inhibit synthesis of prostaglandin E_2 (PGE_2), which acts as a stimulator of indoleamine-2,3-dioxygenase [IDO; (30)] and mediates inflammatory response (31). IDO activation, in turn, promotes the conversion of tryptophan along the kynurenine pathway instead of serotonin and may explain the serotonin depletion and neurodegeneration hypotheses of depression (20, 32). Interestingly, depressed patients showed increased serum PGE_2 (33).

Several reviews and meta-analyses have evaluated the efficacy of anti-inflammatory treatments in MDD patients, mostly concluding an overall limited beneficial effect for clinical outcome (34–39). These overview articles included four important trials that investigated the efficacy of celecoxib (a COX-2-inhibitor) augmentation to sertraline, fluoxetine, or reboxetine and showed a greater decline of symptom severity as compared to add-on placebo [although both groups showed a significant symptom reduction; (40–43)]. Given the notion that inflammatory activation is present only in a subgroup of patients, a critical point in former analyses is the evaluation of efficacy without addressing differential inflammatory levels and the evaluation of the relation between inflammatory compounds and response across treatment arms. Consequently, potential differentiating effects of inflammatory status by treatment were lost, which is reflected by the variability of, discrepancy between, or lack of positive individual study results (4, 44). Thus, it is necessary to investigate efficacy of different treatment regimens with respect to the levels of inflammatory compounds. A systematic review revealed that higher biomarker levels (IL-6, CRP, TNFα) were associated with treatment resistance to predominantly serotonergic acting drugs and that response improved using mainly noradrenergic or dopaminergic acting drugs, as well as using anti-inflammatory drugs (45). Further, when levels of these biomarkers were low, response to several anti-inflammatory agents was even lower as compared to placebo (45). Thus, studies need to compare levels of inflammatory biomarkers depending on response status per treatment arm is needed to gain more insight into patient profiles, which may help to individualize treatment options.

Here, we evaluated data from a trial designed to investigate the relation between levels of inflammatory compounds and

response to add-on placebo vs. add-on celecoxib to standard selective serotonin reuptake inhibitor (SSRI) sertraline in MDD patients before reaching the state of treatment resistance (patients with no more than two unsuccessful treatments). In this report, we exploratively investigated serum MIF, neopterin, and TNFα levels because these compounds represent markers of macrophage and inflammatory activation. An earlier study by our group found elevated MIF levels in MDD patient, but it did not focus on patient subgroups (22). Other than that, to our knowledge MIF and neopterin have not yet been investigated in this context. Additionally, TNFα levels emerged as predictor of response to a TNF antagonist in depression (46), thus we were interested in studying this effect with celecoxib. Further, we also analyzed efficacy data independent from biomarker levels, as well as patient characteristics that are known to be related to inflammation and cardiovascular risk [i.e., smoking status, sex, body mass index (BMI), and age; (47–49)].

MATERIALS AND METHODS

This phase IIa study used a randomized, double-blind, placebo-controlled, parallel group design was used. Considering a high and variable placebo response in depression, a placebo control was chosen. Patients and investigators/study staff were blinded to treatment allocation. Psychiatric inpatients were sampled by convenience and randomly assigned in a 1:1 ratio by fixed block randomization to 6 weeks of either sertraline plus placebo or sertraline plus celecoxib treatment. The study was approved by the ethics committee of the medical faculty of Ludwig-Maximilians-University Munich (project-nr. 234-09). The trial was performed in compliance with the standards of good clinical practice and in accordance with the Declaration of Helsinki and its subsequent revisions. All participants provided written informed consent.

Participants

Patients aged between 18 and 60 years were included if they had been diagnosed with MDD by a psychiatrist (DSM-IV-TR) and had a baseline Montgomery Åsberg Depression Rating Scale [MADRS; (50)] score of 20 or above (indicating moderate to severe depression). Exclusion criteria were as follows: comorbid psychotic depression, bipolar disorder, addiction, schizoaffective disorder, schizophrenia, and other psychiatric disorders if their symptomatology was predominating. Also excluded were patients taking concomitant psychotropic drugs or anti-inflammatory pain medication such as COX-2-inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), or paracetamol; pregnant or breastfeeding women; patients with history of cardiovascular disease or heart disease, or with current cardiovascular disturbances; and patients with inflammatory or other relevant diseases were excluded from the study. For the full inclusion and exclusion criteria see **Supplementary Table A**.

Measures

Serum levels of MIF, neopterin, and TNFα were assessed at baseline and endpoint (week 6). Blood was drawn in fasting condition. All parameters were determined by enzyme-linked immunosorbent assay (ELISA) with standard curve by using the quantitative sandwich enzyme immunoassay technique (MIF, TNFα) and competitive enzyme immunoassay technique (neopterin), according to the instruction of the kit manufacturer (MIF ELH-MIF, RayBio®, Peachtree Corners, USA; TNFα HSTA00D, R&D Systems, Minneapolis, USA; neopterin EIA-2949, DRG International, Inc., Springfield, USA) and analyzed using MARS data analysis software (BMG Labtech, Ortenberg, Germany). BMI was calculated as body weight (in kg) divided by the square of body height (in m). Depression severity was assessed by trained raters at baseline and endpoint (6 weeks) with the MADRS (50, 51). Response was defined as a reduction of MADRS score of at least 50% on at endpoint, depending on the individual baseline score, and remission was defined as a score of 7 or lower at endpoint (52).

Treatment Protocol

All patients who were taking antidepressant medication prior to the study underwent a 3-day wash-out period before the start of trial treatment. In case of premedication with long half-life, a longer period since the last treatment was necessary to be included (see **Supplementary Table A**). If needed, lorazepam was administered up to 4 mg/day during wash-out and for the first 2 weeks, up to 3 mg/day for the third week, up to 2.5 mg/day for the fourth and fifths weeks, and up to 1.5 mg/day for the sixth week. Zopiclone was administered up to 7.5 mg/nightly during the wash-out period (one patient had 15 mg) and the study time, if needed. At baseline, eligible patients were randomized to one of the following treatment arms: 50–100 mg sertraline daily (one tablet/unblinded) plus celecoxib twice daily (one capsule/200 mg/blinded) or 50–100 mg sertraline daily (one tablet/unblinded) plus placebo twice daily (one capsule/blinded) The placebo capsule contained 308 mg microcrystalline cellulose coated by hard gelatin and was of the same size, weight, color, and shape as the celecoxib capsule. If a higher clinical benefit was expected, a dose of 150 mg sertraline was allowed.

Statistics

SPSS software (IBM SPSS Statistics 25) was for statistical analyses used. The trial data presented here were planned as secondary per-protocol analyses. In this report, the primary outcomes were response and remission independently of inflammatory status and cytokine serum levels according to the response and remission status. Secondary analyses, investigated the role of BMI, age, sex, and smoking status. Endpoint parameter values were subtracted from baseline parameter values to reflect the change of values over time. Therefore, positive values represent a decrease and negative values represent an increase of levels over time. Descriptive analyses are given as mean and standard deviation or median and interquartile range for continuous variables, and as frequencies (percentages) for categorical variables. Testing for significances was performed using Chi-square test for categorical data (in case of 5 or fewer observations in the cells of the contingency table, Fisher's exact test was performed), Student's *t*-test for continuous data that met the criteria for parametric testing (in case of inhomogeneous variances, Welch-test is reported; in case of non-normality,

Mann-Whitney-*U*-test was performed), or linear regression. To compare levels of inflammatory compounds between subgroups, Mann-Whitney-U-test for independent samples and Wilcoxon signed-rank test for dependent samples were used if data did not meet assumptions for parametric testing and/or small subsamples were tested. The significance level was set at 5% (two-sided) for all tests. As analyses were exploratory and subsamples were small, adjustment for multiple testing and correction for possible confounding variables were neglected in the primary analyses. Hence, the data reported here are preliminary. Due to statistical power limitations, trends are also reported ($p < 0.10$). The flow chart is given in **Supplementary Figure 1**.

RESULTS

Patient Characteristics and Levels of Immune Parameters

Table 1 shows the descriptive demographic data, depression severity, and baseline biomarker levels in each treatment group. **Table 2** shows the levels of immune parameters at baseline and endpoint in the placebo- and celecoxib-treated groups, as well as for responders/remitters and non-responders/non-remitters. Because drug treated patients were randomized but sample is of limited size, demographic data and baseline parameter values were tested for differences between the treatment arms. No statistically significant differences emerged for MADRS baseline score, MADRS endpoint score, percentage MADRS score reduction over time, age, baseline BMI, BMI at endpoint, sex distribution, smoking status, and immune parameter values at baseline between placebo and celecoxib groups (see **Supplementary Table B**).

Response and Remission Rates

Table 3 shows the proportions of responders and remitters in each treatment arm. No significant differences emerged for the distribution of response rates ($\chi^2 = 0.62$; $p = 0.43$) or remission rates ($\chi^2 = 0.49$; $p = 0.49$) between the two treatment groups. Both treatment groups showed a significant decline of MADRS scores over time (placebo: $T = 12.81$; $SE = 1.31$; $p < 0.001$; 95% $CI = [14.07; 19.51]$; celecoxib: $T = 7.86$; $SE = 2.00$; $p < 0.001$; 95% $CI = [11.53; 19.94]$). In responders, the MADRS score decreased slightly more in the celecoxib group than in the placebo group, although the difference was not significant ($T = -1.15$; $SE = 1.75$; $p = 0.26$; 95% $CI = [-5.61; 1.60]$). In non-responders, the MADRS score decreased slightly more in the placebo group than in the celecoxib group, but this difference was also not significant ($T = 1.01$; $SE = 2.45$; $p = 0.33$; 95% $CI = [-2.81; 7.77]$).

Predictive Capability of Biomarkers for Response and Remission

MIF

Figure 1 shows the results for MIF at baseline. In the placebo group (sertraline only), responders showed a trend for lower MIF levels at baseline compared with non-responders, and remitters showed significantly lower MIF levels at baseline than non-remitters. In the celecoxib group (sertraline plus celecoxib), responders showed a trend for higher MIF levels at baseline

TABLE 1 | Descriptive characteristics of patients with MDD.

	Sertraline + placebo		Sertraline + celecoxib	
MADRS baseline *Md (IQR)*	29.00 (4.00)	*N* = 23	28.00 (8.00)	*N* = 19
MADRS endpoint *M (SD)*	12.35 (7.75)	*N* = 23	13.20 (7.21)	*N* = 20
% MADRS score reduction *M (SD)*	58.91 (23.05)	*N* = 23	52.16 (26.25)	*N* = 19
Age *M (SD)*	38.78 (10.71)	*N* = 23	39.25 (12.75)	*N* = 20
BMI baseline *M (SD)*	23.28 (3.46)	*N* = 23	23.31 (3.22)	*N* = 20
BMI endpoint *M (SD)*	22.58 (3.26)	*N* = 21	22.82 (2.93)	*N* = 19
Sex women *N* (%)	12/23 (52.17)		9/20 (45.00)	
Smoking yes *N* (%)	7/23 (30.43)		11/20 (55.00)	
MIF (pg/ml) *Md (IQR)*	3484.00 (5002.25)	*N* = 22	4306.00 (4638.25)	*N* = 18
Neopterin (ng/ml) *Md (IQR)*	0.85 (0.47)	*N* = 22	0.73 (0.49)	*N* = 19
TNFα (pg/ml) *Md (IQR)*	0.78 (0.81)	*N* = 19	0.76 (0.71)	*N* = 15

BMI, body mass index; MADRS, Montgomery Åsberg Depression Rating Scale; Md, median; IQR, interquartile range; M, mean; SD, standard deviation. MADRS score at baseline was available in only 19 of the 20 patients in the celecoxib group but was available in all 20 patients at endpoint assessment.

TABLE 2 | Baseline and endpoint biomarker levels according to treatment and response status.

	Baseline *Md (IQR)*	Week 6 *Md (IQR)*	*Z (N)*	*p*
MIF (pg/ml)				
Placebo	3484.00 (5002.25)	2102.00 (2862.50)	−1.61 (22)	0.11
Celecoxib	3406.00 (4638.25)	4197.50 (3826.75)	−0.28 (18)	0.78
Responder	3484.00 (4948.25)	3078.00 (4820.00)	−0.60 (26)	0.55
Non-responder	4092.00 (5301.50)	3519.00 (2888.00)	−0.73 (13)	0.46
Remitter	2663.00 (3.697)	1207.50 (2893.00)	−1.96 (12)	0.05^{+}
Non-remitter	4321.50 (5545.75)	3736.50 (4160.50)	−0.18 (28)	0.86
Neopterin (ng/ml)				
Placebo	0.85 (0.47)	0.79 (0.44)	−0.52 (22)	0.60
Celecoxib	0.73 (0.49)	0.88 (0.21)	−2.15 (19)	0.03*
Responder	0.72 (0.50)	0.86 (0.38)	−1.21 (26)	0.23
Non-responder	0.87 (0.38)	0.91 (0.36)	−1.67 (14)	0.10
Remitter	0.80 (0.48)	0.81 (0.47)	−0.08 (12)	0.94
Non-remitter	0.76 (0.51)	0.88 (0.36)	−2.12 (29)	0.03*
TNFα (pg/ml)				
Placebo	0.78 (0.81)	0.92 (0.89)	−1.25 (19)	0.21
Celecoxib	0.76 (0.71)	0.78 (1.02)	−0.34 (15)	0.73
Responder	0.71 (0.85)	0.68 (0.94)	−0.57 (21)	0.57
Non-responder	0.96 (0.96)	1.08 (0.96)	−0.11 (13)	0.92
Remitter	0.75 (0.90)	0.63 (0.59)	−0.15 (10)	0.88
Non-remitter	0.81 (0.86)	1.07 (1.00)	−0.69 (24)	0.49

*Md, median; IQR, interquartile range; Z-test statistic Wilcoxon signed rank test. *$p < 0.05$ and $^{+}p < 0.10$.*

compared with non-responders, but no significant difference emerged between remitters and non-remitters. Statistical test results are shown in **Table 4A**. At endpoint, MIF levels were not significantly different between responders and non-responders in the placebo group but were significantly lower in remitters compared with non-remitters. In the celecoxib group, responders did not differ statistically from non-responders at endpoint, but remitters showed a trend for lower MIF levels than non-remitters. Statistical test results are shown in **Table 4B**.

Regarding the change of MIF levels over time, in the placebo group non-responders showed a trend for a decrease to endpoint ($Z = -1.86$; $p = 0.06$), but no differences of MIF change between placebo responders and non-responders were found. However, the celecoxib group showed a trend for differential change of MIF levels over time: Remitters showed a decrease while non-remitters showed an increase ($U = 14.00$; $p = 0.07$). Furthermore, the change in MIF levels was significantly different in non-responders and non-remitters to celecoxib (increase) compared with non-responders and non-remitters to placebo (decrease; $U = 6.00$; $p = 0.03$ and $U = 51.00$; $p = 0.03$, respectively).

Neopterin

No significant differences in neopterin levels emerged at baseline and endpoint between responders and non-responders as well as remitters and non-remitters in either treatment arm (see **Supplementary Tables C.1**, **C.2**). Investigating change over time, only non-responders to celecoxib showed a trend for an increase of neopterin levels ($Z = -1.95$; $p = 0.05$).

TNFα

No significances emerged for TNFα levels at baseline. At endpoint, only lower levels were found in placebo responders

TABLE 3 | Response and remission rates of MDD patients in each treatment arm.

	Responder	Non-responder
Sertraline + placebo % (*N*)	69.6 (16)	30.4 (7)
Sertraline + celecoxib % (*N*)	57.9 (11)	42.1 (8)
	Remitter	**Non-remitter**
Sertraline + placebo % (*N*)	34.8 (8)	65.2 (15)
Sertraline + celecoxib % (*N*)	25.0 (5)	75.0 (15)

TABLE 4A | Comparison of baseline MIF levels of different response statuses in the two treatment arms.

Sertraline +	Responder (*Md*)	Non-responder (*Md*)	*U*	*p*
placebo	2853.00 pg/ml	4743.00 pg/ml	29.00	$<0.10^{+}$
	Remitter (*Md*)	Non-remitter (Md)	*U*	*p*
	501.00 pg/ml	4240.00 pg/ml	15.00	$<0.01^{**}$
Sertraline +	Responder (*Md*)	Non-responder (*Md*)	*U*	*p*
celecoxib	4961.00 pg/ml	1901.00 pg/ml	15.00	0.07^{+}
	Remitter (*Md*)	Non-remitter (*Md*)	*U*	*p*
	4209.00 pg/ml	4403.00 pg/ml	24.00	0.40

Md, median; U-Mann-Whitney-U-test statistic. $^{**}p < 0.01$ *and* $^{+}p < 0.10$.

TABLE 4B | Comparison of endpoint MIF levels of different response statuses in the two treatment arms.

Sertraline +	Responder (*Md*)	Non-responder (*Md*)	*U*	*p*
placebo	1066.00 pg/ml	2924.00 pg/ml	37.00	0.28
	Remitter (*Md*)	Non-remitter (Md)	*U*	*p*
	480.00 pg/ml	2924.00 pg/ml	8.00	$<0.01^{**}$
Sertraline +	Responder (*Md*)	Non-responder (*Md*)	*U*	*p*
celecoxib	3970.00 pg/ml	4814.00 pg/ml	32.00	0.92
	Remitter (*Md*)	Non-remitter (*Md*)	*U*	*p*
	3579.00 pg/ml	4587.00 pg/ml	15.00	0.09^{+}

Md, median; U-Mann-Whitney-U-test statistic. $^{**}p < 0.01$ *and* $^{+}p < 0.10$.

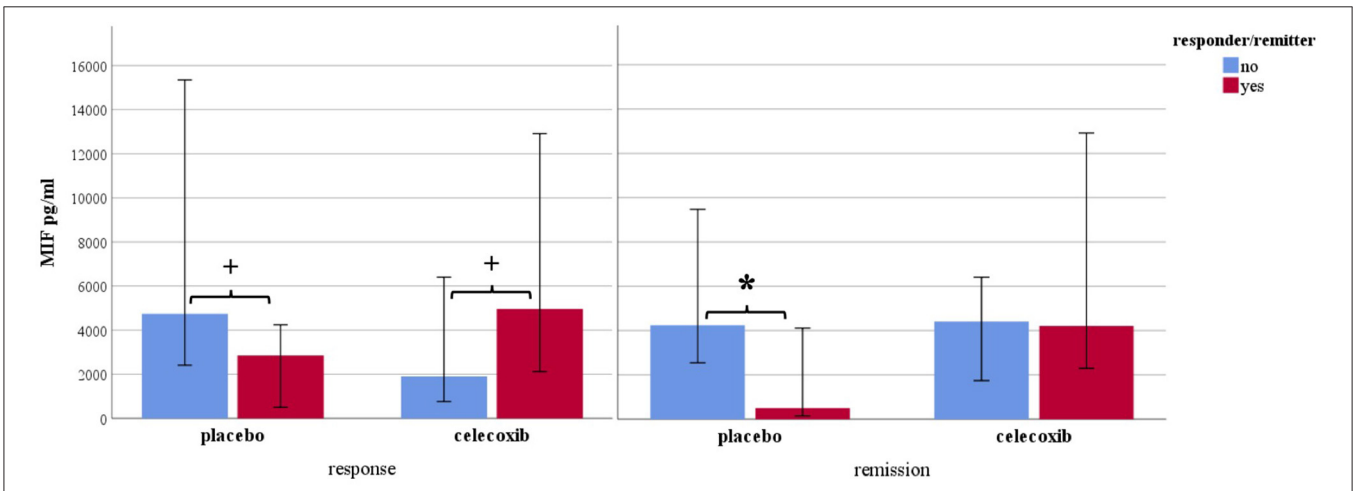

FIGURE 1 | Macrophage migration inhibitory factor levels at baseline according to response and remission status. Median levels are displayed in pg/ml; 95% confidence interval error bars are displayed; *placebo* sertraline plus placebo group; *celecoxib* sertraline plus celecoxib group. $^{*}p < 0.05$ and $^{+}p < 0.10$.

compared with placebo non-responders. For further details, see **Supplementary Tables D.1**, **D.2**. Investigating change over time, placebo non-responders showed a trend for an increase of TNFα levels over time ($Z = -1.86$; $p = 0.06$). In contrast, celecoxib non-responders showed a decrease of TNFα levels over time ($Z = -2.21$; $p = 0.03$). In the celecoxib group, responders showed differential course over time (increase) compared with non-responders (decrease; $U = 8.00$; $p = 0.03$). In non-responders, celecoxib-treated patients showed a decrease of TNF α levels over time whereas placebo-treated patients showed an increase ($U = 1.00$; $p < 0.01$).

Patient Characteristics Associated With Inflammation

The most interesting and relevant results were obtained for MIF. Therefore, possible confounders were further investigated.

BMI

No significant correlation was found between BMI and MIF baseline levels (Spearman-*Rho* = 0.23; $p = 0.16$). We found no difference in the change of BMI over time according to treatment ($T = -0.54$; $p = 0.59$) or response/ remission status across treatments ($T = -0.44$; $p = 0.66$ and $T = 0.15$; $p = 0.88$, respectively) occurred. However, in the placebo group a decrease in BMI over time significantly predicted a lower response (% MADRS score reduction) [$R^2 = 0.29$; $F_{(1, 19)} = 7.85$; ß = −22.37; $p = 0.01$].

Age

We found a significant positive correlation between age and MIF levels (Spearman-*Rho* = 0.34, $p = 0.03$) at baseline. No difference emerged in age according to response/ remission status ($T = -0.56$; $p = 0.58$ and $T = -1.04$; $p = 0.31$, respectively) across treatments. However, higher age significantly predicted a lower response (% MADRS score reduction) in the placebo group [$R^2 = 0.19$; $F_{(1, 21)} = 4.76$; ß = −0.93; $p = 0.04$].

Sex

Men had significantly higher MIF levels ($U = 115.00$; $p = 0.02$) at baseline than women. Response/remission status were independent from sex (response: $\chi^2 = 0.10$; $p = 0.75$; remission: $\chi^2 = 0.19$; $p = 0.67$). Response status was also statistically independent from sex in the placebo and celecoxib subgroups ($p > 0.99$ and $p = 0.65$, respectively). However, in the celecoxib group men showed a numerically lower reduction in MADRS score than women. Regarding changes in biomarker level over time, women as compared to men showed a significantly different course of MIF levels (women: increase, men: decrease; $U = 122.00$; $p = 0.04$). This difference emerged only in the placebo group ($U = 25.00$; $p = 0.02$) and was seen in responders as a trend ($U = 47.00$; $p = 0.06$).

Smoking Status

No significant differences in baseline MIF levels emerged between smokers and non-smokers ($U = 164.00$; $p = 0.44$). Furthermore, response/remission status were independent from smoking status (response: $\chi^2 = 0.14$; $p = 0.71$; remission: $\chi^2 = 0.14$; $p = 0.71$). Response status was also independent from smoking status in the placebo and celecoxib subgroups ($p = 0.63$ and $p = 0.66$, respectively).

Adjusted Analysis

Because MIF levels were associated with age and sex, we performed preliminary regression analyses with age and sex as covariates to further investigate the predictive capability of response status on the expression of MIF levels. In the placebo group, a trend emerged for predicting of MIF levels at baseline by response status, age, and sex [$R^2 = 0.32$; $F_{(3, 18)} = 2.82$; $p = 0.07$]. For remission, a significant model was obtained [$R^2 = 0.37$; $F_{(3, 18)} = 3.56$; $p = 0.04$]. Similarly, the model predicting MIF levels at endpoint was significant [$R^2 = 0.41$; $F_{(3, 18)} = 4.19$; $p = 0.02$]. In the celecoxib group, a trend emerged for the prediction of MIF levels at baseline by response status, age, and sex [$R^2 = 0.38$; $F_{(3, 13)} = 2.60$; $p < 0.10$]. Data on the individual predictors are given in **Supplementary Table D**. The results of the adjusted analyses are mostly in accord with the data given under 3.3. Since some models revealed a significant R^2 but partly lacking significance of single predictors, collinearity diagnostics were performed. No indication for multicollinearity was present in any of the models. Thus, this effect may result from the collective impact of more or less nearly significant predictors.

DISCUSSION

Overall, no difference in response or remission rates between the two treatment arms were found. One reason for this result might be that celecoxib add-on to sertraline is not superior to sertraline plus placebo. In contrast to our findings, a meta-analysis of previous trials found a superior effect of add-on celecoxib to standard antidepressant treatment over add-on placebo without taking inflammatory status into account (53, 54). Noteworthy, the magnitude of symptom reduction and the obtained response rates varied among the studies, even despite using the same celecoxib dose (41, 53). The studies with very high response rates to celecoxib had a higher women to men ratio in the celecoxib group than the other studies and our study (41, 42, 53). According to previous literature, inflammatory activation is associated with depression severity particularly in women (55), thus leading to a higher potential of benefitting from anti-inflammatory therapy. In line with our study, another trial investigating add-on celecoxib to SSRI compared to placebo plus SSRI in drug-naïve depressed women and found no difference in reduction of depression severity at endpoint (41). However, the authors found a superior effect of celecoxib after half the treatment phase, i.e., 4 weeks, suggesting that celecoxib might accelerate symptom reduction during early treatment phases (41). Although celecoxib add-on was not superior to standard treatment in our study, both groups had a reasonably large proportion of responders and both treatment groups showed a significant decline of depression severity over time. As compared to one of the investigated trials in the meta-analysis, we found a similar response rate in the celecoxib group while both studies used sertraline and the same celecoxib dose (43). However, the non-superiority of add-on celecoxib in our trial questions the clinical benefit that the

previous studies concluded. Because only a subgroup of patients exhibits an increased pro-inflammatory profile and patients were not stratified for inflammatory state before receiving anti-inflammatory treatment, it may be expected to find no substantial difference in response rates. This assumption is supported by studies on other (add-on) anti-inflammatory treatments, which showed that depressed patients with a low inflammatory status exhibited even lower response rates after anti-inflammatory therapy than patients with a low inflammatory status who had received (standard therapy plus) placebo (45). Therefore, patients with low levels of inflammation may have contributed to lower response rates to celecoxib. Furthermore, our study found much higher response and remission rates in the sertraline group than were found in the control groups in most of the other trials (53). In our study, almost all patients were without current premedication and about half the patients were experiencing their first episode of depression. Sertraline is a potent antidepressant and drug-naïve patients respond better to treatment than patients who have received multiple treatments (2), which together may explain the high response and remission rates to sertraline plus placebo in our study. Furthermore, many of the above-mentioned studies included outpatients, whereas our sample consisted only of inpatients (41–43). A high placebo effect may arise from receiving extensive care and attention in a hospitalized setting. Generally, given the variability of previous study results, explanations for differences of our results are rather speculative. Regarding change of biomarker levels over time, significant results for non-responders and responders or non-remitters and remitters across treatment arms, and for sertraline with add-on placebo or add-on celecoxib across response status are lacking not indicating any general effects of treatment or response status for change of biomarker levels.

Our study goes beyond the current state of knowledge by looking into response status in subgroups. A dependency of inflammatory biomarker levels and response/remission to different treatment becomes apparent, at baseline and during the course of treatment. However, a clear pattern was only observed for MIF, which is consistent with previous literature. Non-responders to sertraline plus placebo showed a trend for higher baseline levels as compared with sertraline responders. This was especially and significantly evident in non-remitters compared with remitters pointing to the well-known treatment resistance to standard serotonergic agents when pro-inflammatory levels are increased (45). Vice versa, responders to celecoxib showed a trend for higher baseline MIF levels as compared with celecoxib non-responders (who had the overall lowest levels), suggesting a beneficial effect of anti-inflammatory medication on clinical outcome when such activation is present. Interestingly, baseline MIF levels in non-responders/non-remitters to sertraline plus placebo were similar to those of responders/remitters to sertraline plus celecoxib. This oppositional relationship has also been found for CRP, IL-6, and IL-1ra (46, 56, 57). Moreover, higher MIF baseline mRNA levels were also shown to predict response to escitalopram or nortriptyline in depressed patients (58). The difference between baseline levels in placebo remitters vs. non-remitters was larger than between responders/non-responders and was still present at the end of the study. We therefore conclude that stronger or faster symptom reduction can be achieved with standard SSRIs when MIF levels are preferably low and high MIF levels after treatment indicate the persistence of clinically relevant depressive symptoms. Celecoxib non-responders and non-remitters showed the highest MIF levels of all subgroups at endpoint (see **Table 4B**). This change was significantly different from that in non-responders and non-remitters in the placebo groups and may point to a subgroup of concern that should be studied in more detail in the future. Taken together, MIF shows a predictive capability for remission (and a trend toward such capability for response) in treatment as usual (SSRI) and a trend toward predicting response to add-on celecoxib.

Regarding relevant patient characteristics, MIF levels were found to be related to age and sex but not to BMI. In the placebo group, weight loss and higher age were associated with treatment resistance. Since age was positively associated with both, MIF levels and treatment failure, the concept of immunosenscence presents a suitable explanation. Immunosenscence describes the changes of the immune system which, among others, is characterized by low-grade inflammation and all of which increase during aging (59). It is thus not surprising that these factors were related in our study. We found no effect of sex on response and no associations with smoking whatsoever. Hence, BMI, sex, and age seem to be linked to MIF levels and/or response status and should be addressed in design and statistical evaluation of future studies.

As for neopterin, baseline levels did not discriminate between responders/remitters and non-responders/non-remitters to either treatment. Previous research found that higher neopterin levels were associated with depression and the number of depressive episodes (21, 60) indicating higher disease severity. This also demonstrates that neopterin levels seem to rise with treatment resistance as the increase in non-remitters shows in our study. Further, this may be especially driven by the celecoxib non-remitters explaining the increase of neopterin levels in the whole celecoxib group (see **Table 2** and **Supplementary Tables C.1**, **C.2**). To our knowledge, no other studies have investigated baseline neopterin in relation to treatment response yet. Our study found no indication that neopterin is a potential biomarker. However, this result should be verified in a study with a larger sample size and greater power. TNFα levels were not significantly different at baseline but at endpoint in the placebo group indicating that treatment resistance to standard SSRI is accompanied by high levels of TNFα. One earlier study found significantly lower TNFα levels at baseline in responders to sertraline (at least 50% MADRS score reduction) compared with non-responders (61). Our data showed the same tendency, but the difference did not reach statistical significance. The authors of the earlier study (61) defined endpoint at 12 weeks which might better separate response status and biomarker levels. Further, Powell et al. (62) found higher levels of TNF gene expression in SSRI (escitalopram) non-responders compared to responders and this difference was even larger at endpoint (8 weeks) than at baseline, supporting our results. However, conflicting results exist (63, 64).

The involvement of the kynurenine pathway in MDD pathophysiology has been receiving growing attention. In fact, pro-inflammatory cytokines such as IFNγ, TNFα, IL-1, and the hormone PGE2, which is stimulated by MIF via COX-2-upregulation, stimulate the activity of the enzyme IDO and consequently the breakdown of tryptophan into potentially neurotoxic kynurenine metabolites (13, 65–68). This results in a lack of serotonin, a widely known characteristic of MDD. Because MIF stimulates PGE2 production, which is counteracted by a celecoxib-related decrease in IDO synthesis, MIF might act as a surrogate for COX-2 and PGE2 activity. Further, TNFα activates IDO (13), also possibly explaining the lacking serotonergic response. Furthermore, kynurenine in turn may lead to more pro-inflammatory cytokine release like TNFα (69) possibly explaining the higher levels of TNFα at endpoint. In fact, in all three markers we observed the same numeric trend of higher levels in non-responders/non-remitters at baseline and at endpoint, though not all the differences reached statistical significance. Nevertheless, this points in the direction of disturbed antidepressant action in those patients. Other previous results show that higher kynurenine/tryptophan ratio (favors kynurenine pathway) was predictive of remission after celecoxib add-on treatment in another sample of MDD patients (70). With perspective on the comorbidity of depression and cardiovascular disease, IDO activity is associated with cardiovascular risk factors (71), and celecoxib has shown beneficial effect on atherosclerotic progression (72).

This study has several limitations. Analyses in small subsamples have limited statistical power and non-significant results may therefore be a matter of type II error, which is we refrained from confident interpretation. However, we did give statistical trends some credibility. There is an ongoing debate on whether the *p*-value should be treated as a strict cut-off, and many scientists favor a non-categorical use today (73). The addition of covariates to a binary predictor in the adjusted analyses further limits power while the smaller sample size already increases variance. Future studies should aim at replicating these findings in larger samples which would also allow for multiple test correction. Due to the preliminary nature of subgroup analyses, and especially the adjusted analyses, results are rather hypothesis generating for future studies. We used convenience sampling so that even though important patient characteristics were equally distributed between the treatment arms other variables might have acted as confounders that were not accounted for. For example, childhood adverse experience was shown to be related to cytokine levels in depression (74). In addition, our sample consisted of inpatients only, so the results cannot be generalized to outpatient settings. Because antidepressant medication has some immunomodulatory effects (75), prior use of such medication might have elicited a modulating effect before the study already. Further, we did not evaluate kynurenine metabolites which will be important for future analyses to demonstrate mechanistic links as discussed above. In general, one important drawback is that there are no established cut-offs yet for categorizing levels of the investigated biomarkers as high or low. Such cut-off values are needed so that patients can be stratified by inflammatory level beforehand to investigate response to a tailored treatment allocation.

CONCLUSION

Celecoxib add-on did not lead to greater response rate at 50% symptom reduction than sertraline plus placebo regardless of inflammatory state, but patients were not stratified beforehand according to their level of pro-inflammatory activation. MIF shows potential for acting as a reliable biomarker indicating treatment responsiveness, especially remission. Response rates may be increased if such biomarkers were used to guide treatment choice or change when their monitoring during treatment indicates non-response. The present study serves as a call for future investigations, in particular on treatment remission in response to anti-inflammatory vs. standard antidepressant treatments, stratifying patients by immune activation in advance. As the trends found for response status cannot be neglected entirely, studies should also reevaluate these finding in larger samples. Therefore, cut-off values should also be established for classifying abnormal immune activation. Moreover, complex models including possible confounding variables should be performed and more biomarkers should be investigated in larger samples to predict response.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Medical Faculty of Ludwig-Maximilians-University Munich, Germany (project-nr. 234-09). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS has prepared the data and conducted the analysis, evaluation, and interpretation of the present work, as well has drafted the present manuscript. BB and GA-H have conducted data acquisition. EW has contributed to planning the study and conducted data acquistion. PZ has conducted the laboratory analyses. RM has contributed to the interpretation of data. HD has contributed to design and planning the study and data interpretation. NM has contributed to design and planning the study, data acquisition, and evaluation. BB, EW, GA-H, PZ, RM, HD, and NM have critically reviewed and revized the content. All authors contributed to the manuscript and approved submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.615261/full#supplementary-material

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