THE SEARCH FOR BIOMARKERS IN PSYCHIATRY

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THE SEARCH FOR BIOMARKERS IN PSYCHIATRY

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EDITORIAL
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Editorial: The Search for Biomarkers in Psychiatry

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Keywords: biomarkers, central biomarkers, peripheral biomarkers, omics, psychiatry

Editorial on the Research Topic

The Search for Biomarkers in Psychiatry

Psychiatric disorders present a high level of complexity in many aspects. Contrary to other diseases, they are classified by diagnostic categories with a broad variety list of symptoms. This categorical organization results in a great clinical heterogeneity among patients diagnosed from the same psychiatric illness. Besides, the high rate of comorbidity among psychiatric disorders is an additional distinguishing factor that makes diagnosis complicated. On the top of that, the limited knowledge of the molecular mechanisms underlying mental disorders, greatly contribute to the reduced efficacy of current pharmacological treatments, being especially poor as the severity of the disease increases. This clinical situation stimulated the searching of biomarkers to improve prevention, diagnosis and treatment of psychiatric disorders. This special issue includes one review article of García-Gutiérrez et al., summarizing the concept and types of biomarkers in psychiatry and providing examples about the most promising results achieved sorted by categories (genetics, transcriptomics, proteomics, metabolomics, and epigenetics). The review includes a final conclusion that remarks the future challenges required to reach the goal of developing valid, reliable and broadly-usable biomarkers for psychiatric disorders and their treatment. Complementary, the opinion article of Vinberg further explored the main limitations and the future perspectives in the searching of peripheral biomarkers in psychiatry.

The potential role of key targets of the immune and endocannabinoid systems are studied in detail in three articles. The brief research report of Larsen et al. provide evidences about an association between alterations in different cytokines and motor activity in an acute psychiatric population, suggesting that some cytokines deserve further exploration as biomarkers for predicting and treating changes in motor activity. Additionally, the review article of Momtazmanesh et al., is a fantastic review summarizing the main cytokine alterations observed in schizophrenic patients, their potential link with certain symptoms and their potential clinical impact. Similarly, the review article of Navarrete et al., provide the most promising results achieved to date that support the potential role of different targets of the endocannabinoid system as biomarkers in several psychiatric conditions.

Besides, the review of Li Z. et al., is an excellent article containing the most recent studies supporting the potential use of circular RNA as biomarkers in two major psychiatric disorders, depression and schizophrenia. Finally, the review of Jurado-Barba et al., covers the potential clinical use of EEG as clinical assessment in alcohol dependence.

A total of seven articles are focused on the identification of biomarkers in major depressive disorder (MDD). Zhao et al., analyse the utility of CACNA1C rs1006737 polymorphism together with exposure to threatening life events as predictive biomarkers for MDD, pointing out this polymorphism as a target for new pharmacological treatments in this psychiatric disorder. Explore the use of high heart rate as biomarker for assessing the outcome of

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reinstatement at work in MDD patients who took a leave of absence, with promising results. Zhao et al., carried out the study of different single nucleotide polymorphisms of FoxO1, A2M, and TGF-B1 in MDD patients, demonstrating a potential association between some of them and the environment in MDD. Authors suggest that these SNPs may be useful for assessing MDD risk improving prevention.

An emerging type of biomarkers is metabolomic alterations. In this respect, Gao et al., focus on studying the role of 36 metabolic biomarkers in depression. The research sheds light on potential important metabolites and enzymes in the underlying molecular mechanisms of depression.

Troyan and Levada, wrote an exciting original article revealing the correlation between serum concentrations of the neurotrophin BDNF and IGF-1 and MDD. Interestingly, changes in these targets are also observed after 8 weeks of treatment with the antidepressant vortioxetine, supporting that both may be useful as biomarkers of diagnosis and treatment outcome in MDD. Similarly, Köhler-Forsberg et al., provide the results of an open label clinical trial in MDD patients supporting the utility of a panel of biomarkers (serotonin 4 receptor PET brain imaging, fMRI, cognitive-, EEG-and peripheral biomarkers) for MDD diagnosis and for predicting pharmacological efficacy. Ho et al., presented a comprehensive update regarding functional near-infrared spectroscopy (fNIRS) as a biomarker for guiding diagnosis and monitoring treatment response in MDD.

Kittel-Schneider et al., covered the emerging role of proteomics profile as a diagnostic tool for distinguish bipolar from unipolar depression. The results support the potential role of the multivariate predictive model proposed by authors as a predictive biomarker model useful to discriminate between these psychiatric entities.

Interestingly, Cross et al., presented a case report suggesting the clinical relevance to discard the presence of infectious diseases, such as Lyme borreliosis or PANDAS, in patients presenting neuropsychiatric symptoms. These results data, although preliminary, support the close connection between the immune system and the brain.

The original article of Li G. et al., point out lower serum uric acid concentrations as a biomarker for predicting depression in stroke patients. These interesting results support the development of additional longitudinal and clinical studies.

Taken together, these articles updated new advances about peripheral and central biomarkers using a variety of methods (proteomics, transcriptomics, genetics, and imaging) in psychiatric clinical conditions and samples to identify alterations with specific traits of the disease or with the outcome of the pharmacological treatments.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Cytokine Alterations in Schizophrenia: An Updated Review

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Schizophrenia, a multisystem disorder with an unknown etiology, is associated with several immune dysfunctions, including abnormal levels of circulating cytokines. In this review, we investigated the changes of cytokines in schizophrenic patients, their connection with behavioral symptoms severity and their potential clinical implications. We also assessed the possible causative role of abnormal cytokine levels in schizophrenia pathogenesis. Based on meta-analyses, we categorized cytokines according to their changes in schizophrenic patients into four groups: (1) increased cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, IL-12, and transforming growth factor (TGF)-β, (2) non-altered cytokines, including IL-2, IL-4, and IL-17, (3) increased or non-altered cytokines, including IL-8 and interferon (IFN)-γ, and (4) IL-10 with increased, decreased, and non-altered levels. Notably, alterations in cytokines may be variable in four different categories of SP, including first-episode and drug-naïve, first-episode and non-drug-naïve, stable chronic, and chronic in acute relapse. Furthermore, disease duration, symptoms severity, incidence of aggression, and cognitive abilities are correlated with levels of certain cytokines. Clinical implications of investigating the levels of cytokine in schizophrenic patients include early diagnosis, novel therapeutic targets development, patient stratification for choosing the best therapeutic protocol, and predicting the prognosis and treatment response. The levels of IL-6, IL-8, IFN-γ, IL-2 are related to the treatment response. The available evidence shows a potential causative role for cytokines in schizophrenia development. There is a substantial need for studies investigating the levels of cytokines before disease development and delineating the therapeutic implications of the disrupted cytokine levels in schizophrenia.

Keywords: schizophrenia, cytokines, inflammation, behavioral symptoms, treatment outcome, antipsychotic agents

INTRODUCTION

Schizophrenia, a multisystem disorder with a global prevalence of 0.33–0.75%, is one of the top 15 causes of disability (1–4). The underlying etiology of this disease is controversial and not fully understood. Increased dopamine-based activities, together with decreased glutamatergic signaling, are the main suggested etiological hypotheses (5). Abnormalities in the immune system, which are associated with schizophrenia, are one of the other etiological hypotheses.

The immune system is composed of innate and adaptive immune responses. The innate immunity is a rapid-acting antigen-independent response, while the adaptive immunity is an antigen-dependent defense mechanism, with the ability to memorize the antigens. The immune response is mainly mediated by cytokines, which are mostly produced by a critical component of the adaptive immunity, T-lymphocytes. These mediators can be divided into 5 groups; (1) pro-inflammatory cytokines; interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1 family, and IL-8, which are involved in initiation and aggravating inflammatory responses (6); (2) T-helper 1 cytokines; IL-2, interferon (IFN)-γ, and IL-12, which create a pro-inflammatory response and function in autoimmune diseases and defense against intracellular parasites; (3) T-helper 2 cytokines; IL- 4, IL-5, and IL-13, which counterbalance the effects of T-helper1 cytokines; (4) T-helper 17 cytokines; Il-17, and IL-23, which are chiefly involve in pro-inflammatory processes and defense against extracellular pathogens; (5) T-regulatory cytokines; IL-10 and transforming growth factor (TGF)-β, which primarily suppress immune responses (7, 8).

The associated immune disorders in schizophrenia have been investigated for more than a century (9–11). These abnormalities include increased activity and density of microglia cells, abnormal profiles of peripheral leukocytes, serum cytokines, and cerebrospinal fluid (CSF) cytokines (12, 13). Based on genome-wide association studies, schizophrenia is also associated with specific major histocompatibility complex (MHC) region genes (14). Furthermore, this disease is significantly linked with enhancers having a strong role in the immune functions, even after excluding the MHC region genes (15). These findings support the clinical and genetic aspects of the connection between schizophrenia and the immune system.

One group of components of the immune system affecting the brain by several mechanisms are cytokines which are either produced outside of the central nervous system (CNS) or within the CNS. Peripheral cytokines, presented in the circulation, can access the CNS and affect it *via* four major ways: (1) binding to specific transporters, (2) stimulating afferent vagal fibers, (3) accessing areas such as circumventricular organs, and (4) passing the damaged blood-brain barrier (BBB) which has an increased permeability (16, 17). Notably, elevated levels of the peripheral markers of BBB damage, such as S100B, indicate BBB damage in schizophrenic patients (18). In addition to the peripheral cytokines, microglia, astrocytes, endothelial cells, and even neurons can produce different cytokines within the CNS (17, 19). Moreover, despite the prevailing view that the brain is an immune-privileged area, several studies have shown that immune responses can be established within CNS by several mechanisms, one of which is transferring the immune cells located within the meninges, which are sources of different immune mediators, into the brain's parenchyma in a pathologic state. These cells physiologically transfer through the blood-meningeal barrier in order to pass the meninges as it is more permeable than BBB (20, 21).

These cytokines can have various roles in neurodevelopment, neuroendocrine activities, and neurotransmission. Their role in neurodevelopment is mainly through affecting microglia which are the chief cells responsible for this task (11, 16, 17, 19). Cell migration, water balance, body temperature regulation and synthesis and release of neurotransmitters can be influenced by these mediators (16).

A role for cytokines in schizophrenia was proposed almost three decades ago (22, 23). Ever since, an increasing number of studies investigated alterations of cytokine levels in schizophrenic patients, their changes following antipsychotic treatment, and their relationship with clinical manifestations. However, not only are the results of these studies controversial, but they also do not clearly answer whether the changes in the levels of these cytokines can have a causative role in the development of psychotic symptoms. Moreover, diagnostic and therapeutic implications of these alterations are not defined.

This review aims to answer four questions: A) What are the changes in the serum levels of cytokines in schizophrenic patients? B) What is the relationship between the levels of cytokines and the severity of clinical symptoms? C) How antipsychotics affect the baseline levels of cytokines? and D) What is the potential role of cytokines as predictors of treatment response?

Using the answers to these questions, we investigate whether abnormal cytokine levels are the culprit in the pathogenesis of schizophrenia and also provide clinical implications in terms of diagnosis and treatment.

ALTERATIONS IN THE LEVELS OF CYTOKINES IN SCHIZOPHRENIC PATIENTS

Alterations in the Levels of Pro-Inflammatory Cytokines

A considerable number of studies, including meta-analyses, found increased levels of IL-6 in different groups of patients including, first-episode and drug-naive (FEDN) psychosis patients and cases with first-episode psychosis (FEP), majority of whom were using antipsychotics (24–28). Similar findings were observed in chronic patients, including those without any significant inflammation (29), patients in an acute relapse or recovering from it, and stable outpatients (27, 30–32). Recently, Hartwig et al. found increased levels of soluble IL-6 receptors in a clinical two-sample Mendelian randomization study, which can be explained as a compensatory response to the increased levels of IL-6 in schizophrenia (33).

Conversely, some studies found no significant changes in the levels of IL-6 in schizophrenic patients (34, 35).

Multiple studies, including meta-analyses, reported elevated levels of TNF-α, one of the other pro-inflammatory cytokines, in

FEDN patients (24, 25), (both adult and pediatric) FEP patients, majority of whom were using antipsychotics (26, 27, 36) and chronically ill patients using antipsychotics, regardless of their status in terms of acute relapse (24, 37). Higher levels of TNF-α have also been reported in chronic patients taking atypical antipsychotics having no major inflammation (29).

On the contrary, Potvin et al. conducted a meta-analysis and found no significant alteration in the levels of TNF-α in *in vivo* and *in vitro* studies (30). Their finding may be explained by the scarcity of studies until 2005. Decreased levels of TNF-α are seen in FEDN patients with a disease duration of under two years (37) and chronic patients with a disease duration of more than five years taking typical and atypical antipsychotics (38, 39).

Regarding the next pro-inflammatory cytokine, IL-8, despite meta-analyses supporting elevated levels of it in FEP patients (27) and chronic patients who are stable or are experiencing an acute relapse or are recovering from one (24, 32), a recent meta-analysis showed no significant alterations in the levels of IL-8 in FEP patients versus HC's (32). This is concordant with the findings of another study in FEP patients, most of whom were medicated (36). Concordantly, in a study in which less than a quarter of patients were taking benzodiazepine and others were drug-naive, except for obese cases who had increased levels of IL-8, other patients showed no significant alterations in the IL-8 level (40).

IL-1 family is one of the major pro-inflammatory cytokines, one of the members of which is IL-1β. A large number of studies, including several meta-analyses, found elevated levels of IL-1ß in FEDN patients (25) and adult and pediatric FEP patients, majority of whom were taking atypical antipsychotics (26, 28, 36), and chronically ill patients who were stable or were experiencing an acute relapse or were recovering from one (24, 27, 32, 37). Interestingly, it seems that the levels of IL-1ß mRNA do not change in FEP patients (36).

In contrast to a substantial number of studies suggesting an increase in the levels of IL-1ß, the meta-analysis conducted by Potvin et al. in 2008 found no significant alterations in the levels of IL-1ß in *in vivo* and *in vitro* studies (30). No significant elevation has been reported in chronic patients with a disease period of more than six years, either (41). Interestingly, a recent study has found decreased levels of IL-1ß in FEDN patients with a disease period of shorter than 2 years (37).

Other members of the IL-1 family may be elevated in schizophrenic patients as well. The levels of IL-1α and its leukocyte mRNA were found to be higher in FEP patients who were mostly medicated (36). However, other investigators did not find the same pattern in long-term chronically ill patients (41). A recent study has reported increased levels of IL-33, one of the other members of IL-1 family, and its soluble receptor (sST2) in FEDN patients and patients in an acute relapse compared to patients in remission or HC's (42). Several studies, including a number of meta-analyses, reported increased levels of IL-1 receptor antagonist (IL-1RA) in patients with first-episode psychosis, whether most of the patients used antipsychotics (43) or whether they were drug-naive (40), in chronic patients who were experiencing an acute relapse or had multiple episodes of schizophrenia (24, 27, 32), and in *in vitro* studies (30).

Alterations in the Levels of T-Helper 1 Cytokines

According to the majority of studies (including meta-analyses), the levels of IL-2, one of the cytokines produced by T-helper 1, do not alter in schizophrenic patients. These studies were performed on FEDN patients, FEP patients (with a history of using antipsychotics in many of them), and chronic patients who were experiencing an acute relapse, were recovering from it or were stable (24, 25, 27, 35, 36).

However, several studies found contradictory results. Some of these studies reported increased levels of IL-2 in FEDN patients with a normal BMI (44), FEP patients, majority of whom were using atypical antipsychotics (28), and chronic patients with stable antipsychotic medication regimens combined with a disease period of more than six years (41). Furthermore, some *in vitro* studies found a substantial decrease in the levels of IL-2 (30). Studies also found a decrease in the mRNA levels of this cytokine in the peripheral blood of chronically ill patients who were using antipsychotics for at least a year (45).

Moreover, several studies (including meta-analyses) found a significant increase in the levels of soluble IL-2 receptor (sIL-2R), levels of which might affect the level of IL-2 by binding with it, in FEDN patients and chronic patients who were stable or were experiencing an acute relapse or were recovering from one (24, 25, 27).

Alterations in IFN-γ, the next T-helper 1 cytokine, levels are very controversial. The correlation between IFN-γ and BMI, previously found in patients with first-episode schizophrenia, may explain this inconsistency (32). Due to this great controversy, the reported changes in the levels of this cytokine in each group of patients are discussed separately.

In FEDN patients, several studies, including a meta-analysis, found no significant alterations in the levels of IFN-γ (40, 46–49) while some other studies, including another meta-analysis, showed increased levels of IFN-γ (24, 50). Conversely, Reale et al. found decreased levels of IFN-γ in FEDN patients (51).

Studies showed elevated levels of this cytokine in adult or pediatric FEP patients, most of whom had a history of using antipsychotic medications (26–28). However, Di Nicola et al. found non-altered levels in adult FEP patients, most of whom were on atypical antipsychotics (36).

Several studies, including some meta-analyses, found elevated levels of IFN-γ in chronic schizophrenic patients who were stable or were experiencing an acute relapse (24, 27, 32). However, no significant disruption was found in patients recovering from an acute relapse (27, 46). Decreased levels of IFN-γ were also reported in patients with acute psychotic symptoms who were drug-naive for at least 6 months (52) and in chronic patients. The details of the medications that were used were not reported in these studies (53, 54).

Investigation of the alterations in the levels of IL-12, one of the other T-helper 2 cytokines, showed that several studies (including meta-analyses) found elevated levels in FEP patients, whether drug-naive or not, and chronic patients who were stable or were experiencing an acute relapse, or were recovering from one (24, 27, 55). In the study by Bedrossian

et al., the patients were treated with clozapine for at least one year (55).

In contrast to the studies reporting elevated levels of IL-12, some other studies reported non-altered levels of this cytokine in FEP (28, 43), and in chronic patients who had a history of using antipsychotic medications (32, 45).

Alterations in the Levels of T-Helper 2 Cytokines

Compared to other T-helper 2 cytokines, there are a larger number of studies performed on the alterations of IL-4. Several studies, including two meta-analyses, found no major differences in the levels of IL-4 between HC's and FEDN or FEP patients, majority of whom were taking atypical antipsychotics, or chronic patients who had schizophrenia for a long time (including treatment-resistant patients) (25, 27, 30, 36, 48, 56). However, like other cytokines, a small number of studies reported inconsistent results. Decreased levels of IL-4 were reported in chronic patients experiencing an acute relapse, during their treatment after the relapse (supported by a meta-analysis) (27), and in stable chronic patients (41, 57). In addition, elevated levels were reported in FEP pediatric patients taking antipsychotics (26) and in adult chronic patients taking clozapine (58).

Few studies have investigated other T-helper 2 cytokines, including IL-5 and IL-13. Increased levels of IL-5 were found in chronic adult patients with multiple episodes of unsuccessful treatment and FEP pediatric patients who were mostly taking antipsychotics (26, 59). Similarly, the levels of IL-13 have been reported to be elevated in adults with multiple episodes of schizophrenia (32, 59).

Alterations in the Levels of T-Helper 17 Cytokines

The literature is inconsistent about IL-17 alterations in schizophrenic patients. A recent meta-analysis found no significant changes in the levels of IL-17 in FEDN patients (60). Non-disturbed levels are also reported in chronic patients experiencing an acute relapse (46).

On the contrary, some studies reported increased levels of this cytokine in FEDN (50) and chronic hospitalized patients who were medication free for at least four weeks (61).

However, decreased levels have also been reported in FEDN patients (46) and in chronic patients using different antipsychotics (62).

One of the other main T-helper 17 cytokines is IL-23, which has been reported to be elevated in FEDN and chronic patients in an acute relapse (8, 61, 63).

Alterations in the Levels of T-Regulatory Cytokines

Goldsmith et al. found reduced levels of IL-10 in FEP patients and chronic patients who were in an acute relapse in their meta-analysis (27). Decreased levels are also reported in FEDN patients (64).

However, this meta-analysis showed that the levels of this cytokine do not change in stable chronic patients (27, 45). Other studies have reported no significant alterations in the levels of IL-10 in comparison with HC in FEDN (44), FEP (36), and chronic patients experiencing an acute relapse (65).

Conversely, many investigators have reported elevated levels of this cytokine in FEDN (49, 66), FEP (26), and chronic patients (41, 57, 67).

Several studies, including meta-analyses, found elevated levels of TGF-β in FEDN, FEP patients (27, 46), and in chronic patients experiencing an acute relapse (47).

However, some studies found other findings. Non-disturbed levels of TGF-β have been detected in FEDN and chronic patients who were medication free for four months before the study (18, 44). Interestingly, some studies found decreased levels of TGF-β in chronic treatment-resistant patients (56).

A summary of alterations in the levels of cytokines in schizophrenia based on the meta-analyses is shown in **Table 1**.

RELATIONSHIP BETWEEN CYTOKINE LEVELS AND SEVERITY OF CLINICAL SYMPTOMS

The levels of cytokines seem to be correlated with both the disease duration and symptom severity. Patients with elevated levels of IL-6, IL-8, and IlL-4 tend to have a longer disease duration and longer hospitalizations (31, 65, 68).

Furthermore, higher levels of IL-6, IL-1ß, IL-33, and IL-17 are associated with more severe positive symptoms (28, 42, 50, 69, 70). In chronic patients using a stable dose of antipsychotics, decreased levels of TNF-α are similarly associated with more severe positive symptoms (38, 71), while no correlation has been found in FEDN patients (37).

TABLE 1 | Summary of alterations in serum levels of cytokines in schizophrenia, based on meta-analyses [18, 20, 21).

Increased levels		Non-altered levels		Increased or non-altered		Increased or decreased or non-altered	
Cytokine	**Type**	**Cytokine**	**Type**	**Cytokine**	**Type**	**Cytokine**	**Type**
IL-6	PI	IL-2	TH1	IL-8	PI	IL-10	TR
TNF-α	PI	IL-4	TH2	IFN-γ	TH1		
IL-1β	PI	IL-17	TH17				
IL-12	TH1						
TGF-β	TR						

IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon; PI, pro-inflammatory cytokine; TH1, T-helper 1 cytokine; TH2, T-helper 2 cytokine; TR, T-regulatory cytokine; TH17, T-helper 17 cytokine.

Exacerbated negative symptoms are seen in patients with elevated levels of IL-6, TNF-α, IL-1ß, IL-8, IFN-γ, IL-4, and TGF-β as well as patients with decreased levels of IL-2 and IL-17 (50, 61, 64, 65, 72–75). Interestingly, the correlation between TNF-α and IL-1ß and negative symptoms is only seen in chronic patients and is not reported in FEDN patients (37). Additionally, in FEDN patients, the levels of IL-10 are negatively correlated with negative symptoms, while in chronic patients, they are positively correlated with these symptoms (54, 76).

Increased levels of IL-6, IL-33, sIL-2R, IL-17, and TGF-β are positively correlated with PANSS (positive and negative syndrome scale) general psychopathology sub-score (42, 50, 61, 65). PANSS is a widely used tool to determine the severity of the psychotic symptoms. It is a clinical interview assessing the severity of positive symptoms, negative symptoms, and general psychopathology in schizophrenic patients *via* 30 items. Higher scores indicate more severe conditions (77). The total PANSS score is positively correlated with the levels of IL-6, sIL-2R, IL-1β, IFN-γ, IL-13, TGF-β1 and IL-17 (61, 65, 78, 79). Interestingly, the levels of IL-6 and IL-17 correlate with the total score in both chronic and FEDN patients, while the levels of IFN-γ correlate with the total score only in FEDN patients (50). Moreover, only in chronic patients, decreased levels of TNF-α are associated with higher general and total sub-scores, and no association has been reported in FEDN patients (37, 38, 71).

Regarding the correlation between IL-8 and severity of symptoms, Dahan and his colleagues did not find any association between the levels of IL-8 and PANSS sub-scores. Instead, they reported that patients with higher levels of IL-8 had higher scores of the Clinical Global Impression (CGI) severity scale (a subjective assessment tool to determine the severity of the mental illness by clinicians) (65, 80). However, the levels of IL-8 are reported to correlate with the PANSS mean score positively (79).

The levels of cytokines may be relevant to behavior disorders as well. Surprisingly, worse cognitive abilities are associated with higher levels of IL-6, IL-1RA, IL-33, and IL-12 or lower levels of TNF-α in chronic patients, and with lower levels of IL-10 in FEDN patients (31, 38, 64, 81–86). Moreover, patients with higher levels of IL-10 had a higher total score of the RBANS (Repeatable Battery for the Assessment of Neuropsychological Status) and a worse performance in the attention domain (83, 87). Furthermore, better performance on the memory and intelligence tests has been reported to be associated with higher levels of IL-2 (72). Aggressive behavior is more common among patients with higher levels of IL-17 and IL-10 (54, 61, 76). In addition, in FEDN schizophrenic patients, depressive behaviors are more prevalent among those who have higher levels of IL-4 and TNF-α (79).

Table 2 summarizes the relationship between levels of various cytokines and severity of clinical symptoms.

EFFECT OF ANTIPSYCHOTICS ON THE BASELINE LEVEL OF CYTOKINES

Altered Pro-Inflammatory Cytokines After Antipsychotic Treatment

Shortly (up to 2 months) after treatment with typical or atypical antipsychotics (such as risperidone), the levels of IL-6 and IL-1β seem to decrease (16, 88, 89). However, in the long term, their levels either rise or do not change compared to the baseline levels (70, 90–92).

The increasing trend of IL-6 and Il-1β can be explained by antipsychotics (especially atypical antipsychotics) side effects such as metabolic syndrome in the long period (93), as increased levels of IL-6, IL-1β, TNF-α, IL-2, IFN-γ, and IL-4 have been reported in patients with metabolic syndrome (94, 95).

Unaltered Pro-Inflammatory Cytokines After Antipsychotic Treatment

After using typical or atypical antipsychotics for up to two months, the levels of TNF-α and IL-8 do not change (24, 70, 89, 91, 96). The IL-8 levels seem to remain unchanged even after three months of therapy with risperidone and haloperidol (90). However, one study found that taking typical or atypical antipsychotics or a combination of them in FEDN patients for seven months causes a significant decrease in the level of IL-8 while patients' BMI also increased (97). Furthermore, it has been reported that the levels of TNF-α significantly increase following

TABLE 2 | Relationship between cytokine levels and severity of clinical symptoms.

Negative symptoms		Positive symptoms		Cognitive/intelligence abilities		Total PANSS/RBANS/CGI score		Incidence of depression	Incidence of aggressive behavior
Positive correlation	**Negative correlation**	**Positive correlation**	**Negative correlation**	**Positive correlation**	**Negative correlation**	**Positive correlation**	**Negative correlation**	**Positive correlation**	**Positive correlation**
IL-6 TNF-α IL-8 IL-1β (PI) IFN-γ (TH-1) IL-4(TH2) TGF-β IL-10	IL-2 (TH1) IL-17 IL-10	IL-6 IL-1β IL-33 IL-17	TNF-α	IL-33 TNF-α IL-2 IL-10	IL-6 IL-RA IL-12	IL-6 IL-8 sIL-2R IIL-1β IFN-γ (only in FEDN patients) IL-13 IL-10 TGF-β1 IL-17	TNF-α (only in chronic patients	IL-4	**IL-17** **IL-10**

IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon; PANSS, The Positive and Negative Syndrome Scale; RBANS, The Repeatable Battery for the Assessment of Neuropsychological Status; CGI, Clinical Global Impression.

taking risperidone for more than three months (possibly because of its side effects resulting in induction of metabolic syndrome) (92), or after taking adjunct mood stabilizers with typical or atypical antipsychotics for an average of six weeks of treatment (98). Interestingly, Amisulpride seems to decrease the levels of TNF-α after six weeks of treatment (52).

The level of the anti-inflammatory cytokine, IL-1RA, is reported to decrease following 6 weeks of treatment with olanzapine or risperidone (48) or 8 weeks of antipsychotic therapy adjusted with patients' clinical status (99). Nonetheless, these studies are in contrast with meta-analyses that found no significant alterations in the levels of this cytokine after an average of eight weeks of treatment (70, 91).

Altered T-Helper 1 Cytokines After Antipsychotic Treatment

The levels of IL-2 seem to decrease in the first month after antipsychotic therapy (atypical or typical antipsychotics or mixed) (89). Particularly, olanzapine and haloperidol decrease the level of IL-2 significantly (70). However, studies with longer average treatment periods found no significant changes in the level of IL-2 (24, 70, 91).

Moreover, the levels of IL-12 seem to significantly increase following the use of risperidone for 7–8 weeks (24, 91). Similarly, an elevation in the levels of IL-12 has been reported after six weeks of treatment with olanzapine and haloperidol (100). Surprisingly, aripiprazole (a third-generation antipsychotic) seems to decrease IL-12 levels in chronic patients after four weeks of treatment (101). This is in contrast with the results of a meta-analysis of studies with a treatment period of 4–10 weeks using typical or atypical antipsychotics that found no significant changes in the level of IL-12 after medication (70).

Unaltered T-Helper 1 Cytokine After Antipsychotic Treatment

IFN-γ levels do not significantly change in the first month following antipsychotic treatment (89). However, findings after antipsychotic therapy for an average of approximately 2 months are inconsistent. Two meta-analyses found decreased levels following treatment with typical or atypical or mixed antipsychotics while a meta-analysis by Miller et al., in which more than half of the included studies had non-standardized antipsychotic treatment, suggested the levels of IFN-γ remained constant in this period (16, 70, 91). Olanzapine seems to be the main medication that decreases IFN-γ levels (70). Interestingly, assessing the effect of atypical antipsychotics for 3 months revealed increased levels of IFN-γ, which may be because of their side effects such as metabolic disorder (73).

T-Helper 2 Cytokines After Antipsychotic Treatment

Several meta-analyses have confirmed no disturbances in the level of IL-4 after an average of two months of antipsychotic treatment (70, 91). In contrast, some studies found that treatment with typical or atypical antipsychotics or a combination of them for 1 month or treatment with risperidone for 10 weeks led to decreased levels of IL-4 (102, 46, 68). Thus, IL-4 might be a trait marker that decreases at first but then increases after a certain time due to the metabolic side effects of antipsychotics, which can result in normal levels in the long term. However, to the best we know, no meta-analysis has evaluated the short-term effects. Furthermore, it has been reported that treatment with atypical antipsychotics for eight weeks causes a significant reduction in the levels of IL-13 (78).

Unaltered T-Helper 17 Cytokines After Antipsychotic Treatment

IL-17 and IL-23 seem to be a trait marker whose level does not change after one month of treatment with typical or atypical antipsychotics or a combination of them compared to the baseline level (47, 63, 89). Assessing the effect of risperidone for 10 weeks has shown the same result (102). Interestingly, one study found that a 4-week treatment course with risperidone decreased the number of T-h17 cells while it had no significant effects on the IL-17 levels (50).

Unaltered T-Regulatory Cytokines After Antipsychotic Treatment

The levels of IL-10 do not change following an average of 8 weeks of treatment compared to the baseline levels (70, 91). However, it has been reported that in chronic patients, treatment with aripiprazole, risperidone, or clozapine for 4–6 weeks increased the IL-10 levels (101, 103). The results of treatment with atypical antipsychotics, particularly risperidone, in FEDN patients are different. In these patients, the levels of IL-10 are lower after treatment compared to baseline. Similarly, an average of eight weeks of treatment does not cause a significant alteration in the level of TGF-β (70, 91). However, a meta-analysis by Miller et al., in which more than half of the studies had non-standardized antipsychotic treatment, found decreased levels of TGF-β after 8 weeks of therapy (24). Four weeks of treatment with aripiprazole in chronic patients and 6 weeks of therapy, mostly with atypical antipsychotics in FEDN patients are associated with decreased levels of TGF-β as well (101, 104). However, elevated levels of TGF-β have been reported after four weeks of typical or atypical or mixed antipsychotic treatment in FEDN patients (46).

THE ROLE OF CYTOKINE LEVEL AS PREDICTORS OF TREATMENT RESPONSE

There is a growing body of evidence on the clinical implications of cytokines in schizophrenia. Considering the lack of predictor biomarkers of the treatment response in psychosis, one of the suggested applications is using cytokines to predict response to treatment (73). Furthermore, the relationship between cytokine levels and response to treatment can support the hypothesis that cytokines may play a role in schizophrenia pathogenesis.

Increased levels of IL-6 and IFN-γ are associated with treatment resistance (105, 73). Treatment resistance is defined by not achieving the remission criteria proposed by the Schizophrenia

Working Group Consensus (106) or Kane's criteria (107). Patients with higher levels of IL-8 and IL-2 experience less improvement in PANSS compared to other patients after twelve weeks of therapy with risperidone or haloperidol (90). Moreover, even though the level of TGF-β is not related to treatment response, TGF-ß1 polymorphism is reported to be associated with PANSS score improvement after antipsychotic treatment (108).

DISCUSSION

Abnormal Cytokine Profiles in Schizophrenic Patients

The results showed that schizophrenic patients had an inflammatory cytokine profile and imbalanced T-helper 1, T-helper 2, and regulatory cytokines. The severity of symptoms and abnormal behaviors in addition to antipsychotic therapies may affect abnormal cytokine levels in these patients. However, there were significant inconsistencies regarding the cytokine profile in the reviewed studies in schizophrenic patients. Six reasons may explain these controversies: 1) Diverse Patients' characteristics; 2) Different sampling methods; 3) Heterogeneous patient populations; 4) The difference between cytokine profile of plasma, serum or whole blood; 5) Different specifications of assay kits; 6) Small sample size.

1. Diverse Patients' characteristics: Several studies did not evaluate factors such as the BMI, diet, diurnal rhythm, smoking habits, psychological stress, and lifestyle in their patients, which may affect the cytokines profile (109–112). However, elevated levels of IL-6 are reported in a study excluding patients with a body mass index (BMI) > 25, which gave rise to the conclusion that the changes of IL-6 did not seem to be related to obesity (44).
2. Different sampling methods: The method and duration of storage and the used anticoagulant may affect the measurement of levels of cytokines. Moreover, as the levels of cytokines are influenced by the circadian pattern, the time of sampling is of great importance. Mornings are suggested to be the best time to take samples (113).
3. Heterogeneous patient populations: In some studies, FEDN patients were not separated from patients with a history of antipsychotic treatment. Not only do antipsychotics affect cytokine levels, but they can also cause weight gain, which is considered a low-grade inflammation (93). Thus, studies in a FEDN population are more reliable.
4. The difference between cytokine profile of plasma, serum or whole blood: The cytokine profile of each of these can be different from the other ones as the coagulation process may trigger release of some inflammatory cytokines (113).
5. Different specifications of assay kits: The quality of antibody used in ELISA kits, kit manufacture, and the operator's skills may affect the measurement (113). For example, Hope et al. indicated that different assay kits could affect the measurement of serum levels of different cytokines. In their study, the IL-6 level was higher than the detection limit of the kit in more than half of the samples (82).
6. Small sample size: A considerable number of the studies had small sample sizes (less than 30 participants in each of their groups) that limited their statistical power (54, 114).

Abnormal Cytokine Profile: Culprit, Consequence, or Simple Association?

The question is whether abnormalities in cytokine levels have a causative role in schizophrenia or are merely associated with the disease? We used the Bradford Hill criteria (115, 116) to answer this question. These criteria assess nine factors, including the strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, analogy, and experimental effect to differentiate causality from association. As for the strength of association, the levels of several cytokines (specifically pro-inflammatory cytokines) are found to be significantly disturbed in schizophrenic patients. The second criterion, consistency, is not met in most of the cytokine level alterations. These changes are not specific either and can cause a wide variety of diseases. However, in the modern context, specificity is a less important factor in determining or refusing causality (116). Temporality, as the next criterion, was assessed by only a few studies. In two studies that measured the levels of cytokines before schizophrenia presentation, individuals who developed the disease in the following years had higher baseline levels of IL-6 compared to others (117, 118). This association is strengthened by the study of Khandaker et al. in 2018, finding a strong association between a genetic variant of IL-6 receptor relating to the levels of IL-6 and CRP and development of schizophrenia (119). Moreover, individuals with higher levels of inflammatory markers, including ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein), were more likely to develop schizophrenia (120, 121). The severity of the psychotic symptoms correlated with abnormalities in the levels of cytokines, particularly pro-inflammatory cytokines. So, the biological gradient criterion was met. Regarding plausibility and coherence, there are three suggested major ways by which pro-inflammatory cytokines can contribute to schizophrenia development. First, they can increase kynurenic acid (a metabolite of tryptophan) formation. This metabolite function as an N-Methyl-D-aspartate (NMDA) receptor antagonist, which based on the glutamate hypothesis of schizophrenia (122), might play a causative role in schizophrenia together with decreased glutamatergic signaling. Second, these cytokines increase oxidative stress leading to increased neurodegeneration, which can be seen in schizophrenia. Third, pro-inflammatory cytokines may disturb neurodevelopment (particularly when there is a prenatal inflammation), increasing the risk of psychosis (12, 123). Furthermore, the interplay of cytokines and neurotransmitters may be one of the mechanisms by which cytokines can play a causative role in schizophrenia. Inflammatory cytokines can affect synthesis of monoamine neurotransmitters, increase reuptake of dopamine, serotonin, and norepinephrine, and influence on the release of neurotransmitters (16). Increased levels of soluble IL-6 receptors can be explained as a compensatory response to increased levels of IL-6 (33), and elevated levels of TNF-α mRNA suggest systemic blood immune cells as the main source

of higher levels of TNF-α (36). These two findings provide more experimental evidence for this causation. In terms of analogy, disrupted cytokine levels can be seen in depression and bipolar disorder (114). There is considerable evidence supporting the causal role of cytokines in depression (124). Finally, decreasing inflammation using adjuvant anti-inflammatory agents is reported to improve the symptoms in schizophrenic patients. Consequently, the experimental effect criterion was met as well (125, 126).

CLINICAL IMPLICATIONS

Early Diagnosis

The association between cytokine alterations and schizophrenia may have potential clinical implications. Currently, the diagnosis of schizophrenia is based on clinical symptoms, and there is no standard diagnostic biomarker that allows early recognition of this disease, while cytokines such as IL-6, TNF-α, IL-1ß, and IL-RA can be used as potential biomarkers for early detection of at least a subgroup of schizophrenic patients (118, 127, 128). Furthermore, IL-6, sIL-2r, TNF-α, IL-1RA, and IL-4t can also be useful in detecting the acute-relapse phase of disease (27, 46).

Novel Therapeutic Horizons

Targeting inflammatory pathways may lead to new treatment options in schizophrenia. A growing body of evidence supports the effect of immune modulators on ameliorating the symptoms of schizophrenia. (12, 129). A recent meta-analysis showed that a variety of medications can reduce the severity of symptoms of schizophrenia. These medications are aspirin (by reducing inflammation by modifying cyclooxygenase-2 enzyme), estrogens (through immunomodulatory effects), minocycline (by inhibiting microglia), and N-acetylcysteine (as an anti-inflammatory agent) (130).

Prediction of Prognosis

Prediction of response to treatment is another application of cytokine levels. Patients with increased levels of IL-6, IL-8, IFN-γ, and soluble TNF-α receptor 1 and decreased levels of IL-2 exhibit less improvement after standard antipsychotic therapy (73, 90, 131). Moreover, patients with higher levels of CRP, an inflammatory marker, tend to have lower quality of life (132).

Patient Stratification for Choosing the Best Therapeutic Protocol

It is possible that patients who have higher levels of pro-inflammatory cytokines benefit more from adding specific drugs to the standard therapeutic regimen. Stratifying patients on this basis can potentially help the physicians choose the best treatment option. A randomized clinical trial study in treatment-resistant depressive patients supports this hypothesis. Depression is also associated with a disrupted cytokine profile. This study showed that in a subgroup of patients who had higher baseline inflammation, the use of infliximab—a TNF-α antagonist—led to better results after the treatment. However, infliximab had no significant effects on another subgroup of patients (133).

Future Studies: Causation, Diagnosis, Prognostic, and Therapeutic Applications

Although current studies provide a wealth of information on the cytokines profile in schizophrenic patients, several major shortcomings cannot be overlooked. There is a substantial need for longitudinal studies investigating the levels of cytokines before the development of clinical manifestations of schizophrenia in individuals with a strong positive family history of schizophrenia. Moreover, the potential confounding factors such as age, sex, smoking, obesity, and individuals' diet should be rigorously controlled in the future studies (134).

More studies also need to be performed on the diagnostic and the prognostic applications of measurement of levels of cytokines. The relationship between severity of symptoms and levels of cytokines in FEDN patients can be one of the examples of the diagnostic applications. Identifying treatment-resistant patients based on their cytokines profile can be noted as the prognostic applications, on which more studies are needed. Last but not the least, future studies should be based on defined categories of schizophrenic patients.

Lastly, there is a significant shortcoming in the studies that investigate the therapeutic applications. As an example, selection of patients for adjuvant therapy with medications targeting immune modulatory pathways can be guided by inflammatory cytokine levels (135). Moreover, the role of adjuvant monoclonal antibody immunotherapy, which in contrast to NSAIDs only targets immune pathways, needs to be more investigated (134).

LIMITATIONS

This was a narrative review. Therefore, all the limitations of narrative reviews may apply to this study (136).

CONCLUSION

Schizophrenia is associated with several abnormalities in the levels of cytokines, which are the mediators of the immune system. A deeper understanding of this association can be useful in clinical practice in terms of early diagnosis and treatment.

AUTHOR CONTRIBUTIONS

SM developed the concept and design, collected the data, drafted the article, critically revised the manuscript for important intellectual content, and approved the final version. AZ-S critically revised the manuscript for important intellectual content and approved the final version. NR supervised the project, critically revised the manuscript for important intellectual content, and approved the final version.

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BRIEF RESEARCH REPORT
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Cytokines in Relation to Motor Activity in an Acute Psychiatric Population

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Background: Deviations in motor activity are important clinical features of several psychiatric disorders in an acute state. Immune activity is associated with several psychiatric disorders and may affect motor activity. We aimed to examine the association between immune activity measured as serum levels of cytokines and deviations in motor activity, in an acute psychiatric setting.

Methods: Data on motor activity and immune markers were available on 277 patients admitted to an acute psychiatric inpatient department. The degree of increased or decreased motor activity was clinically assessed at admission. Serum concentrations of the following immune markers were measured: interleukin (IL) -1β, IL-4, IL-6, IL-10, tumor necrosis factor (TNF) -α, interferon (IFN) -γ, and transforming growth factor (TGF) -β.

Results: Scores of increased motor activity were negatively correlated with IFN-γ (rho = −0.128, p = 0.033) in an acute psychiatric population. There was also a trend towards an association between motor activity and TGF-β (rho = 0.118, p = 0.050). In a multiple-linear-regression model correcting for age, gender, and body-mass index (BMI, kg/m^2), the association did not remain significant. No significant correlations between motor retardation and circulating cytokines were found.

Conclusions: After adjustment for potential confounders our study did not reveal any significant association between cytokines and motor activity. However, there is an indication of increased Th17 and decreased Th1 responses in relation to increased motor activity in line with the few previous reports in the field. The phenomenon however needs further exploration.

Keywords: cytokines, psychomotor retardation, agitation, depression, psychosis, acute psychiatric care

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INTRODUCTION

Altered motor activity is gaining increased interest within psychiatric research and may be a prominent finding in an acute psychiatric setting (1, 2). Traditionally, increased or abnormal activity is seen in ADHD, tic disorders, affective disorders, anxiety, and schizophrenia (3). Motor symptoms may also characterize different subtypes of unipolar depression and predict treatment response (2, 4).

Evidence supports a role of immune activity in the etiology and pathogenesis of psychiatric disorders (5). Several studies have demonstrated altered systemic levels of cytokines in patients with schizophrenia, bipolar disorder, and unipolar depression compared to healthy controls (6). These cytokine alterations may also be more prominent in an acute psychiatric setting (6).

Immune activity often is classified into different profiles based on effector mechanisms and characterized by a set of cytokines promoting those effector mechanisms. Th1 profile is characterized by cytokines such as interferon (IFN) -γ and tumor necrosis factor (TNF) -α and mediates potent responses to viruses. Th2 profile is characterized by interleukin (IL) -4 and IL-10. Th2 mediates certain B-cell responses (e.g., immunoglobulin-E production) and opposes Th1. Th17 is characterized by cytokines such as IL-17 and TGF-β and mediates other effector mechanism in the immune system (7).

Cytokines may be the factor mediating altered motor activity in certain psychiatric conditions (8). One mechanism by which cytokines may influence motor activity, is through alterations in neural activity and dopamine metabolism in the basal ganglia (9). It is also shown that treatment with cytokines such as IFN-α induces psychomotor retardation and depressive symptoms in patients with hepatitis (10). Finally, an association between motor activity, psychomotor retardation, and cytokines in outpatients with major depression has been described (11). Agitation is an important clinical syndrome consisting of several symptoms and signs, including increased motor activity. In patients with Alzheimer's disease, a previous study demonstrated that increased IL-1β was associated with agitation (12). However, few previous studies have investigated the association between increased motor activity only and immune markers.

The aim of this study, was to assess the association between circulation levels of cytokines, motor retardation, and increased motor activity in a sample of patients with a variety of severe mental disorders admitted to an acute psychiatric department. Because changes in motor activity are more common symptoms in certain diagnostic groups, unipolar depression and non-affective psychosis were chosen as subgroups.

MATERIALS AND METHODS

Setting and Participants

This cross-sectional study was conducted in the acute psychiatric inpatient wards of St. Olav's University Hospital, Trondheim, Norway. All acutely admitted inpatients between September 2011 and March 2012 were asked to participate. At the time of inclusion, the psychiatric department served a catchment area of 228.000 inhabitants (≥18 years old) and represented the only psychiatric inpatient acute unit in the area. Of the total 654 admitted patients in the inclusion period, 382 (58.4%) patients were included in the study. The study was approved by the regional committee for ethics (REC Central number 2011/137) and registered at ClinicalTrials.gov (NCT01415323). All patients gave their written informed consent prior to inclusion. The inclusion process was conducted by specialists in clinical psychology or psychiatry in order to secure that all included patients had the mental capacity to give their consent. The study was conducted according to the Declaration of Helsinki.

Exclusion Criteria

The following exclusion criteria were applied: (1) chronic or ongoing infections, (2) comorbid autoimmune diseases, (3) C-reactive protein (CRP) levels above 35 mg/L, or (4) lack of patient consent. When patients had multiple admissions, we only included the first admission in our analyses.

Diagnostic Evaluation

Patients were diagnosed according to the International Classification of Diseases-10 (ICD-10) Criteria for Research (13). The diagnoses were set in a consensus meeting in the treatment staff, always including at least two senior psychiatrists of whom one had personally examined the patient. For subgroup analyses, we included patients with non-affective psychosis (ICD-10 F20–29) and unipolar depression (ICD-10 F32 and F33).

Assessments

Sociodemographic history, comorbid medical conditions, smoking status, substance abuse, and psychiatric symptoms were recorded after an interview by a staff member. In addition, participants were screened in a general medical examination and routine blood tests, including CRP and leukocyte count. Height and weight were measured for calculation of the body mass index (BMI, kg/m^2).

The degree of motor retardation and increased motor activity was assessed by an experienced clinician using the Symptomatic Organic Mental Disorder Assessment Scale (SOMAS). SOMAS is a 5-item scale developed to assess atypical depressive symptoms. Item B rates the degree of motor retardation, and item C rates the degree of increased motor activity when the patient was most dysthymic during the previous 24 h (14). Both items are modified from the Positive and Negative Syndrome Scale (PANSS), where item B was modified from PANSS item "motor retardation" (general psychopathology scale, item G7), and item C was assessed from PANSS item "hyperactivity" (positive scale, item P4).

For analyses, patients were subdivided into two groups: with or without increased motor activity according SOMAS item C. If the patients were scored as ≥2 on SOMAS item C, they were grouped as motor active. Similarly, patients were separated into the two groups with or without motor retardation according to SOMAS item B. A score on SOMAS item B ≥ 3 was set to group the patients as motor retarded. In order to simplify the interpretation of findings on SOMAS item B, it was reverse-coded. Therefore, a higher score on both SOMAS item B and C would be interpreted as more severe symptoms of motor retardation or increased motor activity. Subgroup analyses were performed on patients with diagnoses non-affective psychosis group and unipolar depression.

Serum Analyses of Immune Biomarkers

Blood samples were collected on 9 ml serum tubes with SiO2 without gel between 08.00 and 13.00 (median at 10:00) at the first working day after admission. Strict instructions regarding fasting were not given, though most patients would be fasting overnight. Samples were immediately cooled on ice, protected from daylight, and centrifuged within 30 min (15 min, 1,500 g, 4°C). Serum samples were stored at −80°C until further analysis in a registered Biobank (Biobank1, St. Olav's University Hospital, Trondheim, Norway). The following parameters were analyzed bymultianalyte profiling Milliplex MAP assays: IL-1β, IL-4, IL-6, IL-10, TNF-α, and IFN-γ (Millipore Corporation, Billerica, MA, US). TGF-β1 was measured by a Bio-Plex Pro TGF-β Assay (Biorad Hercules, CA, US). Intra- and interassay coefficients of variance were less than 10%. The range of detected values was IL-1β: 0.06-198.72 pg/ml; IL-4: 0.92-286.01 pg/ml; IL-6: 0.10-576.42 pg/ml; IL-10 0.10-1125-33 pg/ml; TNF-α: 0.70-268.89 pg/ml; IFN-y: 0.04-1529.70 pg/ml; and TGF-β: 13.07-415.61 ng/ml. The number of samples and percentage under the detection limit was as follows: IL-1β 236 (74.2%), IL-4: 242 (76.1%), IL-6: 177 (55.7%), IL-10:177 (55.7%), TNF-α: 11 (3.5%), IFN-y: 68 (21.4%), and TGF-β: 0.

Statistical Analyses

Statistical analyses were done using SPSS version 24.0 for Windows. The level of significance was set at $p \leq 0.05$, and all analyses were two-tailed. Significant findings were adjusted for multiple testing with the Bonferroni correction (α/k where k = the seven tested cytokines giving $\alpha/k = 0.007$). Data normality was assessed by using a Kolmogorov-Smirnov test. The distribution of all serum cytokines was skewed, and only TGF-β became normally distributed after logarithmic transformation. Descriptive statistics were calculated by using chi-square tests for categorical variables and student's independent samples t-tests or Mann-Whitney U test (depending on distribution) for continuous variables. The Spearman correlation coefficient was calculated for the relationship between cytokines and SOMAS. Additionally, we examined the difference in cytokine levels between the groups with and without increased motor activity by using student's independent samples t-test or Mann-Whitney U test if the data were not normally distributed. The same statistical methods were applied when comparing cytokine levels between the groups with and without motor retardation.

RESULTS

Sociodemographic, Clinical, and Inflammatory Characteristics of the Sample

Of the total 382 patients included in the study, 24 were excluded due to infection or autoimmune diseases. This left us with 358 patients for whom serum samples were available, and cytokines were analyzed in 318 patients. For 277 of these 318 patients we also had complete measures for altered motor activity (increased or reduced motor activity). The main ICD-10 diagnostic categories in the 277 patients were unipolar depression (22.7%), substance-use disorders (15.9%), schizophrenia (9.7%), bipolar disorder (12.7%), neurotic, stress-related, and somatoform disorders (10.1%), and personality disorders (8.3%), and other diagnoses (20.6%).

The demographic, clinical, and immune data for the total study population, the non-affective psychosis group, and unipolar depression group are given in **Table 1**.

Relation Between Serum Inflammatory Markers and Measures of Motor Activity

When all patients were analyzed together, scores of increased motor activity were significantly negatively correlated with IFN-γ (rho = −0.128, p = 0.033, **Table 2**). In addition, we found a trend

TABLE 1 | Demographic and clinical parameters.

	All patients N = 358	Non-affective psychosis N = 48	Unipolar depression N = 73	*p*-value[a]
Age (years), mean ± SD	38.9 ± 14.8	40.1 ± 11.8	40.1 ± 14.9	0.756[b]
Gender (female), N (%)	174 (49)	19 (40)	42 (58)	0.053[c]
Smoking, N (%)	175 (49)[d]	25 (66)	28 (25)	**0.045[c]**
BMI (kg/m^2), mean ± SD	25.5 ± 5.9[e]	27.6 ± 6.2	24.9 ± 5.4	**0.015[f]**
Higher education (above high school), N (%)	50 (14)	2 (4)	14 (19)	**0.017[c]**
Unemployment (incl. sick leave), N (%)	255 (71)	41 (85)	42 (58)	**0.002[c]**
Alcohol use upon admission, N (%)	99 (28)	9 (19)	15 (21)	0.808[c]
Substance abuse, N (%)	78 (22)	8 (17)	11 (15)	0.813[c]
Motor retardation score[g], mean ± SD	1.6 ± 0.7	1.5 ± 0.8	1.7 ± 0.9	0.205[f]
Motor activity score[g], mean ± SD	1.5 ± 0.9	1.6 ± 1.0	1.3 ± 0.5	0.084[f]
Number with blood samples, N (%)	318 (89)	40 (83)	68 (93)	0.088[c]

[a]*Comparison between non-affective psychosis and unipolar depression. Significance with a p-value < 0.05 is indicated in bold text.*

[b]*Independent students' samples t-test.*

[c]*Chi-square test.*

[d]*Missing 60 (10 within non-affective psychosis group and 11 within unipolar depression group).*

[e]*Missing 89 (15 within non-affective psychosis group and 11 within unipolar depression) observed (data not shown).*

[f]*Mann-Whitney U test.*

[g]*Motor retardation and motor activity were assessed by items form a Symptomatic Organic Mental Disorder Assessment Scale (SOMAS). Both items were scored on a scale from 1–5. BMI, body mass index; SD, standard deviation.*

TABLE 2 | Correlation coefficients (rho) between serum cytokines, motor retardation, and motor activity[a].

	IL-1β	IL-6	TNF-α	IFN-γ	IL-10	IL-4	TGF-β
All patients							
-Motor retardation	0.112	0.066	–0.027	0.090	0.061	–0.043	–0.042
-Motor activity	0.001	–0.010	–0.034	–0.128*	–0.002	0.057	0.118
Non-affective psychosis							
-Motor retardation	0.154	0.165	0.002	0.191	0.194	–0.181	–0.153
-Motor activity	0.052	0.251	0.029	0.000	0.085	0.080	–0.054
Unipolar depression							
-Motor retardation	–0.089	–0.003	–0.047	–0.007	–0.056	0.059	0.173
-Motor activity	–0.046	–0.133	–0.181	–0.173	–0.239	–0.002	0.107

*$p \leq 0.05$.

[a]*Motor retardation and motor activity were scored on a scale from 1–5.*

IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

towards a positive correlation between TGF-β and increased motor activity (rho = 0.118, p = 0.050). No significant correlations where found between cytokines, motor retardation, and increased motor activity analyzing the two subgroups non-affective psychosis and unipolar depression (**Table 2**). In a multiple-linear-regression model correcting for age, gender, and BMI, the associations between increased motor activity and IFN-γ (beta = −0.112, t = −1.726, p = 0.086) and TGF-β (beta = 0.123, t = 1.904, p = 0.058) did not remain significant. No findings remained significant after correcting for multiple testing with the Bonferroni correction.

Comparisons of Cytokine Levels Based on the Presence or Absence of Increased Motor Activity and Motor Retardation

Mean log-transformed values of TGF-β were significantly higher in patients with increased motor activity compared to those with no increase in motor activity (11.31 ± 0.34 vs. 11.20 ± 0.45, t = −2.11, df = 236.41, ES = 0.016, p = 0.036) (**Figure 1**). The increased-motor-activity group had significantly lower levels of IFN-γ compared to the group without increased motor activity (2.27 ± 11.52 vs 6.00 ± 20.04, U = 7213, d = 0.13, p = 0.032) (**Figure 2**). These findings were however not significant after the Bonferroni correction for multiple testing. No other differences in cytokine levels between groups reached the level of significance. When comparing cytokine levels between the two groups with and without motor retardation, no statistical differences were detected (data not shown).

DISCUSSION

After correcting for multiple testing and confounders, we did not find any significant association at the 0.05-level between motor activity and cytokines. However, a trend towards an association between increased motor activity and lower serum levels of IFN-γ and higher levels of TGF-β in patients admitted to an acute psychiatric ward was seen. No statistically significant association

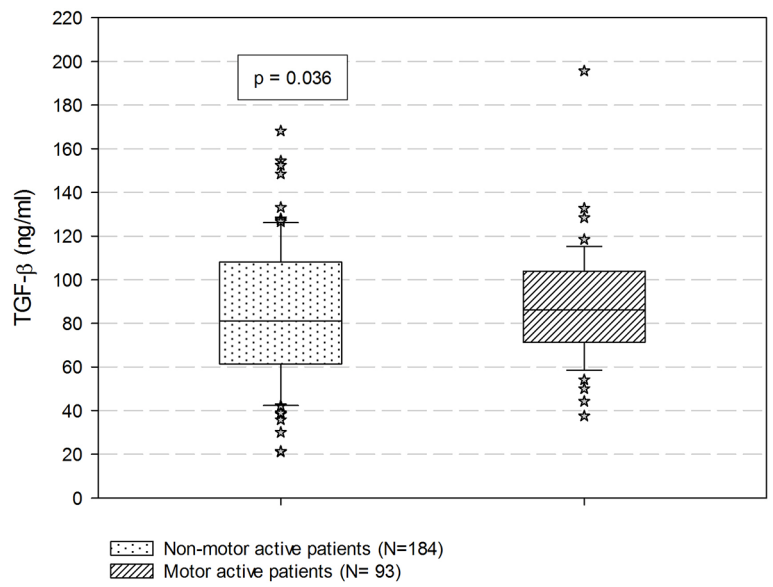

FIGURE 1 | Comparisons of serum TGF-β based on the prevalence of increased motor activity. P-value is estimated by student's independent samples t-test with log transformed values. Data are expressed as median with percentiles. TGF-β, transforming growth factor β.

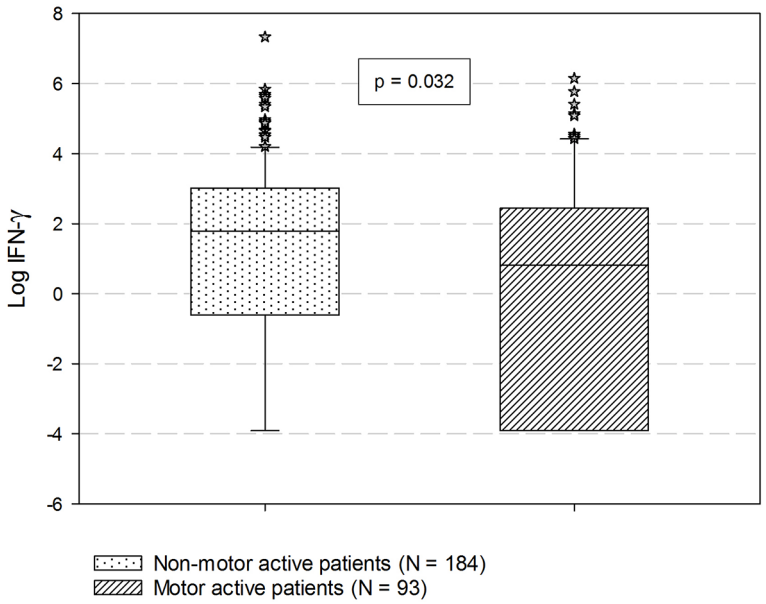

FIGURE 2 | Comparisons of serum IFN-γ based on the prevalence of increased motor activity. P-value is estimated by Mann-Whitney U test. Data are expressed as median with percentiles. IFN-γ, Interferon-γ.

between motor retardation and cytokines was seen. To our knowledge, this is the first report on the relation between immune markers and motor activity in an acute inpatient psychiatric population.

Aggression is a psychiatric sign associated with increased motor activity (15). In a recent study on inpatients with schizophrenia, aggressive behavior was associated with increased levels of Th17 cytokines TGF-β, IL-17, and IL-23 (16). In the present study, we examine motor activity only, but our finding of a trend towards increased TGF-β may be in line with the report on aggressive behavior. However, our findings did not remain significant after corrections for multiple testing and confounders. One might therefore also interpret this previous finding as not being in line with our study.

The present study did not show any significant difference in cytokine levels in relation to motor retardation. This may be somewhat surprising as other studies have indicated a relation between motor retardation and Th1 cytokines. Increased Th1 response has been demonstrated in association with low number of steps per day (17) as well as reduced psychomotor speed (11) in outpatients with major depression. Also, treatment with IFN-α increases the risk of depression and reduced psychomotor speed in patients with hepatitis C (18) and is associated with higher degree of motor retardation in depression (19). However, none of these studies were conducted in an acute psychiatric population, making our results not directly comparable. It is possible that other inflammatory factors are stronger in the acute population masking an association between Th1 and retardation.

There are several limitations and strengths to the current study. We did unfortunately not calculate power for this part of the study. The degree of motor retardation and increased motor activity was assessed by items from SOMAS, which is validated for this purpose. However, the items are published in a previous study (14, 20), and is also shown to correspond with findings in actigraphy (2). For future studies actigraphy should be included. Finally, even though alterations in cytokines in relation to motor activity were influenced by age, gender, and BMI, cytokines still may be clinically important.

Several of the serum cytokines had a high percentage of samples below the detection limit. Although this was not the case for IFN-γ and TGF-β, it may have affected the analyses of other pro-inflammatory cytokines, increasing the risk for type-II error. Further, the results for subgroup analyses are limited by a relatively low number of participants in the subgroups non-affective psychosis and unipolar depression and by the lack of a healthy control group. However, it is interesting as it suggests that alterations in immune activity are more related to symptoms than diagnostic group.

The study population was also relatively heterogeneous with the possibility of confounding factors. Thus, all findings need replication and should be interpreted with care.

We were however able to include severely ill patients in acute states with a variety of psychiatric diagnoses. The blood samples were drawn during the first 24 h of the admission, the period in which the symptoms were most prominent. In addition, the clinic recruiting the study participants is the only acute psychiatry inpatient service in the catchment area, reducing the effect of socioeconomics. All patients in the area needing acute psychiatric services were admitted to this unit. Also, our total sample size is relatively large, compared to other studies in the field.

CONCLUSIONS

Our study comparing levels of immune markers in an acute setting did not reveal any significant associations between altered motor activity and cytokine levels. However, a trend towards a Th17 profile among patients with increased motor activity was seen. The finding should be further explored because it may have implications for predicting and treating deviations in motor activity.

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 Regional Committees for Medical and Health Research Ethics (REC) Central Regional, Trondheim, Norway. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL did all statistical analyses, took a leading role in selection of analyses and interpretation of the results, and wrote the original draft for the manuscript. AS did all the laboratory analyses on cytokines. AS took an equal part in planning and discussing the choice of analyses, interpretation of results and writing of the manuscript. VI supervised on statistics and took an equal part in choice and discussion of the statistical analyses as well as writing of the manuscript. AV initiated the study and headed the inclusion of patients in the clinic. AV took an equal part in interpretation of the results and writing of the manuscript. SR collaborated in initiation and performance of the study in the clinic. SR together with JL took a leading role in choice of laboratory analyses as well as interpretation of the data and in writing of the manuscript.

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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.2019.00920/full#supplementary-material

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ORIGINAL RESEARCH
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CACNA1C rs1006737, Threatening Life Events, and Gene–Environment Interaction Predict Major Depressive Disorder

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Introduction: *CACNA1C rs1006737* is a novel variant in discovery of replicable associations in major depressive disorder (MDD). However, there have been no specific studies considered effect of environmental pathogens to date examining its clinical significance. In this study we investigated the interaction effect between *CACNA1C rs1006737* polymorphism and threatening life events (TLEs) in MDD and carried out a meta-analysis of published findings.

Methods: A total of 1,177 consecutive participants were genotyped. Information on exposure to TLEs, socio-demographic data, and history of psychological problems among first-degree relatives was collected. MDD was diagnosed according to the Chinese version of the 24-item Hamilton Rating Scale for Depression.

Results: There was a significant interaction effect between *CACNA1C rs1006737* polymorphism and TLEs in MDD. A dose–response relationship was found between *CACNA1C rs1006737* genotypes and TLEs in MDD. The results of the meta-analysis showed that *CACNA1C rs1006737* genotypes interacted with TLEs in MDD.

Conclusion: *CACNA1C rs1006737* genotype and previous exposure to TLEs interact to influence the risk of developing MDD. We propose that *CACNA1C rs1006737* may represent a target for novel pharmacological therapies to prevent or treat MDD.

Keywords: *CACNA1C*, polymorphism, threatening life events, gene–environment interaction, major depressive disorder

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INTRODUCTION

Predisposition to complex diseases is not solely conferred by genetic factors; it is also influenced by environmental exposure. Delineating their respective contributions is a major challenge in the study of complex diseases (1–5). Gene–environment interactions (G × E) are thought to account for a large fraction of the unexplained variance in heritability and disease risk (6, 7). However, disease risk due either to environmental exposure and/or its interactions with genotype remains poorly understood (8, 9).

Major depressive disorder (MDD) is an example of a complex disease for which G × E are likely important. An early study on G × E in MDD showed that a length polymorphism (*SLC6A4*) in the promoter region of the *5-HTT* gene mediates the response to stressful life events (10). The *rs1006737* polymorphism in the third intron of the gene encoding the Cav1.2 subunit of the L-type voltage-gated calcium channel gene (*CACNA1C*) (Chromosome12:2345295), which is highly expressed throughout the forebrain (11), has been attributed to G × E. Cav1.2 couples a transient increase in membrane permeability to cell membrane depolarization and gene transcription and plays a critical role in dendritic development, neuronal survival, synaptic plasticity, memory formation, learning, and behavior (12–16). *In vitro* studies have shown that disease-associated increases in Ca^{2+} influx *via* Cav1.2 channels can alter gene expression (17) and contribute to activity-dependent dendrite retraction (18), which can occur in response to chronic stress (19).

Threatening life events (TLEs) precede the onset of depressive episodes more frequently than expected by chance (20); TLEs were shown to cluster before the onset of a depressive episode or an exacerbation of symptoms (21, 22). Although a single stressor may have relatively minor effects, the cumulative effects of multiple stressors (23) can lead to psychiatric disorders, consistent with a dose–response effect (24).

It was recently reported that the *CACNA1C rs1006737* polymorphism mediates the influence of TLEs on human MDD (25). However, this has been contradicted by another study (26). Neither of these investigations addressed the specificity of the *CACNA1C rs1006737* and dose-response effects of TLEs. Examining the perceived threat level of TLEs in the context of *CACNA1C rs1006737* genotype may provide more detailed insight into the nature of genetic effects on stress response.

Measurements of environmental risk can vary across studies; a meta-analysis is one tool for determining whether a result transcends inter-study variation, and is widely used in the field of psychiatric genetics, which has been plagued in recent years by non-reproducibility (27). By pooling data from several studies, a meta-analysis maximizes the power to detect significant effects and avoids overemphasizing estimates from any single study (28).

In this report, we investigated G × E effects between *CACNA1C rs1006737* genotype, TLEs, and MDD in a large clinical sample. A meta-analysis was also carried out to evaluate the current evidence for these interactions.

MATERIAL AND METHODS

Study Population

From November 2014 and December 2017, 590 patients with MDD (420 women and 170 men) were recruited for the study (mean age: 44.22 ± 13.45 years) along with 587 age- and sex-matched control subjects without a history of neuropsychiatric disorders. Both patients and controls subjects were from the same geographic area in Northern China and were of Chinese Han ethnicity, and provided written, informed consent before participation in the study. The study was approved by the Ethics Committee of Harbin Medical University.

Independent Measures

Participants completed three questionnaires: a socio-demographic questionnaire, the Chinese version of the 24-item Hamilton Rating Scale for Depression (HRSD-24), and the Life Events Scale (LES). The socio-demographic questionnaire was used to collect detailed information about socioeconomic background and medical history including individual and family psychiatric history. The HRSD-24 is a reliable tool that has been used in several studies to assess depressive symptoms (29–31). Patients above the threshold (21 points) were included in the study. The LES was used to evaluate negative life events; this self-rating questionnaire consists of 48 items in three areas—i.e., family life (28 items), work-related problems (13 items), and social and other aspects (seven items) (32).

Genotyping

Genomic DNA was extracted from venous blood samples using the AxyPrep Blood Genomic DNA Miniprep kit (Axygen, Union City, CA, USA) and the single nucleotide polymorphism (SNP) rs1006737 of the *CACNA1C* gene was detected by PCR amplification using primers designed with Primer 5.0 software, which had the following sequences: 5'-AAGTTCC ATTCCATCTCAGCCCGAA-3' (forward) and 5'-TGTT TTCAGAGCCGGAGACCTCACA-3' (reverse). SNP analysis was performed using SNaPshot according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using R Studio 1.1.423. The χ^2 test was used to evaluate differences in the distributions of independent variables. Genotype frequencies were tested for Hardy–Weinberg equilibrium. The Bonferroni method was used for multiple-testing correction of genetic association in univariate analysis and the significance level was set at $P < 0.01$ (0.05/5). G × E were examined with a logistic regression model. Four predictor variables were used: *CACNA1C* rs1006737 genotype (*GG*, *GA*, or *AA*), sex, family history, and either the presence/absence or number of TLEs. The dependent variable was the onset of an episode of MDD. Study power was calculated with QUANTO 1.2.4 (http://hydra.usc.edu/gxe/).

For analyses incorporating TLEs, the TLEs were coded so that 0 represented no TLE occurrence and values of 1, 2, 3, or ≥4 represented the occurrence of TLEs that were minor, low-moderate, high-moderate, and severe, respectively. To simplify the interpretation of interactions, the number of TLEs was coded using four dummy variables (X1, X2, X3, and X4). If there was no TLE, all four were coded as zero. For example, if there was one, two, or three TLEs, X1, X2, and X3, respectively, were coded as 1. Thus, the coding for three TLEs was: X1 = 1, X2 = 1, X3 = 1, and X4 = 0. This method of coding dummy variables known as thermometer coding does not alter the model results but is simpler yet mathematically equivalent to contrasts (33); compared to typical indicator variables, it greatly simplifies the

model selection process. Removing a level of a standard indicator variable requires recoding the data and a likelihood ratio test; with thermometer coding, the task is not different from removing other independent variables.

We conducted a meta-analysis by pooling results from previous G × E studies of the *CACNA1C rs1006737* polymorphism, TLEs, and MDD with findings from the present study. The Lipták–Stouffer z-score approach was used to obtain an aggregate value for the significance level of tests weighted by sample size, and a sensitivity analysis was conducted by recomputing effect size after systematically removing each study in turn. To gauge potential publication bias, we calculated fail-safe N and its ratio (34, 35).

RESULTS

Frequencies of Independent Variables

Demographic and genotypic data for the study population are shown in **Table 1**. About half of subjects had TLEs, and one in nine had a family history of psychological problems among first-degree relatives. Approximately half of subjects had the *G/G* genotype, one in three the *G/A* genotype, and the remaining subjects the *A/A* genotype. Genotype frequencies were in Hardy–Weinberg equilibrium among both cases and controls.

Associations With MDD

There were significant differences in genotype (χ^2 = 6.36, P = 0.04), homozygosity (χ^2 = 5.47, P = 0.01), TLEs (χ^2 = 64.27, P = 1.105e^{-14}), and family history (χ^2 = 66.55, P = 3.408e^{-16}) distribution between patients with MDD and controls (**Table 2**). TLEs (P < 0.01) and family history (P < 0.01) were still associated with MDD after Bonferroni correction. On the basis of sample size of the study, the power for association study of *CACNA1C rs1006737* was 98.92%.

Interaction Between TLE Occurrence and *CACNA1C rs1006737* Genotype in the Prediction of MDD

In our initial analyses, which considered only the presence or absence of TLEs, a full model was first generated including *GG/GA/AA* genotype, sex, family history, and the occurrence of TLEs. We then selected the optimal model based on the Akaike information criterion. The dominant mode of action that combined the effects of *GA* and *AA* genotypes showed an improvement in fit. This best-fit model suggested significant main effects of family history (β = 8.56, s.e. = 0.37, P = 1.29e^{-08}) and TLE occurrence (β = 2.08, s.e. = 0.15, P = 1.26e^{-06}) but not of sex (β = 0.83, s.e. = 0.13, P = 0.17) or genotype (β = 0.91, s.e. = 0.17, P = 0.55) for predicting MDD (**Table 3**). However, a significant genotype × TLE interaction was found (β = 1.81, s.e. = 0.27, P = 0.02). Estimates based on this model indicated that family history and TLE exposure could influence the prediction of MDD, and that genetics alone cannot predict MDD but can modify the risk effect conferred by exposure to TLEs. On the basis of sample size of the study, the power was 87.59% to detect a significant effect of rs1006737× TLE interaction on MDD under dominant genetic model.

Interaction Between Number of TLEs and *CACNA1C rs1006737* Genotype in the Prediction of MDD

Based on the evidence for an interaction between *CACNA1C rs1006737* genotype and TLE exposure in the prediction of MDD, we explored how this polymorphism alters the dose-response relationship between number of TLEs and risk for MDD onset.

We generated a full model encompassing the dominant action model (*GG* vs. *GA/AA*), TLEs, sex, and family history. Two of the four possible interactions with genotype and number of TLEs were retained. The final model for the prediction of MDD included sex, family history, minor threat, low-moderate threat, high-moderate

TABLE 1 | Summarized frequencies of socio-demographic and independent variables.

Variables	Frequencies
Socio-demographic variables	
Gender	
Female	802 (68.1%)
Male	375 (31.9%)
Mean age	43.6 (s.d. 11.55)
Independent variables	
CACNA1C(rs1006737) genotypes	
G/G	770 (65.4%)
G/A	365 (31.1%)
A/A	42 (3.5%)
Exposure to threatening experiences	
No TLE	592 (50.2%)
1 TLE	284 (24.2%)
2 or more TLEs	301 (25.6%)
Family history of psychological problems among first-degree relatives	
FH+	102 (9%)
FH–	1,075 (91%)

FH, family history; s.d., standard deviation; TLE, threatening life events.

TABLE 2 | Association between depression and genetic or environmental factors.

	Case	Control	χ^2	df	*P*
Genotypes			6.36	2	0.04
G/G	384 (65)	386 (66)			
G/A	177 (30)	188 (32)			
A/A	29 (5)	13 (2)			
Homozygous			5.47	1	0.01
*G/**	561 (95)	574 (98)			
A/A	29 (5)	13 (2)			
Alleles			1.08	1	0.29
G	945 (80)	960 (82)			
A	235 (20)	214 (18)			
Threatening life events			64.27	2	1.105e–14*
No	229 (39)	363 (62)			
1	167 (28)	117 (20)			
2 or more	194 (33)	107 (18)			
Family history			66.55	1	3.408e–16*
Negative	499 (85)	576 (98)			
Positive	91 (15)	11 (2)			

**P <0.01 after Bonferroni correction.*

TABLE 3 | *CACNA1C* genotype interaction with threatening life experiences.

	β (95% CI)	SE	P
Sex	0.83 (0.63–0.99)	0.13	0.17
Family history	8.56 (4.34–19.46)	0.37	1.29e–08
No/Any TLE (E)	2.08 (1.55–2.81)	0.15	1.26e–06
CACNA1C genotype (*G*)	0.91 (0.64–1.26)	0.17	0.55
Gene (*G*) × Environment (E)	1.81 (1.06–3012)	0.27	0.02

threat, main effects of *CACNA1C rs1006737* genotypes, and the interaction between genotype and TLE values of 1 and 3 (**Table 4**).

The main effects of *CACNA1C rs1006737* genotypes (β = 0.90, s.e. = 0.17, P = 0.55), sex (β = 0.84, s.e. = 0.13, P = 0.20), and a TLE of 2 (β = 0.85, s.e. = 0.26, P = 0.56) were non-significant in this final model. Conversely, those of a TLE of 1 (β = 0.1.81, s.e. = 0.18, P = 0.001) or 3 (β = 2.16, s.e. = 0.31, P = 0.01) and family history (β = 8.35, s.e. = 0.37, P = $2.05e^{-08}$) were significant. Importantly, we found that the *CACNA1C rs1006737* genotype interaction with a TLE of 1 (β = 2.02, s.e. = 0.17, P = 0.04, γ = 2.37) was significant, with individuals harboring the *AA* and *GA* genotypes showing greater sensitivity to the depression-inducing effects of a TLE of 1 than those with the GG genotype. We also observed a significant interaction between genotype and a TLE of 3 (β = 0.28, s.e. = 0.61, P = 0.03, γ = 0.13)—that is, high exposure to TLEs was associated with an increase in risk for MDD in all genotypes. Interaction coefficients (γ) ranging from a TLE of 1 to 3 indicated a low exposure–gene effect between exposure level and *CACNA1C rs1006737* polymorphism in MDD. In contrast, the interaction between *CACNA1C rs1006737*genotypes and a TLE of 2 (β = 1.54, s.e. = 0.52, P = 0.40) was non-significant in this final model (**Table 4**). Estimates based on this model indicated that family history and TLE exposure still have main effects on the prediction of MDD, and that genetics can modify the risk effect conferred by exposure to TLEs from a minor threat to a high-moderate threat.

Meta-Analysis

We included studies in our meta-analysis that met three criteria: the study had to be published in a peer-reviewed journal, and include genotypic information on the *CACNA1C rs1006737* gene as well as a measure of TLEs. After searching the PubMed, Wolters Kluwer, and Web of Science databases, we identified two previous studies that met all three criteria (8.9). The results of these studies were pooled with the present findings to assess the interaction between *CACNA1C rs1006737* polymorphism and TLEs in MDD in a total of 8,728 subjects (**Table 5** and **Figure 1**). The significance of the results remained robust in the sensitivity analysis when each study was removed in turn, with the exception of one study (0.0001 < P < 0.01). To render the outcome in the analysis non-significant (P = 0.05), an additional four unpublished or undiscovered studies with average sample size of n = 2909 and a non-significant result (P = 0.50) would be required. This yielded a fail-safe ratio of one study excluded for each one included in the meta-analysis.

DISCUSSION

Prior studies documenting the interaction effect between *CACNA1C rs1006737* genotypes and TLEs in MDD have reported a positive interaction effect (25) or contrary findings (26). However, neither of these studies focused on a single marker, resulting in low *a priori* probability and power. Additionally, the influence of the *CACNA1C rs1006737* polymorphism on the dose-response relationship between TLEs and risk for MDD was not reported in either study. Here we attempted to replicate the prior finding that *CACNA1C rs1006737* genotypes modified the depressogenic effects of TLEs. Our second goal was to clarify the dose-response relationship between TLEs and *CACNA1C rs1006737* genotypes in MDD. Finally, we carried out a meta-analysis of the interaction between *CACNA1C rs1006737*genotypes and TLEs in MDD to evaluate the current evidence.

Our main findings were that *CACNA1C rs1006737* genotypes and TLEs were independently associated with MDD, and that *CACNA1C rs1006737* genotypes significantly modified the risk conferred by TLEs for MDD; moreover, a dose-response relationship was found to exist between *CACNA1C rs1006737* genotypes and TLEs in MDD, with the meta-analysis confirming an interaction between these variables.

Previous association studies of MDD have suffered from low rates of reproducibility. Instead of a main effect of genotype on phenotype, G×E effects were reported. Since the latter are more difficult to detect than the former (36, 37), replications may be expected to be more rare and have a specific value when they do occur. We first analyzed the interaction between TLE occurrence and *CACNA1C rs1006737* genotypes in the prediction of MDD and found a better-fitting model after adjusting for potential confounds such as sex and family history. There is conflicting evidence regarding the impact of sex on depressive disorder, with

TABLE 4 | *CACNA1C* genotype interactions with threat exposure.

	β (95% CI)	SE	P
Sex	0.84 (0.65–1.09)	0.13	0.20
Family history	8.35 (4.22–19.01)	0.37	2.05e–08
Minor threat	1.81 (1.26–2.59)	0.18	0.001
Low-moderate threat	0.85 (0.51–1.45)	0.26	0.56
High-moderate threat	2.16 (1.18–3.97)	0.31	0.01
CACNA1C genotype (*G*)	0.90 (0.64–1.26)	0.17	0.55
Gene (G) × Minor threat	2.02 (1.03–4.06)	0.34	0.04
Gene (G) × Low-moderate threat	1.54 (0.56–4.39)	0.52	0.40
Gene (G) × High-moderate threat	0.28 (0.08–0.93)	0.61	0.03

TABLE 5 | Studies on the interaction between *CACNA1C* polymorphism, threatening life events, and depression included in the meta-analysis.

Source, year	No. of Subjects	1-Tailed P Value	P Value After Study Exclusion
Lavebratt et al. (26)	2,743	0.77	0.0001
Dedic et al. (25)	4,808	0.001	0.41
Zhao et al. (34)	1,177	0.01	0.01
Total:	**8,728**		
Average sample size:	**2909**	**0.003**	

P = 0.003 stands for overall effect size which is significant.

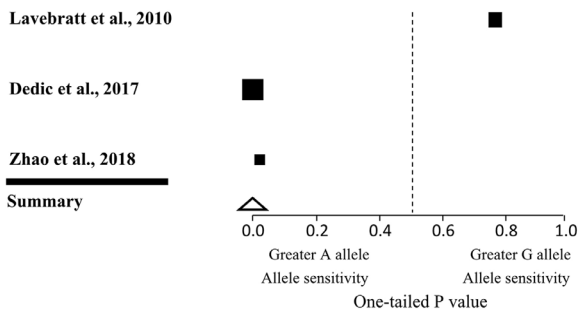

FIGURE 1 | Forest plot of three human observational studies on the interaction effect of *CACNA1C* genotype and life stress on depression. Squares indicate the one-tailed P value for each study, with lower values denoting greater stress sensitivity of *A* allele carriers and higher values corresponding to greater stress sensitivity of *G* allele carriers. The size of the box reflects the relative sample size. The triangle indicates the overall result of the meta-analysis.

some studies reporting it as valid for both sexes (38–41), and others suggesting an effect only in women (42, 43) or the inverse effect in men (44). We found a significant sex difference between patients with MDD and controls. Family history of psychological problems is associated with both exposure (45) and outcome (46); we also found a significant difference in family history between patients with MDD and controls, and therefore included this parameter in the model. In the regression analysis, family history remained significant but not for sex and TLEs, and had a main effect but not for genotype. Importantly, a genotype × TLE interaction was observed. These findings suggest that the *CACNA1C rs1006737* polymorphism does not have a main effect on MDD by itself, but does in combination with TLEs.

We then analyzed the interaction between number of TLEs and *CACNA1C rs1006737* genotypes in the prediction of MDD. Sex and family history were retained in the model, although the effect of sex was non-significant. Our results showed that individuals with the *AA* or *GA* genotype had greater sensitivity to the depressogenic effects of a TLE of 1 than those with the *GG* genotype, and that high exposure to TLEs was associated with a marked increase in the risk for MDD for all genotypes. The dose–response effect analysis revealed a low exposure-gene effect. Exposure levels were measured based on retrospective reporting by participants, and bias in this data could have influenced the dose-response effect. In many cases, cumulative measurements can be obtained by making repeated measurements over time, which enhances power to detect G × E (47). The dose–response effect in our study suggests that elucidating the mechanism underlying the progression from genetic variation to MDD requires more precise measures of environmental risk factors and stressful experiences.

A strength of this study is that it included a meta-analysis as well as an analysis of original data. The former provided evidence of a *CACNA1C rs1006737* genotype × TLE interaction effect in MDD. However, our previous meta-analysis of G × E (34, 35) showed that a subgroup analysis stratified by type of stressor, study design, or subjects' ancestry should be carried out wherever possible in order to reduce confounds for the G × E effect.

Our study also had some limitations. Firstly, data on environmental pathogens were collected from subjects' retrospective reports, which has risks such as forgetting, revisionist recall, and bias due to cognitive dysfunction or low mood (48). Secondly, mistreatment in childhood was not considered as an independent environmental risk factor separate from TLEs although it can affect the development of neural circuitry and is therefore a good candidate to study G × E effects in mental disorders (49, 50).

In conclusion, we provide evidence supporting an effect modification by the *CACNA1C rs1006737* genotype on the risk of MDD conferred by previous exposure to TLEs. Thus, *CACNA1C rs1006737* is an example of a gene that influences vulnerability to MDD not by a main effect on risk but rather by modulating sensitivity to the negative effects of the environment. Future work will include genome-wide association studies data to test for G × E interactions.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because the datasets were also used in another study which is not published yet. Request to access the datasets should be directed to YY, yanjie1965@163.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MZ and JY conducted the statistical analyses and wrote the first draft of the manuscript. XQ and XY provided expertise in MDD search. ZQ and XS collected the data. LW and EZ did the experiment. YY and DC designed this study and provided expertise. All authors were involved in modifying the secondary-analysis design and editing the manuscript. All authors contributed to and have approved the final manuscript.

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Lower Serum Uric Acid Is Associated With Post-Stroke Depression at Discharge

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Background: Serum uric acid (SUA) has been shown to play an important role in the pathophysiology of mood disorders including 3- and 6-month post-stroke depression (PSD). This study aimed to investigate whether SUA levels on admission were associated with PSD at discharge.

Methods: A total of 498 stroke patients were consecutively recruited from Tongji Hospital. Clinical and laboratory test data were collected on admission. They were categorized into equal tertiles according to the distribution of SUA and the number of patients. PSD status was evaluated by DSM-V criteria and 17-item Hamilton Rating Scale for Depression at discharge.

Results: The optimal cut-off points of SUA were: (T1) 80.00~300.80 μmo1/L, (T2) 300.81~391.67 μmo1/L, (T3) 391.68~710.0 μmo1/L. A total of 232 patients (46.59%) were diagnosed as PSD at discharge. Significant differences were found between the PSD and non-PSD groups in SUA tertiles of patients (P = 0.00). After adjustment for conventional confounding factors, the odds ratios of PSD were 5.777 (95% CI = 3.463~9.637, P = 0.00) for the lowest tertile and 4.153 (95% CI = 2.492~6.921, P = 0.00) for the middle tertile of SUA, as compared with the highest tertile. In restricted cubic spline regression, continuous SUA showed linear relation with PSD risk at discharge after 300 μmol/L.

Conclusions: Lower SUA levels on admission were found to be associated with PSD at discharge and the threshold effect was also revealed. For stroke patients, doctors should pay attention to the baseline SUA for screening high-risk PSD at discharge in clinical practice.

Keywords: post-stroke depression, serum uric acid, restricted cubic spline regression, antioxidants, threshold effect

INTRODUCTION

Post-stroke depression (PSD) is the most frequent neuropsychiatric sequela after stroke, affecting about 40% of stroke patients (1, 2). It is well known that PSD was associated with reduced quality of life (QoL), poorer functional outcome, as well as increased cost of treatment, burden of family caregiver, and mortality (1, 3–5). Moreover, a recent meta-analysis reported that patients with early PSD had a mortality risk about 1.5 higher than non-depressed individuals, considering both short- and long-term mortality (6). The depression effects on cardiovascular risk, such as unhealthy lifestyle behaviors and lower adherence to treatment, the higher stroke severity, possible negative effects of a SSRI treatment on survival, and non-natural causes of death like suicide may be the potential interpretations (6). Although the importance of PSD has been well documented and there are validated screening tools for PSD, many PSD patients cannot be diagnosed and rate of refusal by busy stroke clinicians to recommend antidepressant treatment remain high. One reason is that there are no reliable objective biomarkers for diagnosing and predicting PSD.

To date, there is a lack of understanding of what the exactly underlying pathophysiological mechanisms of PSD are (7). Among these discovered biological factors, oxidative and nitrosative stress pathways are regarded as the most conclusive factors because there are low antioxidant levels and high metabolic rates and levels in the brain (8, 9). As we know, the brain is a site of excessive reactive oxygen species (ROS) production and especially vulnerable to oxidative stress, as it accounts for 20% oxygen consumed by the body (10). Hence, brain neurons damaged by oxidate stress may lead to altered membrane structure and function, which may further affect the expression of membrane receptors leading to increased risk of depression (11, 12). Serum uric acid (SUA) is an important antioxidant which is the end product in the degradation of the purine nucleotides adenine and guanine, accounting approximately 60% of the total antioxidant capacity in plasma (13). Meanwhile, SUA is a low-cost indicator which can be easily obtained on admission. A recent meta-analysis has shown that subjects with major depressive disorders (MDD) have levels of the antioxidate uric acid (UA) lower than healthy controls (14). Moreover, previous studies have shown that the lower SUA level is associated with both PSD and depression (9, 15). However, the assessment on the association between SUA and PSD only aimed at 3- and 6-month outcomes. It is still lacking objective and quantitative biological factors to assess PSD at discharge. When the early onset of PSD is ignored by clinicians, it will adversely affect the early rehabilitation after discharge, which is the most important stage for stroke rehabilitation. Thus, we need effective biological predictors in order to more efficiently identify and diagnose PSD at discharge, and then intervene to promote early recovery of stroke.

Therefore, considering that SUA is proven to be an effective predictor of 3- and 6-month PSD, we hypothesized that the SUA may also serve as a predictor of patients with PSD at discharge. The aim of this study was to assess the association between SUA on admission and the PSD outcome at discharge.

METHODS

Study Design

All first-ever stroke patients were consecutively recruited within 7 days of the onset of symptoms from the Tongji Hospital which is located in Wuhan City, Hubei Province, China, between May 2018 and October 2019. SUA was obtained within 24 h after admission. There are few clinical concerns about the decreased odds ratio (OR) value in PSD for each unit change of UA, which could be obtained by taking SUA as a continuous measure. Therefore, we divided the SUA into tertiles to observe whether any enhanced performance could be quantified while maintaining statistical effect in each category, according to the patients' amount and the skewed distribution of the raw SUA values (7, 16). All patients involved in this study or their family members gave written informed consents according to the Declaration of Helsinki. The approval of the study for experiments was obtained from the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

The registration number of this prospective cohort study was ChiCTR-ROC-17013993. The URL of the publicly accessible website on which this trial is registered is: http://www.chictr.org.cn/index.aspx. The original protocols used for this *post hoc* analysis did not include SUA as a potential predictor.

Inclusion and Exclusion Criteria

All suspected stroke patients were confirmed by magnetic resonance imaging or computerized tomography within 7 days after admission and the following inclusive criteria were used: (i) males and females, age ≥ 18 years old, first-ever diagnosed stroke patients, including ischemic and hemorrhagic stroke; (ii) hospitalized within 7 days after stroke onset; (iii) written informed consent was provided.

The exclusive criteria were as follows: (i) brain dysfunction caused by other non-vascular causes, such as primary brain tumors, subdural hematoma, paralysis after seizures, metastatic encephaloma, brain trauma, etc.; (ii) history of depression (previous treatment history or clinical diagnosis), dementia and/or other psychiatric illness; (iii) communication problems due to aphasia, dysarthria, disturbance of understanding or consciousness (a Mini-Mental State Examination score was <19 points, in particular the MMSE score of illiterate patients was <17 points); (iv) unable to complete the follow up; (v) Transient ischemic attack and subarachnoid hemorrhage; (vi) with other concomitant neuropsychiatric diseases, such as Parkinson's disease and epilepsy.

Serum samples were collected at room temperature on admission, then centrifuged at 3,500 r/min for 10 min, which could be used to measure the levels of serum biomarkers. Depressive symptoms were measured at discharge, while the baseline sociodemographic information, clinical characteristics, and routine laboratory indicators were collected on admission.

Data Collection and Follow-Up

Standard patient demographic data was collected with a case report form at baseline, covering gender, age, body mass index

(BMI), marital status, degree of education, and vascular risk factors, including smoking, drinking, and history of stroke, diabetes mellitus, hypertension, hyperlipidemia, coronary heart disease, and surgery. The stroke severity was assessed within 24 h of hospital admission by well-trained doctors using the National Institutes of Health Stroke Scale (NIHSS) score. Barthel Index (BI) score, mRS score, and hospitalization days were also included into the variables. The concentrations of SUA, serum albumin (ALB), homocysteine (Hcy), hypersensitive C-reactive protein (Hs-CRP) were measured by standard autoanalyzer techniques with a Roche automatic analyzer (cobas c 701) in clinical lab of Tongji Hospital. The inflammatory factors, including IL-1β, IL-6, IL-10, IL-18, TNF-α, BDNF, and IFN-γ, were measured using a solid-phase sandwich enzyme-linked immunosorbent assay kit (CUSABIO, China) according to the manufacturer's specifications in Kindstar Company, Wuhan. To minimize assay variance, all samples were analyzed on the same day in duplicate in a random order by a technician blind to the clinical diagnoses; the intra-assay coefficients were <5%.

Psychological Measurement

All psychological evaluations were performed by two experienced psychiatrists (X.S. and W.S.) who were blinded to other clinical and laboratory findings after receiving standardized training. The interrater reliability reached an acceptable level. PSD was diagnosed by a psychiatrist at discharge according to DSM-V criteria. Seventeen-item Hamilton Rating Scale for Depression (HRSD) was used to measure the degree of PSD at discharge. The DSM-V diagnostic criteria (Depressive Disorder Due to Another Medical Condition) was met, and HRSD score≥7 at discharge, which was regarded as the primary endpoint. Patients were divided into PSD group and non-PSD group according to whether they had PSD outcome or not. The validity and reliability of the Chinese HRSD version had been proven in previous studies (17).

Statistical Analysis

Results were expressed as percentages for categorical variables and as medians [interquartile range (IQR)] or means ± standard deviation (S.D.) for the continuous variables, depending on the normal or nonnormal distribution of data by Kolmogorov-Smirnov test. Proportions were compared using the Chi-squared test, Student's t-test, and analysis of variance (ANOVA) were employed for the normally distributed variables, while the Mann–Whitney U-test was used for the asymmetrically distributed variables. Statistical comparisons among SUA stratification were assessed by Pearson's Chi-square test or Fisher's exact test for categorical variables, and continuous variables were evaluated by Kruskal–Wallis test or ANOVA. After adjusting for main baseline variables identified in the univariate logistic regression analysis and traditional confounders related to PSD, the OR values and 95% confidence intervals (95% CIs) for PSD risk were obtained by multivariate-adjusted binary logistic regression. The restricted cubic spline (RCS) regression was used to test the linear association between PSD and SUA as a continuous measure with three knots.

All statistical analysis was performed with R version 3.5.2 and SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was identified as a two-tailed *P* values less than 0.05 ($P < 0.05$). The R packages "rms", "Hmisc", "Formula" and "ggplot2" were applied.

RESULTS

Baseline Characteristics of all Patients in SUA Tertiles

A total of 707 stroke patients from Tongji Hospital between May 2018 and October 2019 were recruited in the study, and 567 patients were eligible for the research. By the time of discharge, there were 69 patients failed to be followed up. Ultimately, we included a total of 498 stroke patients, which consisted of 379 males (76.10%) and 119 females (23.90%). Their mean age was 57.17 ± 10.88 years (**Table 1**). We divided all cases into three groups according to tertiles of SUA levels, which ensured the most categories with adequate number of patients per subgroups between the range of 80.00 and 710.00 μmo1/L (T1, 166 patients; T2, 166 patients; T3, 166 patients).

The cut-off values for this stratification on the SUA level into tertiles were: (T1) 80.00~300.80 μmo1/L, (T2) 300.81~391.67 μmo1/L, (T3) 391.68~710.0. **Table 1** summarized the characteristics of the patients by the tertiles of SUA, including the sociodemographic, clinical, and laboratory characteristics. Patients with the low, moderate, and high SUA were significantly different in following sociodemographic and clinical variables: age, gender, BMI, smoking, drinking, history of diabetes and hypertension, HRSD score at discharge, baseline NIHSS score, BI score, and mRS score. The majority of patients had drinking (60.64%) and smoking habits (59.64%) and the lowest and middle SUA tertiles were significantly associated with higher HRSD score than the highest tertile ($P = 0.00$). And there were statistical differences observed for ALB, Hcy, Hs-CRP, IL-1β, and IL-18 in the SUA tertiles of patients (all *P* values < 0.05). Some significant differences in baseline characteristics were reasonable because the patients were consecutively recruited.

Baseline Characteristics of Patients in PSD Group and Non-PSD Group

The baseline characteristics between PSD and non-PSD groups are presented in **Table 2**. In this study, 232 (46.59%) patients were diagnosed as PSD at discharge. Compared with non-PSD group, PSD patients were more likely to be older and with lower educational level and BI scores, higher proportion of coronary artery disease history, surgery history, HRSD scores, baseline NIHSS, and mRS scores.

We also divided the SUA levels into hyperuricemic and non-hyperuricemic groups, in which hyperuricemia is defined as 416.5 μmol/L (7.0 mg/dl) or higher in males, and 357 μmol/L (6.0 mg/dl) or higher in females. The association between

TABLE 1 | Baseline characteristics of patients with stroke according to SUA tertiles.

Variables	All patients	SUA tertiles			*P*-value
		Tertile 1, n = 166 (80.0~300.80)	Tertile 2, n = 166 (300.81~391.67)	Tertile 3, n = 166 (391.68~710.0)	
UA, median (IQR)	328.0 (275.7, 410.00)	253.00 (215.25, 276.00)	328.00(328.00, 361.63)	410.00 (410.00, 448.00)	0.000
Demographic parameters					
Age (years)	57.17 ± 10.88	58.25 ± 9.88	58.01 ± 11.34	55.23 ± 11.17	0.019
Females, n (%)	119 (23.90)	57 (34.34)	34 (20.48)	28 (16.87)	0.000
BMI (kg/m^2)	24.96 ± 3.54	24.25 ± 3.27	25.05 ± 3.69	25.59 ± 3.53	0.002
Married, n (%)	483 (96.99)	161 (96.99)	160 (96.39)	162 (97.59)	0.814
Education level					0.111
Junior middle school and below, n (%)	306 (61.45)	114 (68.67)	99 (59.64)	93 (56.02)	0.051
Senior high/polytechnic school, n (%)	126 (25.30)	38 (22.89)	42 (25.30)	46 (27.72)	0.601
Bachelor and above, n (%)	66 (13.25)	14 (8.43)	25 (15.06)	27 (16.27)	0.077
Vascular risk factors					
Smoking, n (%)	297 (59.64)	79 (47.59)	96 (57.83)	122 (73.49)	0.000
Drinking, n (%)	302 (60.64)	87 (52.41)	104 (62.65)	111 (66.87)	0.021
History of diabetes, n (%)	106 (21.29)	45 (21.11)	41 (24.70)	20 (12.05)	0.002
History of hypertension, n (%)	273 (54.82)	88 (53.01)	103 (62.05)	82 (49.40)	0.058
History of hyperlipidemia, n (%)	109 (21.89)	27 (16.27)	45 (27.11)	37 (22.29)	0.057
Coronary artery diseases, n (%)	51 (10.24)	17 (10.24)	19 (11.45)	15 (9.04)	0.769
History of previous stroke, n (%)	92 (18.47)	25 (15.06)	32 (19.28)	35 (21.08)	0.349
History of surgery, n (%)	158 (31.73)	61 (36.75)	54 (32.53)	43 (25.90)	0.101
Clinical characteristics					
NIHSS score, median (IQR)	3 (2, 6.5)	4 (2, 8)	4 (2, 7)	3 (1, 5)	0.001
BI score, median (IQR)	85 (45, 100)	72.5 (40, 100)	80 (48.75, 100)	95 (65, 100)	0.000
mRS score, median (IQR)	2 (1, 4)	3 (2, 4)	3 (1,4)	2 (1, 3)	0.000
HRSD score, median (IQR)	7 (4, 12)	9 (5,13.25)	9.0 (4.75, 13.00)	5 (3, 7)	0.000
Hospitalization days, median (IQR)	9 (7, 16)	9 (7,14)	10 (7, 16)	9.5 (6, 16)	0.926
Serum biochemicals					
Albumin, median (IQR)	41.07 (39.60, 42.60)	40.65 (38.18, 42.63)	41.07 (40.10, 42.80)	41.07 (41.07, 42.13)	0.013
Hcy, median (IQR)	15.10 (11.70, 15.3)	12.60 (10.30, 15.10)	15.10 (12.15, 15.10)	15.10 (14.50, 16.95)	0.000
Hs-CRP, median (IQR)	3.85 (1.10, 8.26)	2 (0.60, 8.26)	3.45 (1.10, 8.26)	8.26 (1.98, 8.26)	0.000
IL-1β, median (IQR)	61.14 (18.62, 135.82)	50.43 (14.27, 135.82)	50.51 (18.77, 135.82)	119.01 (28.25,163.83)	0.003
IL-6, median (IQR)	4.48 (1.93, 9.47)	3.63 (1.86, 9.30)	5.03 (2.08, 9.47)	4.75 (1.88, 9.47)	0.730
IL-10, median (IQR)	8.72 (2.30, 23.46)	8.08 (2.10,18.17)	9.26 (2.27, 23.80)	11.26 (2.48, 29.68)	0.268
IL-18, median (IQR)	1,773.41 (568.82, 3,520.77)	1,503.89 (397.79,3,520.77)	1,641.50 (575.27, 3,520.77)	2,552.29 (780.13, 3,534.55)	0.043
TNF-α, median (IQR)	36.73 (18.19, 49.53)	36.75 (19.32, 51.51)	38.53 (19.10, 54.59)	33.32 (15.33, 43.33)	0.062
BDNF, median (IQR)	3.60 (1.96, 5.78)	3.21 (1.63, 5.92)	3.67 (2.18, 5.72)	3.86 (2.14, 6.13)	0.251
IFN-γ, median (IQR)	4.43 (1.73, 9.72)	4.50 (1.54, 8.99)	4.41 (1.83, 8.40)	4.32 (1.71, 9.79)	1.000

BMI, body mass index; BI, Barthel Index; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; SUA, serum uric acid; Hcy, homocysteine; Hs-CRP, hyper-sensitive C-reactive protein; TNF, tumor necrosis factor; BDNF, brain derived neurotrophic factor; IFN, interferon; IL, interleukin.

hyperuricemic and PSD was explored by univariate and multivariate adjusted logistic regression models. We could speculate that hyperuricemia might protect from PSD from the regression results (OR ≈ 0.42, 95% CI ≈ 0.26~0.69, $P = 0.001$).

Moreover, we considered the ALB level as the representative index of overall nutritional status. There were differences in ALB levels across the SUA tertiles, as shown in **Table 1**. The *post hoc* test after Mann-Whitney U test declared that the ALB in lowest tertile SUA group was significantly less than the middle and the highest tertile SUA groups (both $P = 0.032$). Moreover, there was no difference in ALB levels between PSD and non-PSD groups ($P = 0.140$, **Table 2**). The subjects in the lowest tertile SUA group may be with worse nutritional status and decreased food intake. It reminds that there may be more risk factors, such as unhealthy lifestyles rather than ALB itself, leading to PSD prior to admission in the lowest SUA tertile group.

Association Between the Level of SUA and PSD

Significant differences were found between the PSD and non-PSD groups in SUA tertiles of patients (all $P < 0.05$, **Table 3**). Indeed, the proportions of patients in the lowest tertile (80.00~300.80 μmo1/L, $P = 0.001$) and the middle tertile (300.81~391.67 μmo1/L, $P = 0.00$) were significantly higher in the PSD groups, whilst the proportion of patients in the highest tertile (391.68~710.00 μmo1/L) was significantly lower in the PSD group ($P = 0.00$) (**Table 3**). In **Table 4**, with all patients taken as a whole, PSD occurrence taken as a dependent variable

TABLE 2 | Clinical and demographic characteristics of patients with PSD and non-PSD.

Variables	PSD patients (n = 232)	Non-PSD patients (n = 266)	*P*-value
SUA	328 (266.08, 348.45)	389 (295.5, 410)	0.000
Demographic parameters			
Age (years)	58.34 ± 10.49	56.14 ± 11.13	0.024
Females, n (%)	59 (25.43)	60 (22.56)	0.453
BMI (kg/m^2)	24.83 ± 3.26	25.08 ± 3.76	0.426
Married, n (%)	225 (96.98)	258 (96.99)	0.995
Education level			0.018
Junior middle school and below, n (%)	155 (66.81)	151 (56.77)	
Senior high/ Polytechnic school, n (%)	56 (24.14)	70 (26.32)	
Bachelor/Junior college and above, n (%)	21 (9.05)	45 (16.92)	
Vascular risk factors			
Smoking, n (%)	133 (57.33)	164 (61.65)	0.326
Drinking, n (%)	139 (59.91)	163 (61.28)	0.756
History of diabetes, n (%)	52 (22.41)	54 (20.30)	0.566
History of hypertension, n (%)	131 (56.47)	142 (53.38)	0.491
History of hyperlipidemia, n (%)	52 (22.41)	57 (21.43)	0.791
Coronary artery diseases, n (%)	31 (13.36)	20 (7.52)	0.032
History of previous stroke, n (%)	46 (19.83)	46 (17.29)	0.467
History of previous surgery, n (%)	84 (36.21)	74 (27.82)	0.045
Clinical characteristics			
NIHSS score, median (IQR)	5 (2, 8)	2 (1, 4)	0.000
BI score, median (IQR)	65 (35, 95)	95 (68.75, 100)	0.000
mRS score, median (IQR)	3 (2, 4)	2 (1, 3)	0.000
HRSD score, median (IQR)	12 (10, 16)	4 (2, 6)	0.000
Hospitalization days, median (IQR)	10 (7, 15)	9 (6, 16)	0.486
Serum biochemicals			
Albumin, median (IQR)	41.07 (39.20, 42.48)	41.07 (40.30, 42.80)	0.140
Homocysteine, median (IQR)	14.65 (11.73, 15.10)	15.10 (11.70, 15.40)	0.353
Hs-CRP, median (IQR)	3.50 (1.10, 8.26)	4.45 (1.10, 8.26)	0.712
IL-1β, median (IQR)	55.51 (18.90, 135.82)	67.32 (18.19, 135.91)	0.467
IL-6, median (IQR)	4.75 (1.84, 9.47)	4.28 (1.97, 9.47)	0.377
IL-10, median (IQR)	8.15 (1.95, 23.24)	9.50 (2.50, 23.64)	0.620
IL-18, median (IQR)	1,681.96 (503.52, 3,520.77)	1,938.46 (680.12, 3,520.77)	0.496
TNF-α, median (IQR)	36.84 (18.32, 50.95)	36.04 (17.66, 48.50)	0.710
BDNF, median (IQR)	3.34 (1.85, 5.75)	3.80 (2.12, 5.93)	0.393
IFN-γ, median (IQR)	4.56 (1.84, 9.73)	4.21 (1.71, 8.71)	0.357

BMI, body mass index; BI, Barthel Index; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; SUA, serum uric acid; Hcy, homocysteine; Hs-CRP, hyper-sensitive C-reactive protein; TNF, tumor necrosis factor; BDNF, brain-derived neurotrophic factor; IFN, interferon; IL, interleukin.

TABLE 3 | SUA tertiles of patients.

Variable	PSD patients (n = 232)	Non-PSD patients (n = 266)	χ^2	*P*-value
SUA			65.672	0.000
Tertile 1, n = 166 (80~300.8 μmol/L)	95	71	11.334	0.001
Tertile 2, n = 166 (300.81~391.67 μmol/L)	102	64	22.095	0.000
Tertile 3, n = 166 (391.68~710 μmol/L)	35	131	65.078	0.000

SUA, serum uric acid; PSD, post-stroke depression.

TABLE 4 | Multivariate adjusted odds ratios for the association between SUA levels and PSD at discharge.

	Tertile	OR[a]	95% CI	*P*-value
Unadjusted	Lowest	5.965	3.667~9.704	0.00
	Moderate	5.008	3.089~8.120	0.00
Model 1[b]	Lowest	6.009	3.682~9.808	0.00
	Moderate	4.776	2.937~7.767	0.00
Model 2[c]	Lowest	6.009	3.674~9.829	0.00
	Moderate	4.808	2.949~7.838	0.00
Model 3[d]	Lowest	5.777	3.463~9.637	0.00
	Moderate	4.153	2.492~6.921	0.00

OR, odds radio; CI, confidence level; PSD, post-stroke depression.
[a]Reference OR (1.000) is the highest tertile of SUA for PSD.
[b]Model 1: adjusted for age, gender, education levels, body mass index, smoking, drinking.
[c]Model 2: adjusted for covariates from model 1 and further adjusted for medical history (coronary artery disease, diabetes mellitus, hyperlipidemia, hypertension, previous stroke, and surgery).
[d]Model 3: adjusted for covariates from model 2 and further adjusted for baseline NIHSS scores, mRS scores, and Barthel Index scores.

and highest tertile taken as the reference was used for SUA in the unadjusted and multivariate adjusted logistic regression models. In unadjusted logistic regression model, the lowest tertile of SUA was independently consistent with a risk predictor of PSD with an unadjusted OR of 5.965 (95% CI = 3.667~9.704, $P = 0.00$). The middle tertile of SUA was independently consistent with a risk predictor of PSD with an unadjusted OR of 5.008 (95% CI = 3.089~8.120, $P = 0.00$).

After adjusting for conventional confounders including age, gender, education levels, BMI, smoking, drinking, history of coronary artery disease, diabetes mellitus, hyperlipidemia, hypertension, previous stroke and surgery, baseline NIHSS, mRS and BI scores, and the lowest tertile of SUA was remained significant independently associated with the prevalence of PSD (model 1: OR = 6.009, 95% CI = 3.682~9.808, $P = 0.00$; model 2: OR = 6.009, 95% CI = 3.674~9.829, $P = 0.00$; model 3: OR = 5.777, 95% CI = 3.463~9.637, $P = 0.00$), as compared with the highest tertile. Similarly, the middle tertile of SUA was remained significant independently associated with the prevalence of PSD (model 1: OR = 4.776, 95% CI = 2.937~7.767, $P = 0.00$; model 2: OR = 4.808, 95% CI = 2.949~7.838, $P = 0.00$; model 3: OR = 4.153, 95% CI = 2.492~6.921, $P = 0.00$).

Furthermore, we used RCS regression model to confirm the linear relationship between continuous SUA levels and PSD at discharge ($\chi^2 = 6.33$, df = 2, $P = 0.0119$ for nonlinearity,

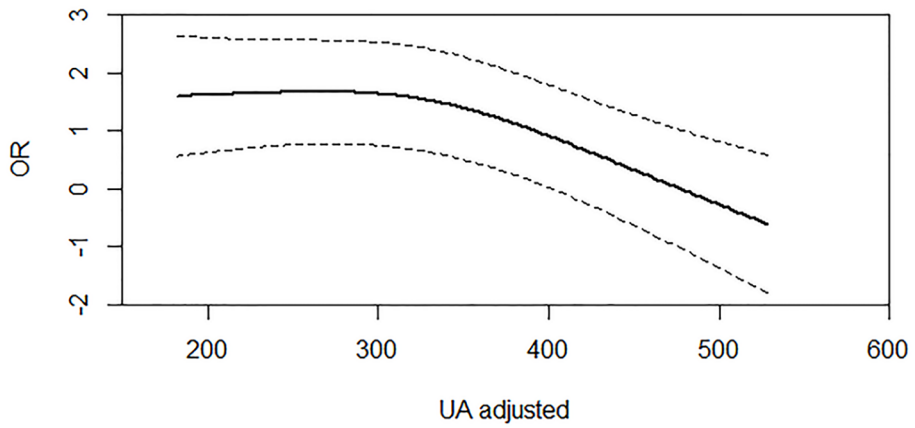

FIGURE 1 | Association of serum UA (uric acid) levels with risk of PSD. Dashed lines are 95% confidence intervals. Odds ratios and 95% confidence intervals derived from restricted cubic spline regression (*P* for nonlinear = 0.0119). Odds ratios were estimated using logistic regression modeling, adjusting for the same variables as model 3 in **Table 4**.

Figure 1). The RCS curve showed that there was nonlinear effect between SUA and PSD outcome. However, the linear association was found between SUA and PSD after the value of around 300 μmol/L. Coincidentally, the cut-off point of RCS curve (300 μmol/L) was approximately the same as the first tertile split points (300.8 μmol/L). It is a reasonable approach to take the tertiles of SUA levels to analyze the association between SUA and PSD risk. There is a protective effect in PSD when the SUA level was more than 480 μmo1/L, while the 95% CI of other SUA values (≤480 μmo1/L) is not completely above or below the value "1" of significance.

DISCUSSION

In this study, we have investigated the association between SUA levels on admission and the development of PSD outcome at discharge using a prospective cohort. Our results suggested that lower levels of SUA on admission were associated with the presence of PSD at discharge, and documented that the risk of PSD in patients significantly declined with the increasing SUA level after 300 μmol/L. Therefore, our findings revealed the SUA value could provide important information for predicting PSD patients at discharge. A total of 46.59% of stroke patients presented with PSD in this study, which is more than the incidence reported in previous studies. This could be potentially explained by that Tongji hospital was the highest-ranked large general 3A hospital in Central China and recruited more serious stroke patients whose the median NIHSS score (IQR) was 3 (2, 6.5) (7). Previous study has reported the higher levels of SUA on admission was associated with the occurrence of 3-month major PSD, while lower level of SUA on admission was closely related to the occurrence of major PSD between 3- and 6-month post-stroke. Six months after acute ischemic stroke, there was no relationship between major PSD and baseline SUA (15). The differences in time of assessment and diagnosis, subjects' samples, and psychiatric assessment methods could explain the discrepancies between our study and their findings.

Compared with the patients without PSD at discharge, our results also demonstrated that the PSD patients had significantly increased stroke severity (higher NIHSS scores) and worse functional outcome (higher mRS scores and lower BI scores), which were consistent with the previous studies (18, 19). The patients from PSD group were also more likely to have history of coronary artery diseases and surgery which may potentially worsen the stroke patients' conditions and increase the risk of occurrence of PSD at discharge (20). Furthermore, the incidence of PSD was higher in older stroke patients, which was also consistent with the previous reports (21). This was probably because older patients were more likely to be involved in a depressive mood when dealing with the stress caused by stroke. The increased risk of PSD at discharge could also be caused by the worsening functional and cognitive impairment in the elderly (22). Previous studies have indicated that proinflammatory factors played important role in the development of PSD (23). However, there were no significant differences in proinflammatory factors between PSD and non-PSD groups which was potentially due to the different definitions of outcomes.

In recent years, the interest of psychiatrist in the association between oxidative stress and depression has grown stronger and relevant studies have shown that the depression is accompanied by increased oxidative stress and decreased antioxidant defenses (24, 25). Unlike previous studies, the present investigation focused on the occurrence of PSD at discharge and attempted to link this outcome with SUA levels on admission (15). Our study may provide an effective biomarker for psychiatrists to diagnose PSD at discharge.

Given that lower SUA had been found in major depressive and anxiety disorders (26), our study reasonably found PSD patients had lower SUA levels on admission. As a crucial central nervous system antioxidant, SUA had been proven to be an

effective predictor in the development of PSD (15). Individuals with lower baseline SUA levels may be more susceptible to oxidative damage in the brain, as a result of lower antioxidant defenses and larger oxygen consumption in brain. This damage could make individuals susceptible to developing depressive mood and has also been suggested as a potential mechanism in the relapse of depression (27). As a free radical scavenger, SUA contributes to over half antioxidant capacity in plasma. As neuronal cell membranes were composed by large amounts of polyunsaturated fatty acids with a large surface area and ROS often react with lipids, optimal neuronal cell function depends on sufficient antioxidants, such as SUA, to remove ROS and protect neuronal cells from oxidative damage. Nanetti et al. found that acute ischemic stroke could cause the strong oxidative stress and generation of free radicals, and that this progression of ischemic injury could be limited by the antioxidant capacity (28). Thus, higher SUA levels could potentially protect neuron integrity and function of stroke patients and decrease the risk of PSD at discharge. Previous studies have also reported that UA has neuroprotective effects due to its potent antioxidant capacity in other diseases, such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (29–31). In acute stage of stroke, UA may exert antioxidant protection against free radical damage. Previous study has shown a negative correlation between SUA levels and stroke outcomes (32). A randomized clinical trial also showed that UA therapy was related to an improved prognosis in patients with hyperglycemia in acute stage through decreasing glucose-driven oxidative stress (33). Moreover, a phase 2 clinical trial with inosine in Parkinson's disease demonstrated a potential efficacy of increased UA on mood disorders as evaluated on the Geriatric Depression Scale. We could speculate that SUA (>480 μmol/L) may exert protective effect in PSD (34).

Moreover, UA is also a marker of purine metabolism as the final product other than antioxidant. The purinergic system has been associated with the pathophysiology of depression and is thought to influence mood, appetite, sleep, cognition, and drive through the neuromodulator adenosine and the neurotransmitter adenosine triphosphate, both of which are upstream metabolites of UA (35). Increased levels of UA are associated with decreased adenosinergic transmission and accelerated purinergic transformation, which could limit the development of depressive disorders (36). Previous studies also suggested that lower SUA levels at the onset of ischemic stroke could indicate a worse prognosis, such as increased risk of PSD, by increasing the neurological damage levels (37, 38). On the other hand, previous study also reported that the lower UA in current MDD may be related to increased exposure to ROS through mitochondrial dysfunction which could deplete UA, if not caused by lifestyle factors (39). As we all know, mitochondrial dysfunction also plays an important role in disorders of purine metabolism which could not provide sufficient UA to counteract increased oxidative stress in PSD (40).

The main strengths of this study are the sufficient sample size, well-established psychiatric diagnoses, prospective cohort study nature, and adjustment for many confounders in different models. Though further longitudinal studies are needed to confirm the association between the SUA level on admission and the risk of PSD at discharge, our study results revealed the threshold effect between them. Moreover, SUA presented affordable routine clinical biomarker which did not incur additional expense as compared with microRNAs which is limited to precise laboratory tests and not appropriate for extensive follow up and clinical application (18). Some limitations of our study should also be acknowledged. First, the stroke patients from a single center and exclusion of patients with alteration of consciousness level and severe speech disturbances may result in biases for the incidence of PSD at discharge. Second, it should be noted that the functioning of antioxidant defenses and oxidative stress is an ongoing dynamic process, the complexities of which cannot be reflected in the measurement of individual peripheral marker level at a single time point. The levels of a single biomarker of SUA may not be representative of the functioning of the whole redox homeostasis system, but nevertheless provide a clue that the system is associated with the psychopathology of PSD. Third, there were five indicators, ALB, Hcy, SUA, Hs-CRP, and hospitalization days, were collected and studied by our post-hoc analysis. The original study protocol was not designed to assess association between these five indicators and PSD risk at discharge. Given the *post hoc* nature of the analysis, the proffered mechanism would likely be speculative. Fourth, the SUA level may be influenced by metabolic profile and the information collection of glycemia, lipid profile, and metabolic syndrome were important issues (41, 42). There is no specific analysis on these issues in our study. Future longitudinal studies should further explore these associations, and SUA's potential as predictor for PSD at discharge.

In conclusion, our study suggested that lower SUA levels on admission were associated with PSD at discharge and the threshold effect was also found by RCS regression model. For stroke patients, doctors should pay attention to the baseline SUA levels for screening high risk PSD patients at discharge in clinical practice. SUA was the potential biomarker for PSD prediction. The limitation of the study includes the study biases and the results should be further confirmed in longitudinal and experimental studies.

DATA AVAILABILITY STATEMENT

The de-identified database used in the current study are available from the corresponding authors on reasonable request.

ETHICS STATEMENT

All patients involved in this study or their family members gave written informed consents according to the Declaration of Helsinki. The approval of the study for experiments was obtained from the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

SZ and ZZ led the study. GL performed the data analysis and implemented the methodology. XS, WS, JM, YL, XQ, XZ, and CZ collected the data. ZZ and GL prepared the original draft. SZ reviewed and edited the final manuscript.

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Proteomic Profiling as a Diagnostic Biomarker for Discriminating Between Bipolar and Unipolar Depression

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Introduction: Affective disorders are a major global burden, with approximately 15% of people worldwide suffering from some form of affective disorder. In patients experiencing their first depressive episode, in most cases it cannot be distinguished whether this is due to bipolar disorder (BD) or major depressive disorder (MDD). Valid fluid biomarkers able to discriminate between the two disorders in a clinical setting are not yet available.

Material and Methods: Seventy depressed patients suffering from BD (bipolar I and II subtypes) and 42 patients with major MDD were recruited and blood samples were taken for proteomic analyses after 8 h fasting. Proteomic profiles were analyzed using the Multiplex Immunoassay platform from Myriad Rules Based Medicine (Myriad RBM; Austin, Texas, USA). Human DiscoveryMAP^{TM} was used to measure the concentration of various proteins, peptides, and small molecules. A multivariate predictive model was consequently constructed to differentiate between BD and MDD.

Results: Based on the various proteomic profiles, the algorithm could discriminate depressed BD patients from MDD patients with an accuracy of 67%.

Discussion: The results of this preliminary study suggest that future discrimination between bipolar and unipolar depression in a single case could be possible, using predictive biomarker models based on blood proteomic profiling.

Keywords: affective disorder, bipolar disorder, major depression (MD), major depressive disorder (MDD), proteome, biomarker, blood, machine learning

INTRODUCTION

Depressive episodes affect up to 322 million people worldwide (Depression and Other Common Mental Disorders. Global Health Estimates. Geneva: World Health Organization 2017, https://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2-eng.pdf). People suffering from a depressive episode can be suffering from either unipolar depression (major depressive disorder; MDD) or bipolar affective disorder (BD) as the underlying cause, depending on whether previous (hypo)-manic episodes have occurred. Unfortunately, this distinction can only be made after the first (hypo-)manic episode has presented. Therefore the most appropriate treatment for the underlying disorder may not initially be prescribed, especially as BD and MDD require fundamentally different pharmacological approaches; BD requires mood stabilizing medication, whereas MDD is treated with antidepressant monotherapy as a first-line treatment (1–3). Patients suffering from BD are often misdiagnosed as MDD and therefore adequate treatment can be delayed for up to several years (4, 5). Inadequate and delayed treatment increases the direct and indirect economic cost of BD, augments individual suffering, and impairs the overall prognosis (6). However, despite distinct treatment approaches, BD and MDD appear to share common molecular pathomechanisms. The gradient of MDD polygenic risk sore has been shown to slide across the mood disorder spectrum, demonstrating an inverse relationship to the mania polygenic risk score (7).

The development of fluid biomarkers that can discriminate between BD and MDD would be highly beneficial, but reliable biomarkers have so far remained elusive. Nonspecific findings have been obtained in many studies, which failed to detect disorder-specific alterations, instead identifying molecular mechanisms implicated in several different psychiatric conditions. For example, several studies have reported dysregulation of the nitrinergic system in BD, but also in ADHD and schizophrenia (8–11). Recent work has additionally suggested that nitric oxide may play a role in the pathophysiology of major depression (12). Another potential cross-disorder mechanism is a dysfunctional hypothalamic-pituitary-adrenal axis (HPA axis), which has been implicated in both BD and MDD (13–15). Moreover, inflammatory processes (including the glucocorticoid system) may also play a role in MDD and BD (16, 17).

Despite several shared neurobiological features of psychiatric disorders, combining different modalities or vast arrays of biomarkers (e.g. using proteomic profiling) has demonstrated potential for providing disorder-specific biomarkers. A previous own study defined a diagnostic panel consisting of 20 protein analytes suitable for the diagnosis of BD (18). Additionally, Chen et al. published a set of 20 differential urine metabolites that could discriminate between BD and MDD (19). Although these initial findings are encouraging, the use of univariate statistical inference does not provide sufficient information to determine discriminative power for individual patients, nor does it quantify generalisation to new data. These initial promising results therefore need to be replicated in additional samples, and more importantly tested for personalized predictive power, before a diagnostic biomarker panel can be used in clinical routine. In addition, modern machine learning approaches may considerably increase model performance by considering multivariate patterns in the data, thereby improving upon classic univariate approaches. In this study, we investigated whether a multivariate machine learning approach using data from multiplexed proteomic assays could accurately be used to discriminate between BD and MDD.

MATERIALS AND METHODS

Study Participants

Bipolar and major depression patients were part of a naturalistic sample recruited from patients treated in our in- and outpatient clinics. The male and female participants were within the age range 18–78 years, had a body mass index (BMI) between 18 and 46 kg/m, and had a test negative for recreational drug screening at the time of sampling. Patients were diagnosed with BD or MDD by two trained psychiatrists (SKS, AR) according to criteria of the International Classification of Diseases–10 (ICD-10), while being treated as inpatients or outpatients at the Department of Psychiatry, Psychosomatic Medicine and Psychotherapy of the University Hospital of Würzburg. Diagnoses were confirmed by the Operational Criteria Checklist for Affective and Psychotic Illness (OPCRIT) (20). Severity of symptoms was assessed using the standard questionnaire-based rating scales Young Mania Rating Scale (YMRS) and Montgomery–Åsberg Depression Rating Scale (MADRS) (21, 22).

Both bipolar I and bipolar II disorder patients were recruited and were in depressed mood states at the time of sample collection. MDD patients also had an acute depressive episode at the time of sample collection. Exclusion criteria included a diagnosis of severe coronary heart disease or cardiac insufficiency (i.e. coronary stent, cardiac bypass surgery angina pectoris, and cardiac insufficiency NYHA>I), severe autoimmune disorders (Hashimoto's thyroiditis excluded), acute or chronic infections, treatment with immunosuppressive/immune-modulating drugs or antibiotics, other severe neuropsychiatric disorders, chronic terminal diseases affecting the brain (such as cancer or hepatic/renal insufficiency), and alcohol or drug addiction (self-reported or taken from hospital discharge letters/general practitioner's letters). Patients were fasting for at least 8 h prior to blood sample collection. For more demographic details as well as somatic disorders and medication taken at sampling point see **Table 1** and **Supplemental Table 2**.

Only study participants who gave written informed consent were enrolled in the study, which complied with the latest Declaration of Helsinki, and was approved by the Ethics Committee of the University of Würzburg.

Sample Collection

Patients were recruited over a total time period of 4 years (2009–2013), and therefore proteomic profiles were analyzed in four

TABLE 1 | Demographic data.

Depressive episode	n=70	n=42
	Bipolar Disorder	Major Depression
BD I/BD II	30/40	N/A
Age (years, mean +/– SD)	43.47 +/– 11.69	44.28 +/– 14.93
Gender (female/male)	44/28	25/16
BMI	27.50 +/– 5.72	27.78 +/– 6.27
Disease duration (years, mean +/–SD)	15.10 +/–11.32	N/A
MADRS sum score (mean +/–SD)	18.0 +/– 1.96	18.47 +/– 8.32
YMRS sum score (mean +/–SD)	2.0 +/– 0.22	N/A
Medication		
Lithium	7	3
Valproic acid	5	0
Other anticonvulsants	1	0
Antipsychotics	17	9
Lithium + Valproic acid	4	0
Valproic acid + antipsychotics	7	0
Other anticonvulsants + antipsychotics	4	0
Lithium + anticonvulsants + antipsychotics	4	0
Lithium + antipsychotics	21	1
Antidepressants only	2	28

BD I, bipolar disorder type 1; BD II, bipolar disorder type II; BMI, body mass index; MADRS, Montgomery-Åsberg Depression Scale; YMR, Young Mania Rating Scale; N/A, not available.

batches. Proteomic analyses were completed in 2010, 2011, and 2013. The maximum storage time for each sample in −80°C prior to analysis was 2 years.

Sample Preparation

Blood samples were taken on the day of clinical assessment (± 24 h). Blood was obtained from the participants by venous puncture in the morning after fasting for 10–13 h (between 7 to 9 am). Serum was collected from fasting patients using Vacutainer (Becton-Dickinson, Franklin Lakes, NJ, USA). Blood clotting time was 2 h at room temperature prior to centrifugation for 15 min at 1.100 *x* g. Samples were stored in low binding Eppendorf reaction tubes (Hamburg, Germany) at −80°C. Sample shipment took place on dry ice.

Multiplex Immunoassay Analysis

Serum from all participants was profiled using the multiplex immunoassay platform at Myriad Rules Based Medicine (Myriad RBM; Austin, Texas, USA), which has been previously described in detail (23). The Human DiscoveryMAP^{TM} was used to measure the plasma concentrations of different proteins, peptides and small molecules (collectively referred to as "analytes"), in a Clinical Laboratory Improved Amendments certified lab. The total number of analytes measured differed between batches, depending on when the study samples were profiled (total range: 190 to 257 analytes). The analytes measured are reported in **Supplemental Table 1** and the concentration of all the analytes for all participants are reported in **Supplemental Table 3** and **Supplemental Table 4**. The raw data of the four different multiplex assays were normalised for batch effects to reduce variability.

Statistical Analysis

Statistical analyses were performed in R (24). We pre-processed the analyte data by excluding analytes with greater than 20% missing values, and imputing missing data. Data points under the lowest limit of detection (LLD) were replaced by the minimum value above the LLD for the specific analyte, and values above the highest detectable limit were replaced with the maximum measured valued within the detectable range. In total, 1.1% of data points were imputed. We $\log_{10}$-transformed data to stabilize the variance. Additional analyses were performed using SPSS (v25, IBM®). The two analytes that appeared to play a significant role in discriminating between unipolar and bipolar depression were analyzed separately using ANCOVA.

Machine Learning Algorithm

In order to discriminate between patients suffering from MDD and BD, we first scaled all features (i.e. analytes) to have zero mean and unit variance. Next, tree ensemble classification was performed using the scikit-learn implementation of the AdaBoost algorithm with default hyperparameters (25, 26). To facilitate training in this extensively imbalanced dataset, we additionally employed Random Oversampling to the training set.

To assess the generalizability of the classifier, we used 10-fold cross-validation. Tenfold cross-validation is the most common standard in the field which ensures low model bias (due to the fairly large training sample) and low variance (due to the reasonably sized test set). Finding a balance between training and test sample size in each iteration is important, particularly because of the fairly small sample size used (for an introduction to the issue of k-fold cross-validation in practice see Bengio et al. (27). In each fold, data from 90% of the sample is used to train the classifier. Categorization of the remaining 10%, which has so far not been seen by the algorithm, is subsequently calculated. This procedure is repeated 10 times, each time leaving out different, nonoverlapping 10% portions of the sample, yielding each subject's categorization. To ensure unbiased test performance estimates in this imbalanced sample, accuracy was computed by calculating the mean of sensitivity and specificity, yielding "balanced accuracy."

To establish whether the observed test accuracy estimate is statistically significant, we ran the entire pipeline 1,000 times with randomly permuted labels and counted the number of permutations which achieved higher accuracy than the one observed with the true labels. The p-value was then calculated by dividing this number by 1,000. If none of the permutation accuracies exceeded accuracy obtained with the true labels, this is denoted as $p<.001$.

To quantify the contribution of each feature, we computed permutation importance scores, calculated as the mean decrease of test accuracy for all samples if a given feature is randomly shuffled 10 times. Generally, permutation importance as used here provides a measure of how much a feature contributed to classification performance while leaving all other features intact. All analyses were performed using the PHOTON framework (www.photon-ai.com).

RESULTS

Patients with MDD and BD did not differ in basic demographic variables (see **Table 1**). However, medication significantly differed, as the majority of BD patients were taking mood stabilizers and the majority of MD patients were taking antidepressants (**Table 1**). Using data from 105 analytes, which was the lowest common denominator of the four batches, a multivariate predictive model was constructed to discriminate between MDD and BD (combined BD I and BD II disorders). The algorithm could discriminate between these two groups on the basis of the proteomic profile with an accuracy of 67% ($p>.001$ with 1,000 permutations) (see **Figure 1**). The analytes which the algorithm used for discrimination and prediction are displayed in **Supplemental Tables 1**, **3**, and **4**. The two analytes Platelet-Derived Growth Factor BB (PDGF-BB) and Thrombospondin-1 (TSP-1) were identified as particularly important for discriminating between BD and MDD in our sample. A subanalysis examining only young BD patients (<35 years) did not increase the accuracy of discrimination (data not shown). Additionally, it was observed that including a set of covariates (such as symptom severity and BD subtype) did not lead to improved accuracy (data not shown). To assess the potential influence of medication, we performed a covariate analysis using PDGF-BB and TSP-1 and included diagnosis (bipolar depression vs. MDD), medication, age, gender and BMI as covariates. No significant differences were found ($p=0.19$, $p=0.47$ respectively; see **Table 2**).

DISCUSSION

Recent studies found that ~10% of patients suffering from a depressive episode subsequently develop BD (4). To date, there are no tests in clinical routine to determine the risk of developing BD in patients experiencing their first depressive episode. However, it would be of great clinical significance to be able to accurately predict the underlying disorder, as pharmacological treatment differs considerably between MDD and BD. In this study, we have demonstrated that a machine-learning algorithm was able to individually discriminate BD (acutely depressed) from MDD (acutely depressed) patients with a moderately good accuracy of 67%, based on their proteomic profile. Based on our data, PDGF-BB and TSP-1 appeared to play a prominent role in this discrimination. Along with other serum analytes, PDGF-BB has previously been reported as associated with lower fractional anisotropy, higher mean diffusivity, and higher radial diffusivity in several brain regions in a sample of depressed BD patients (28). Furthermore, PDGF-BB was found to be increased in BD patients suffering a depressive episode after treatment with a combination of sleep deprivation, lithium and bright light therapy (29). PDGF-BB was also found to have low intra-individual variability when measured with different methods and in serum and plasma, suggesting that this marker may be technically reliable (30). Physiologically, PDGF receptors have been reported to play a role in glutamatergic signaling (31), which is thought to be dysregulated in subtypes of affective patients (32).

TSP-1 may be involved in synaptogenesis (33). Electroconvulsive therapy (ECT) is used to treat therapy-resistant MDD and BD depression, and was found to increase TSP-1 mRNA and protein expression in a rat model. However, chronic antidepressant treatment in this animal model appeared to have no effect on TSP-1 (34). Preclinical data has additionally suggested that TSP-1 may play a role in bidirectional neuron-astrocyte communication, dysregulation of which could be a pathomechanism for the development of mental illnesses (35). There are also several in vitro studies demonstrating that the mood stabilizer valproate can induce TSP-1 protein expression and thrombospondin-1 (*THBS-1*) gene expression in different cell and animal models (36–38). This is in contrast to our results, which showed no difference in TSP-1 expression between patients treated with valproate and patients treated with the other mood stabilizers and antidepressants. However, valproate may only exert its main effect on TSP-1 expression in the central nervous system, with its effects not detectable in the periphery. With regards to human in vivo data, a recent study reported decreased TSP-1 serum levels in female patients with MDD compared to healthy controls and male MDD patients. However, ECT treatment did not influence TSP-1 levels, leading the authors to conclude that serum TSP-1 may be a state marker of female MDD rather than a trait marker (39). Nonetheless, a technical issue in both our study and the one performed by Okada-Tsuchioka et al. is that TSP-1 concentration was measured in serum and not plasma. As thrombocytes release TSP-1 in high concentrations, the TSP-1 generated by other cells may be masked (40). Future studies should therefore measure plasma TSP-1.

Several studies have previously attempted to discriminate between MDD and BD patients using fluid biomarkers. A recent study from Chen and colleagues analyzed urinary metabolic phenotypes and demonstrated that a panel of six urinary metabolites could potentially be used to discriminate between the two disorders (19). In a study comparing cytokine

TABLE 2 | TSP-1 and PDGF-BB levels.

	Disorder	N	Mean (µg/L)	Std. Deviation +/–	ANCOVA, p
PDGF-BB	Bipolar Disorder	70	15,811.67	5,828.79	
	Major depression	42	16,641.46	6,515.39	0.19
TSP-1	Bipolar Disorder	70	16,498.75	8,180.89	
	Major depression	42	17,424.39	4,829.24	0.47

PDGF-BB, platelet-derived growth factor BB; TSP-1, thrombospondin-1; ANCOVA, analysis were calculated including diagnosis, age, gender, and medication as variables.

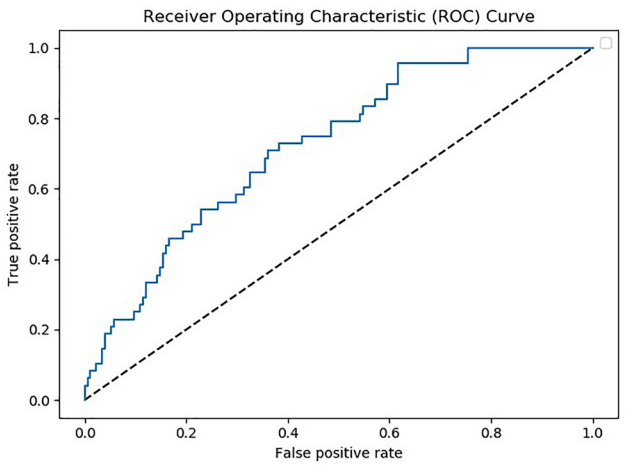

FIGURE 1 | Receiver operating characteristic (ROC) curve depicting true positive versus false positive rates for the major depressive disorder (MDD) vs. bipolar disorder (BD) classification. Note that MDD was denoted as the positive class.

concentrations between remitted BD and MDD patients and healthy controls, higher concentrations of soluble Interleukin-6 receptor (sIL-6R), C-reactive protein (CRP), soluble Tumor-Necrosis-Factor-receptor-1 (sTNF-R) and Monocyte-chemoattractant-protein -1 (MCP-1) were shown in BD compared to MDD (41). Frye et al. used a similar approach to our current and previous studies but with a smaller sample size of MDD patients, BD patients and healthy controls, and observed differences in several serum proteins between groups. To date, the best diagnostic accuracy (>0.8) for discriminating between BD I patients and healthy controls was shown by growth-differentiation factor 15 (GDF-15), retinol-binding protein (RBP-4) and transthyretin (TTR). However, in the same study no marker could be identified that accurately discriminated between BD and MDD, which would be the most clinically relevant diagnostic biomarker (42). GDF-15 and RBP-4 were not included as analytes in our studies and therefore no data comparisons can be made. However, Frye and colleagues also found six proteins to be significantly increased in depressed BD and MDD patients, and these were found to differ from those of our own previous multi-center study with the exception of MMP-7 (18). PDGF-BB and TSP-1 were also not found to be significant markers in our previous study, although our previous samples were derived from BD patients in all episodes (including euthymic), currently depressed and euthymic MDD patients, which could explain the differences in results. Whereas in this smaller sample we only compared acutely depressed bipolar patients vs. current major depression. This inconsistency could be therefore due to the differences in the samples which were investigated with respect to current episode and subtypes as well as to different proteins included in the analysis. Our current study additionally improves upon previous approaches, as it is the first to move beyond group-statistical inference to provide single-subject predictions. As individualized prediction is a key requirement for clinical application, our results support the clinical utility of multivariate predictive analytics approaches in the field.

A current limitation of using fluid peripheral biomarkers is that concentrations measured in the periphery do not necessarily reflect pathophysiological processes in the central nervous system. However, our primary aim is to develop a biomarker that can discriminate between disorders, and not identify underlying disease pathomechanisms. We therefore believe that the most important factor is whether biomarker expression varies sufficiently between individuals to allow for discrimination between different disorders, and not whether the biomarker is in directly involved in disease aetiology. However, for most single metabolites, the differences between groups are statistically significant but not great enough for single prediction [for examples, see (9, 39, 43, 44)]. We therefore suggest that currently the most promising approach for individual prediction is to measure several analytes simultaneously in the form of a biomarker panel, with additional machine learning, rather than measuring only a few select proteins.

Machine-learning algorithms in the development of diagnostic biomarkers have so far mainly been used in neuroimaging studies. There are several preliminary studies demonstrating the potential of this approach, for applications such as identifying individuals at high-risk of BD (45) and for defining subphenotypes of BD (46). However, machine learning may impair the algorithm's ability to derive a high performing model, and preclude the use of more sophisticated approaches, potentially rendering our results an artificially low estimate of the true accuracy. Despite these limitations, the sample size used for evaluation in machine learning entails fairly small test sets, potentially increasing variance of performance estimates (although we employed cross-validation).

To conclude, the initial results obtained from our study are promising. However, larger samples of patients are needed to replicate the results, thereby supporting the development of diagnostic biomarkers which can be used in clinical routine. We are aware that in this hypothesis generating study we could only examine a discovery sample. The necessary next step is to validate our findings in a second, independent dataset which is however not readily available. We are currently reaching out to conduct according replication studies which will finally be the touchstone whether or not the pilot data presented here holds true or not.

LIMITATION

The results of our study have several limitations. First, in the subanalysis, sample sizes were small. Second, as all patients were medicated an influence of mood stabilizing medication and antidepressants on serum proteins cannot be excluded. Further studies using increased sample sizes and including drug-naïve BD and MDD patients should be performed to overcome this methodological weakness. However, studies on drug-naïve patients are difficult to conduct due to ethical issues.

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 Ethics Committee of the University of Würzburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK-S and AR recruited the patients and collected the sample. SK-S wrote the paper draft and did parts of the analysis. FH and SB conducted the multiplex proteomic analysis. FH did the preanalysis of the data and added to the manuscript draft. TH conducted the machine learning analysis and added to the manuscript draft. SB, AR, and RM took part in writing the final manuscript. RM revised the language of the final revised manuscript.

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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.2020.00189/full#supplementary-material

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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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REVIEW
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Endocannabinoid System Components as Potential Biomarkers in Psychiatry

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The high heterogeneity of psychiatric disorders leads to a lack of diagnostic precision. Therefore, the search of biomarkers is a fundamental aspect in psychiatry to reach a more personalized medicine. The endocannabinoid system (ECS) has gained increasing interest due to its involvement in many different functional processes in the brain, including the regulation of emotions, motivation, and cognition. This article reviews the role of the main components of the ECS as biomarkers in certain psychiatric disorders. Studies carried out in rodents evaluating the effects of pharmacological and genetic manipulation of cannabinoid receptors or endocannabinoids (eCBs) degrading enzymes were included. Likewise, the ECS-related alterations occurring at the molecular level in animal models reproducing some behavioral and/or neuropathological aspects of psychiatric disorders were reviewed. Furthermore, clinical studies evaluating gene or protein alterations in *post-mortem* brain tissue or *in vivo* blood, plasma, and cerebrospinal fluid (CSF) samples were analyzed. Also, the results from neuroimaging studies using positron emission tomography (PET) or functional magnetic resonance (fMRI) were included. This review shows the close involvement of cannabinoid receptor 1 (CB1r) in stress regulation and the development of mood disorders [anxiety, depression, bipolar disorder (BD)], in post-traumatic stress disorder (PTSD), as well as in the etiopathogenesis of schizophrenia, attention deficit hyperactivity disorder (ADHD), or eating disorders (*i.e.* anorexia and bulimia nervosa). On the other hand, recent results reveal the potential therapeutic action of the endocannabinoid tone manipulation by inhibition of eCBs degrading enzymes, as well as by the modulation of cannabinoid receptor 2 (CB2r) activity on anxiolytic, antidepressive, or antipsychotic associated effects. Further clinical research studies are needed; however, current evidence suggests that the components of the ECS may

become promising biomarkers in psychiatry to improve, at least in part, the diagnosis and pharmacological treatment of psychiatric disorders.

Keywords: endocannabinoid system, cannabinoid receptor (CB1r, CB2r), endocannabinoid, biomarker, psychiatry, diagnosis, treatment

INTRODUCTION

Psychiatric disorders are one of the main causes of disability in the general population (1). According to a recent estimation, psychiatric disorders account for 32.4% of years lived with disability (YLDs) and 13% of disability adjusted life-years (DALYs), leading the global burden of disease (2). Despite this, we still have a great lack of knowledge about its neurobiological basis, and clinically applicable biomarkers have been elusive. During the last decades, an increasing effort has been made in the search of biomarkers in psychiatry to help in the diagnosis and prediction of disease progression or treatment response. However, a clinical biomarker should be validated, sensitive, specific, feasible, and easily reproducible, characteristics that make difficult the implementation in this field (3–5).

The endocannabinoid system (ECS) components (receptors, ligands, synthesizing and degrading enzymes) have gained a special interest because of their critical neuromodulatory involvement in a plethora of functional mechanisms in the central nervous system (CNS), including emotional regulation, motivational behavior, and cognitive function (6, 7). The wide distribution of ECS in the brain, together with the effects derived from its pharmacological modulation on mood or cognition with exogenous cannabinoid compounds, mainly those contained or derived from the *Cannabis sativa* plant, suggests that the identification of the functional role of ECS elements in certain psychiatric disorders could be a breakthrough to improve diagnosis and treatment (8–11).

Therefore, this review summarizes the findings regarding the potential involvement of ECS components as biomarkers, mainly in terms of the discovery of new therapeutic approaches, but also from the point of view of its diagnostic, prognostic and predictive application. For that purpose, studies on animal models and patients have been collected focusing on the most prevalent psychiatric conditions, including anxiety disorders (3.8%) (12), depressive disorders (3.4%) (12), schizophrenia (0.3%) (12), bipolar disorder (0.6%) (12), post-traumatic stress disorder (7.8%) (13), attention-deficit hyperactivity disorder (2.2%) (14), and eating disorders (0.2%) (12).

A BRIEF OVERVIEW OF THE ENDOCANNABINOID SYSTEM COMPONENTS

ECS regulates a number of physiological functions and mediates the crosstalk between different neurotransmitter systems, therefore representing a key player in the control of behavioral responses (15, 16). ECS is a ubiquitous lipid signaling system distributed throughout the organism that participates in multiple intracellular signaling pathways (17, 18). Cannabinoid receptors, endogenous ligands or endocannabinoids (eCBs), and their synthesizing and degrading enzymes are the main components of the ECS (**Figure 1**) present in the central and peripheral nervous system (15, 19) and in many other peripheral tissues regulating distinct functions (20).

The CB1 receptor (CB1r) is the most abundant G protein-coupled receptor in the brain (21). Physiological actions of endocannabinoids in the CNS are mediated by the activation of CB1r (22). Their expression in the CNS is widespread and heterogeneous and has crucial roles regulating brain function and disease processes (23–25). CB1r is abundant in the basal ganglia, cerebellum, in corticolimbic regions including the prefrontal cortex (PFC), nucleus accumbens (Nac), and hippocampus (Hipp), and in

Abbreviations: ECS, endocannabinoid system; eCBs, endocannabinois; CB1r, cannabinoid receptor 1; CB2r, cannabinoid receptor 2; CSF, cerebrospinal fluid; PET, positron emission tomography; fMRI, functional magnetic resonance; BD, bipolar disorder; PTSD, posttraumatic stress disorder; ADHD, attention deficit hyperactivity disorder; YLDs, years lived with disability; DALYs, disability adjusted life-years; CNS, central nervous system; PFC, prefrontal cortex; NAc, nucleus accumbens; Hipp, hippocampus; Amy, amygdala; PVN, paraventricular nucleus; Hyp, hypothalamus; VTA, ventral tegmental area; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; NAPE-PLD, N-acylphosphatidylethanolamine specific phospholipase D; FAAH, fatty acid amide hydrolase; GPCRs, Gq protein-coupled receptors; DAGL, diacylglycerol; MAGL, monoacylglycerol lipase; ABHD, alpha/beta-hydrolase domain; DSM-5, diagnostic and statistical manual of mental disorders, 5th version; TRPV1, transient receptor potential cation channel subfamily V member 1; dlPAG, dorsolateral periaqueductal gray matter; NO, nitric oxide; WT, wild-type; DMH, dorsomedial hypothalamus; BLA, basolateral amygdala; EPM, elevated plus maze; LHb, lateral habenula; LC, locus coeruleus; DRN, dorsal raphe nucleus; NA, noradrenaline; 5-HT, serotonin; HPA axis, hypothalamus–pituitary–adrenal axis; CRH, corticotropin-releasing hormone; CRHR1, corticotropin-releasing hormone type 1 receptor; 5HTT, serotonin transporter; HDAC, histone deacetylase; CUS, chronic unpredictable stress; CB2xP, transgenic mice overexpression CB2r in the brain; BCP, beta-caryophyllene; THC, tetrahydrocannabinol; MDD, major depressive disorder; BDNF, brain-derived neurotrophic factor; TST, tail suspension test; FST, forced swimming test; CMS, chronic mild stress; FSL, Flinders sensitive line rats; WKY, Wistar Kyoto rats; cKO, conditional knockout; SSRIs, serotonin selective reuptake inhibitors; 5HTT, serotonin transporter; rTMS, repeated transcranial magnetic stimulation; ECT, electroconvulsive therapy; ACC, anterior cingulate cortex; PPI, prepulse inhibition; NMDAr, N-methyl-D-aspartate receptors; MAM, methylazoxymethanol; SHR, spontaneously hypertensive rat; CNR1P1, cannabinoid receptor interacting protein; Nrg1, neuregulin 1; DLPFC, dorsolateral prefrontal cortex; STG, superior temporal gyrus; qRT-PCR, quantitative real time polymerase chain reaction; FEP, first episode psychosis; PBMCs, peripheral blood mononuclear cells; PANSS, positive and negative syndrome scale; SUD, substance use disorder; CBD, cannabidiol; ACTH, adrenocorticotropin hormone; CS, corticosterone; SPS, single prolonged stress; DLS, dorsolateral striatum; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; SEA, steraoylethanolamide; AN, anorexia nervosa; BN, bulimia nervosa; NPY, neuropeptide Y; POMC, proopiomelanocortin; CART, cocaine-amphetamine-regulated transcript; ARC, arcuate nucleus; FID, food intake disorders; BMI, body mass index.

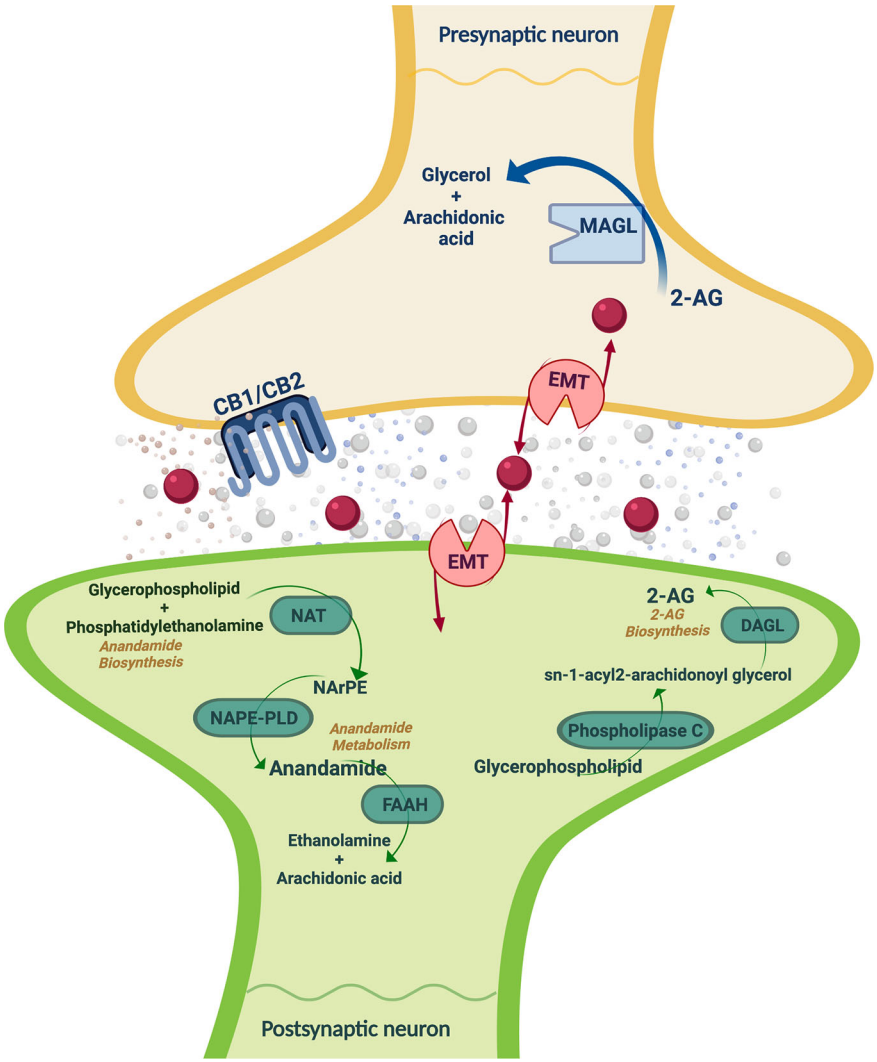

FIGURE 1 | Schematic representation of the main ECS components, including the metabolizing routes of the eCBs. CB1/CB2, cannabinoid receptors 1 and 2; 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; DAGL, EMT: endocannabinoid membrane transporter; NAT, N-acyl transferase; NArPE, N-arachidonoyl phosphatidylethanolamine; NAPE-PLD, N-acylphosphatidylethanolamine specific phospholipase D; DAGL, diacylglycerol lipase. Image created with BioRender.

brain areas related to stress responses, such as the central amygdala (Amy) and the paraventricular nucleus (PVN) of the hypothalamus (Hyp) (21, 26, 27). Furthermore, CB1r is also located in terminals of peripheral neurons and glial cells, as well as in the reproductive system (*i.e.* uterus, ovary, testis, prostate), some glandular systems (adrenal gland), adipose tissue, heart, liver, lung, bone marrow, thymus, and the microcirculation (20, 26, 28–33).

CB2 cannabinoid receptor (CB2r) was initially considered as a peripheral cannabinoid receptor due to its high expression in the rat spleen (34) and leukocyte subpopulation in humans (32), participating in the regulation of the immune system (35). The first findings identified the presence of CB2r in the CNS only under pathological conditions such as in senile plaques in Alzheimer's disease (36), activated microglial cells/ macrophages in multiple sclerosis, spinal cord in amyotrophic lateral sclerosis (37) and in the vicinity of tumors (38). However, Van Sickle and colleagues revealed that CB2r is expressed in neurons of the brainstem of mice, rats, and ferrets under normal conditions (39). This finding was key to increase the interest of CB2r in the regulation of brain function. Different studies identified CB2r in several brain regions including the frontal cortex, striatum, basal ganglia, Amy, Hipp, and the ventral tegmental area (VTA) (40–44). Interestingly, in some of these brain regions, CB2r was detected not only in the microglia (45) but also in the neurons (44, 46, 47).

The eCBs are lipid messengers acting as paracrine, autocrine, and probably endocrine mode, because their lipid nature allows them to diffuse and cross membranes (15, 17, 18, 48, 49). eCBs are agonists of

CB1r and CB2r that are not accumulated in secretory vesicles but rather synthesized under tonic or phasic (on demand) modes, and released to the extracellular space following physiological and pathological stimuli (50). The two main eCBs are derivatives of polyunsatured fatty acids, N-arachidonoylethanolamine (anandamide, AEA) (51), and 2-arachidonoylglycerol (2-AG), being the most abundant eCBs in the brain (52). Firstly, AEA synthesis is produced by the N-acylphosphatidylethanolamine specific phospholipase D (NAPE-PLD) that hydrolyzes N-arachidonoyl phosphatidylethanolamine localized in cell membranes (49, 53). The AEA half-life is very short because of its quick uptake by a high affinity AEA membrane transporter distributed in the neurons and glia (54). AEA is inactivated by fatty acid amide hydrolase (FAAH) present in many organs and in the brain at postsynaptic location (55, 56). FAAH is a serine-hydrolase enzyme bound to intracellular membranes that metabolizes AEA into arachidonic acid and ethanolamine (57). Secondly, 2-AG participates in the CB1r-dependent retrograde signaling and is an intermediate metabolite for lipid synthesis providing arachidonic acid for prostaglandin synthesis (57). Neuronal membrane depolarization or the activation of Gq protein-coupled receptors (GPCRs) triggers the synthesis of 2-AG (49). The diacylglycerol precursors come from the hydrolysis of membrane phosphatidylinositol by phospholipase C, β or δ. The degradation of these precursors by diacylglycerol lipases (DAGL-α and DAGL-β) drives 2-AG synthesis (58, 59). The DAGLα isoform synthesizes the greatest amount of 2-AG, whereas DAGLβ synthesizes 2-AG under certain circumstances (54). Monoacylglycerol lipase (MAGL) is a serine-hydrolase enzyme mainly found in presynaptic terminals that catalyzes 2-AG into arachidonic acid and glycerol (55, 60). Also, the α/β-hydrolase domain 6 (ABHD6) and domain 12 (ABHD12) degrade 2-AG (49, 57).

THE ENDOCANNABINOID SYSTEM IN PSYCHIATRY: SEARCHING FOR POTENTIAL BIOMARKERS

The ECS is one of the most widely distributed neurotransmitter systems in the human brain, with a critical neuromodulatory role that motivates the interaction with other neurotransmitter and neurohormonal systems (61). Accumulating evidence points out the pivotal role of the ECS in the regulation of cognitive and behavioral functioning, suggesting its therapeutic potential in psychiatry (9, 11, 62). Furthermore, it is worth to mention that psychiatric disorders are accompanied by disturbances in the ECS components, as detailed below. Taken together, these facts suggest the potential usefulness of cannabinoid receptors, endocannabinoid ligands and degrading or synthesizing enzymes as biomarkers to move towards improved diagnostic criteria and therapeutic approaches in psychiatry.

The literature review consisted of an exhaustive search for scientific information in the Medline database (PubMed), which was always focused on the following ECS components as potential biomarkers in psychiatry: CB1r, CB2r, AEA, 2-AG, FAAH and MAGL. A total of seven search boxes were employed according to the total of psychiatric conditions included in the review: anxiety, depression, schizophrenia, bipolar disorder, post-traumatic stress disorder, attention-deficit and hyperactivity disorder, and eating disorders. These terms were combined with the term 'cannabinoid' by the Boolean operator 'AND'. All the results for each search were critically analyzed by the authors to decide the inclusion or exclusion of each reference according to the adequacy of its content with the subject matter of the study. Finally, no PubMed filters were applied to maximize the selection of all the available and appropriate information.

Anxiety Disorders

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), anxiety disorders share features of excessive fear and anxiety and related behavioral disturbances. Fear is the emotional response to real or perceived imminent threat, whereas anxiety is an emotional anticipatory response to future potential threatening or stressful situations, triggering symptoms of negative affective, somatic, behavioral and cognitive components (63). The ECS plays a prominent role in the stress response and anxiety, as it is widely documented mainly by animal studies (64–67). However, our knowledge on the precise molecular mechanisms of the ECS signaling in humans is insufficient (68, 69). In the last years, compelling evidence for the involvement of ECS in anxiety has been accumulated that suggests new therapeutic leads through the discovery of potential biomarkers.

Animal Studies

A large body of literature supports the involvement of CB1r as a potential biomarker in anxiety disorders (70–72). CB1r is widely distributed in brain areas associated with emotional regulation and stress responsiveness such as PFC, Hipp, Amy, and Hyp (19). Previous pharmacological studies evaluated the effects of different cannabinoid compounds after either systemic or intracerebral administration in rodents exposed to several animal models of anxiety (73, 74). In addition, it is important to highlight the pivotal role of CB1r in the effects of anxiolytic drugs such as benzodiazepines. Indeed, our group demonstrated that the CB1r antagonist, AM251, completely abolished the anxiolytic effects and significantly reduced the amnesic and the sedative actions induced by alprazolam (75). A very similar result was recently obtained regarding the AM251-induced blockade of the anxiolytic effects of alprazolam (76). On the other hand, the enhancement of CB1r-mediated endocannabinoid function increases the anxiolytic action of diazepam (77).

Accumulated evidence points out that CB1r manipulation produces a bidirectional effect on anxiety-related behavior (78, 79). CB1r activation decreases anxiety at lower doses (80), whereas anxiogenic effects occur at higher doses or after CB1r blockade (70, 81–87). However, several factors could modify this general assumption such as regional endogenous tone, age, sex, species differences, type of test, previous exposure to stressful situations, or dosage of cannabinoid receptor agonists or antagonists. In addition, the underlying mechanisms involved in the bidirectional effects of CB1r pharmacological modulation remain poorly understood. Among the available evidences

addressing this aspect, one study revealed that CB1r in the cortical glutamatergic neurons mediates the anxiolytic effect of CP-55,940 cannabinoid agonist at low doses, whereas anxiogenic actions of higher doses are related with CB1r and $GABA_B$ receptors in GABAergic terminals (88). A growing body of evidence also suggests that the anxiogenic effects of moderate to high cannabinoid doses appear to be mediated by the interaction between endocannabinoid and endovanilloid systems, specifically through the activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) vanilloid receptors (89). In this regard, the combination of high WIN-55,212 doses in the dorsolateral periaqueductal gray matter (dlPAG) with the TRPV1 antagonist capsazepine abolished the anxiogenic effect (90). Furthermore, the anxiolytic effects of high doses of the cannabinoid agonist ACEA combined with an antagonist of TRPV1 in the rat prelimbic medial prefrontal cortex (PL) suggested the critical interaction between both systems (91). Moreover, the co-administration of intra-dlPAG AEA at higher doses with a nitric oxide (NO) scavenger (carboxy-PTIO) restored the anxiolytic profile, leading to the hypothesis that the increase in anxiety-like behavior mediated by TRPV1 receptors is due to subsequent NO formation (92).

Deletion of *CNR1* gene in mice ($CB1^{-/-}$ mice) has been another important tool to elucidate the role of this cannabinoid receptor in anxiety. Many studies have demonstrated the clear anxiety-like behavior of male $CB1^{-/-}$ mice (70–72, 83, 93, 94), although there are some negative results (95). Among the multiple mechanisms involved in the anxious phenotype shown by $CB1^{-/-}$ mice, significant age-dependent alterations in the metabolism of endocannabinoids could be pointed out (96). Interestingly, $CB1^{-/-}$ female mice do not have an anxious phenotype in comparison with female wild-type (WT) subjects. This finding supports an interaction between sex and the ECS at early stages of development that is critical for establishing adult anxiety-like behavior (97). Indeed, these sex-specific effects were also described under pharmacological blockade of CB1r (98). Furthermore, our group described that the effects of the anxiolytic drugs bromazepam and buspirone were missing in $CB1^{-/-}$ mice (99), suggesting a critical role of CB1r that was related with the control of GABAergic responses mediated by $GABA_A$ and $GABA_B$ receptors (100).

Recent studies provide relevant information regarding the specific brain regional involvement of CB1r-mediated anxiolytic actions. In this sense, the intra-dlPAG administration of AEA, ACEA (selective CB1r agonist) or AM404 (AEA reuptake inhibitor) induced anxiety-like responses that were blocked by AM251 (CB1r antagonist) (101). Similarly, AEA-mediated CB1r activation produces anxiolytic-like actions in the dlPAG employing a panic-like animal model (102, 103) or the Vogel conflict test (104). In addition, facilitation of 2-AG-mediated signaling in the dorsomedial hypothalamus (DMH) significantly reduced panic-like responses in Wistar rats, an effect that was reversed by the CB1r antagonist AM251 (105). Furthermore, activation of CB1r by 2-AG in the basolateral amygdala (BLA) has a critical role in the effects of stress-induced glucocorticoid release on suppression of synaptic GABAergic inhibition (106). Interestingly, pharmacologically-induced elevations of AEA or 2-AG in the BLA decrease anxiety in the elevated plus maze (EPM) test under conditions of low emotional arousal while are ineffective when the level of emotional arousal increased (107). Moreover, electron microscopy revealed CB1r expression in the rat lateral habenula (LHb), mediating the actions of increased 2-AG levels after acute stress exposure, while its blockade by SR141716 (rimonabant) significantly reduced anxiety-like behavior (108). In another study, WIN-55,212 was locally administered in the lateral septum (LS) of male Wistar rats, producing a CB1r-mediated anxiogenic response in the EPM paradigm since AM251 blocked this effect (109). Also, the role of CB1r functional manipulation in anxiety behavior regulation and the effects on subsequent signaling pathways in relevant corticolimbic areas such as PFC, AMY, NAc, and Hipp (110–116) have been evaluated.

A better understanding of the functional connections of the ECS with other neurotransmitter or neurohormonal systems is relevant to understand the role of ECS components as potential biomarkers in psychiatry. According to previous studies, CB1r is located in the locus coeruleus (LC) and in the dorsal raphe nucleus (DRN), and it regulates noradrenaline (NA) and serotonin (5HT) release, respectively, by the modulation of GABAergic and glutamatergic terminals (117, 118). In addition, the dopaminergic and opiodergic systems of the Amy may also be involved in the anxiolytic-like effects induced by the activation of CB1r (119, 120). Furthermore, the involvement of the ECS in the regulation of the hypothalamus–pituitary–adrenal (HPA) axis after stress exposure attracted special attention in the last years (121). Gray and cols. recently found that the stress-related neuropeptide corticotropin-releasing hormone (CRH), acting through the CRH type 1 receptor (CRHR1), reduces AEA levels in the PFC and the Amy by increasing the hydrolysis of FAAH and by increasing 2-AG levels. These data suggest that stress-related elevations in CRH signaling induce persistent changes in eCB function, impairing its tonic regulation on stress and enhancing anxiety responses (122, 123).

Genetic studies pointed out interesting results regarding the involvement of polymorphisms or epigenetic modifications of CNR1 as susceptibility/risk biomarkers to develop anxiety disorders. Lazary and cols. analyzed the interaction of the promoter regions of the serotonin transporter (5HTT; SLC6A4) and CNR1 genes on anxiety. Specific constellations of CB1r and 5HTT promoters were closely associated with high or low synaptic 5HT concentrations, which could result critically in the vulnerability to experience an anxiety disorder (124). Hay and cols. employed CRISPR/CAS9 technology to disrupt a highly conserved regulatory sequence (ECR1) of the gene encoding CB1r (CNR1). This manipulation significantly reduced CNR1 expression in the Hipp, but not in the Hyp, and induced a sex-dependent anxiogenic effect (125). In addition, a connection between ECS and epigenetic mechanisms was proposed. The exposure to immobilization stress increases anxiety-like behavior, an effect blocked by histone deacetylase (HDAC) inhibitors. Interestingly, the CB1r antagonist rimonabant

attenuated the anxiolytic-like effects of the HDAC inhibitors, suggesting an association between epigenetic mechanisms and ECS signaling (126). Furthermore, in mice exposed to a chronic unpredictable stress (CUS) there were reduced levels of histone H3K9 acetylation (H3K9ac) associated with CB1r encoding gene (127).

Since the direct pharmacological modulation of CB1r has provided some disappointing results, in recent years much attention has been paid to the therapeutic role of functional manipulation of the endogenous cannabinoid ligands AEA and 2-AG by inhibiting enzymatic degradation (FAAH and MAGL, respectively) or blocking reuptake (128–132). AEA plays a crucial role in emotional control (133). Inhibition of its degradation by FAAH or its reuptake induces a robust anxiolytic effect (134–142). Indeed, stress exposure induces anxiety-like behavior and reduces AEA brain levels (143) by increasing FAAH activity in the Amy (144), a brain region closely involved in AEA-mediated emotional regulation (145). According to the effects observed in FAAH *knockout* mice ($FAAH^{-/-}$ mice), as well as with the administration of URB597 (FAAH inhibitor), preservation of CB1r function regulating GABA transmission in the striatum may be one the mechanisms involved in the anxiolytic actions of FAAH inhibition (146, 147). Environmental experimental conditions are critical to observe the anxiolytic effect of FAAH inhibition, only present under high stressful or aversive stimuli (148). Interestingly, the dual blockade of FAAH and TRPV1 represents another therapeutic approach to reduce anxiogenic behavior (149). In addition, co-administration of an ineffective dose of URB597 with an ineffective dose of diazepam led to a synergistic anxiolytic action (77).

The endocannabinoid 2-AG also presents a close involvement in emotional regulation linked with signaling in hippocampal glutamatergic neurons (150). In the last years, several evidences support the anxiolytic actions associated with the inhibition of 2-AG enzymatic degradation by means of MAGL (151–153). A link with the HPA axis has been proposed, since the elevation of 2-AG levels was accompanied by a dramatic increase in plasma corticosterone, effect that is probably mediating its anxiolytic actions (154). In addition, increased 2-AG levels in the NAc of mice previously exposed to chronic social defeat stress are associated with an anxiolytic effect and the enhancement of synaptic plasticity (155). Furthermore, the enhancement of 2-AG levels in the dlPAG by the local injection of 2-AG or the hydrolysis inhibitor, URB602, prevented NMDA-induced panic-like response in Wistar rats (156). Interestingly, genetic deletion of MAGL in mice induced an anxiety-like phenotype (157), whereas mice lacking DAGLα showed a high anxiety-like phenotype, strengthening the critical involvement of 2-AG in emotional regulation (158, 159).

Another relevant endocannabinoid biomarker is the CB2r. The first studies demonstrating the role of this receptor in the regulation of anxiety-like behavior were performed in our laboratory employing transgenic animals overexpressing CB2r in the brain (CB2xP mice). Increased expression of CB2r was significantly correlated with reduced anxiogenic-related behaviors. Interestingly, CB2xP mice presented an impaired HPA-axis response to restraint stress, as well as increased $GABA_{A\alpha 2}$ and $GABA_{A\gamma 2}$ gene expression probably accounting for the lack of anxiolytic action of alprazolam in these animals (160). Furthermore, a pharmacological approach to evaluate acute and chronic effects of the activation (JWH133, CB2r selective agonist) or blockade (AM630, CB2r selective antagonist) of CB2r revealed opposite effects. Importantly, chronic CB2r blockade induced a significant anxiolytic effect that was associated with an upregulation of CB2r, $GABA_{A\alpha 2}$ and $GABA_{A\gamma 2}$ in the cortex and the amygdala (160). In line with these results, the acute activation of CB2r by the administration of β-caryophyllene (BCP) induced an anxiolytic effect that was completely abolished by AM630-mediated CB2r blockade (161). Recently, Robertson and cols. described that CB2r gene expression is rapidly increased in the Hipp after social stress exposure (social defeat) (162). Genetic manipulation experiments allowed the deepening in the cell-specific functional involvement of CB2r in the Hipp, dissecting the effects of CB2r gene expression disruption in hippocampal neurons or microglia on the regulation of anxiety behavior (163). Moreover, the functional role of CB2r in VTA dopaminergic neurons was also explored. Surprisingly, deletion of CNR2 in VTA dopaminergic neurons induced a very significant anxiolytic effect (47).

Human Studies

In 1981, Fabre and McLendon published the first evidences regarding the anxiolytic properties of cannabinoid compounds. In this study, the synthetic cannabinoid nabilone was administered to 25 patients, producing a significant improvement in anxiety (164). Nowadays, there is a large body of evidence regarding cannabis consumption and regulation of anxiety behavior (165), although the underlying mechanisms are poorly understood. A recent study addressed this issue by combining fMRI and positron emission tomography (PET) in 14 patients following an oral dose of delta-9-tetrahydrocannabinol (THC) while they were performing a fear-processing task. The results suggested that the acute effects of cannabis on anxiety in males are mediated by the modulation of amygdalar function by THC and the extent of these effects are related to local availability of CB1r (166). On the other hand, several clinical trials using rimonabant to treat obesity showed psychiatric side effects such as increased anxiety behavior, depression or even suicidality (167). In spite of the presence of important confounding factors that probably were not appropriately taken into consideration (*e.g.* psychiatric comorbidity in obese patients), rimonabant was withdrawn from the market, and the enthusiasm in its therapeutic usefulness significantly decreased. Interestingly, a recent report suggested that rimonabant increases anxiety only under an aversive/anxiogenic situation (public speaking), without modifying baseline anxiety behavior (168). Alternative pharmacological approaches to modulate CB1r are now under investigation. Neutral antagonists, peripherally restricted ligands, and allosteric modulators may provide promising results [for a recent review (169)].

The elucidation of genetic variations of different endocannabinoid components involved in the vulnerability to develop anxiety-related disorders has recently gained great

interest. In this regard, Gonda and cols. evaluated the interaction between four categories of stressful life events and specific genetic variations in the CNR1 rs7766029 polymorphism, for the development of depression and anxiety. The results suggested that CNR1 rs7766029 interacted significantly with financial but not with other types of life events to increase the vulnerability to develop depression and anxiety (170). In addition, allelic variants of the gene encoding FAAH have been involved in the regulation of anxiety-related behaviors. First, the disturbances of FAAH genetic variation in AEA hydrolysis appear related with alterations in frontolimbic circuits (171), with an age-dependent effect accounting for differences between the adolescence and childhood life stages (172). Second, an interaction between genetic variations of FAAH and corticotropin-releasing hormone receptor type 1 (CRHR1) has been described in relation with the risk to develop anxiety disorders (173, 174). Third, reduced FAAH activity in patients carrying the A allele of the FAAH rs324420 (C385A) polymorphism significantly increases the vulnerability to develop anxiety and depression when exposed to repetitive childhood trauma (175). Moreover, a functional variant of gene encoding CB2r (Cnr2) appears to interact with FAAH gene, increasing the sensitivity for childhood trauma when both are dysfunctional (176).

Depressive Disorders

Major depressive disorder (MDD) has been one of the leading causes of years lived with disability (YLD) during the last three decades (1). According to the World Health Organization estimation for 2015, the number of people living with depression in the world is 322 million, and it is a major contributor to suicide deaths (177). DSM-5 states that the common feature of depressive disorders is the presence of sad, empty, or irritable mood, accompanied by somatic and cognitive changes that significantly affect the individual's capacity to function. MDD is being characterized by distinct changes in affect, cognition, and neurovegetative functions with episodes lasting for at least 2 weeks. Additionally, five or more symptoms have to be present during the same episode, with at least one of the symptoms being either depressed mood or anhedonia (63). Nowadays, pharmacological treatment of MDD entails relevant limitations such as delayed onset of antidepressive actions and appearance of important side effects. The limited success of drug discovery in the context of depression is ultimately linked to an inadequate understanding of the underlying biology of this disorder. In this sense, there is evidence to suggest that the ECS is impaired in MDD providing a unique opportunity to identify potential diagnostic and therapeutic biomarkers.

Animal Studies

Martin and cols. employed the $CB1^{-/-}$ mice and exposed them to the CUS procedure. Their findings showed that $CB1^{-/-}$ were more vulnerable to CUS-induced depressive-like responses and presented an increase susceptibility to develop anhedonia (94). Some years later, it was shown that the increased despair behavior in $CB1^{-/-}$ mice was critically associated with down-regulated brain-derived neurotrophic factor (BDNF) levels in the Hipp. Also, local administration of BDNF in the Hipp of these animals reversed the depressive-like phenotype (178). A complete genetic screening by mRNA microarray hybridization revealed a differential gene expression pattern related to the high depressive-like behavior of $CB1^{-/-}$ mice at basal conditions (179). According to the results derived from the studies employing $CB1^{-/-}$ mice, it was proposed that $CB1^{-/-}$ mice could represent a validated and appropriated model to evaluate depressive-like disorders (180).

In the tail suspension test (TST) and forced swimming test (FST), acute AM251 injection induced an antidepressant effect, decreasing the immobility time in both behavioral paradigms (181). Similar results were obtained by both the acute and chronic administration of rimonabant in Wistar rats and BALB/c mice employing the FST and the chronic mild stress (CMS) paradigms, respectively (182). However, other results reveal that the activation of CB1r mediates antidepressant effects (183–187), and even that chronic rimonabant administration produces a depressogenic effect (188). Interestingly, McLaughlin and cols. showed that CB1r located in the dentate gyrus of the Hipp was responsible for the antidepressant effects of the CB1r agonist HU-210 (189). In addition, a very recent study elegantly discovered a circuit-specific CB1r-mediated modulation of glutamatergic transmission that shapes the information flow from BLA to the NAc (190). In this study, the authors consider if the reduction of CB1r in the NAc may be used as a biomarker for MDD diagnosis and point out that this aspect needs to be further determined by evaluation of CB1r levels in the NAc of MDD patients (190).

The evaluation of ECS components disturbances in animal models of depressive disorders provided relevant information. Hill and cols. showed that male Long–Evans rats exposed to the CUS presented increased CB1r binding site density in the PFC while decreased in the Hipp, Hyp and Nac, and lower levels of AEA were found in all these brain regions (191). Furthermore, sex-dependent effects of CUS were analyzed in Sprague-Dawley rats, obtaining lower and higher CB1r protein expression in males and females, respectively, whereas increased FAAH levels were present in both sexes (192). In addition, further studies employing the CUS procedure specifically focused on CB1r-mediated signaling, revealing significant loss of function disturbances in the NAc (193) and in the LHb (194). Moreover, apart from stress-related animal models, Flinders Sensitive Line (FSL) or Wistar Kyoto (WKY) rats are well-known genetic rat models of depression that were recently used to exhaustively analyze disturbances in different components of the ECS in specific brain regions and plasma (195, 196).

Enhancement of endocannabinoid signaling has been postulated as a new promising pharmacological strategy in the treatment of stress-related disorders (*e.g.* anxiety or depression) (197). Accordingly, a significant reduction in depressive-like behavior was found after the administration of the FAAH inhibitors URB597 (196, 198, 199) or PF3845 (200). In addition, the inhibition of MAGL by the administration of

JZL194 also yielded similar antidepressant effects in the CUS animal model of depression. Interestingly, JZL194-mediated effects may be related with an enhancement of adult neurogenesis and long-term synaptic plasticity in the dentate gyrus of the Hipp, probably activating mTOR signaling pathway (201). Furthermore, a recent study evaluated the effects of JZL195, a dual inhibitor of FAAH and MAGL, in WKY rats. JZL195 elevated the endocannabinoids and BDNF levels in the ventral striatum and reduced the depressive-like phenotype in female WKY rats (202).

In spite of the limited available results, CB2r is also critically involved in emotional regulation (203). Probably, the first evidence suggesting the role of CB2r in depression was a significant reduction of these receptors in the striatum, midbrain, and Hipp in an animal model of depression (204). Afterwards, a study showed the antidepressant effects of the CB2r-seletive agonist GW405833 in rats (205). Interestingly, our group further evaluated CB2r involvement in depressive-like behavior regulation using genetic and pharmacological approaches. Mice overexpressing CB2r (CB2xP) presented decreased depressive-like behaviors under basal conditions or after the exposure to a CUS procedure. In addition, the chronic administration of AM630 blocked the CUS-induced depressogenic effect in stressed mice, effect associated with an upregulation of CB2r and BDNF in the Hipp (43). Recently, similar results were obtained by CB2r functional activation through the administration of the CB2r agonists JWH133 (206) and β-caryophyllene (207). Furthermore, the specific deletion of CB2r in midbrain DA neurons in DAT-Cnr2 conditional knockout (cKO) mice significantly increased depressive-like behavior (47).

Crosstalk of ECS with other neurotransmitter or neurohormonal systems plays a pivotal role in the effects produced by antidepressant drugs. In this regard, interactions with the serotonergic system represent a critical point due to the widely recognized clinical therapeutic usefulness of antidepressants targeting serotonin (*e.g.* serotonin selective reuptake inhibitors, SSRIs). SSRIs fluoxetine and escitalopram modify the concentrations of different ECS components under basal conditions (208–210) or in an animal model of depression (211). Furthermore, low doses of WIN-55,212 produced antidepressant-like actions that appeared to be mediated by 5HT (212), and $CB1^{-/-}$ mice have decreased levels of 5HT transporter (5HTT) (213). Moreover, co-administration of a subeffective dose of fluoxetine potentiated the effect of subeffective doses of AEA, AM404 or URB597 (214). In addition, ECS also interacts with other systems involved in emotional and stress regulation such as the HPA axis (215), glutamatergic (216), opioidergic (217), and cholinergic (218) systems.

On the other hand, it is relevant to highlight that nonpharmacological approaches such as repeated transcranial magnetic stimulation (rTMS) improve depressive-like behavior, at least in part, by modulating the ECS. Recent studies performed in rodents exposed to CUS and subsequently treated with rTMS revealed that: 1) rTMS increases BDNF production and hippocampal cell proliferation to protect against CUS-induced changes through its effect on CB1r (219); 2) rTMS antidepressive effects are at least partly mediated by increasing hippocampal 2-AG and CB1 receptor expression levels (220); and 3) high-frequency rTMS induces its antidepressant effect by upregulating DAGLα and CB1r (221). In addition, electroconvulsive therapy (ECT) significantly reduced AEA content and FAAH activity in the PFC of Sprague-Dawley rats, as well as decreased and enhanced binding site density of the CB1r in the PFC and Amy, respectively (222).

Human Studies

Besides the preclinical clues supporting the critical role of ECS in depression, currently there is a broad body of evidence available from clinical studies. Among them, those evaluating alterations in different ECS components in *post-mortem* brain tissue or plasma samples have provided compelling results. The first evidence revealed that CB1r protein expression was decreased in the anterior cingulate cortex (ACC) of patients with major depression (223). Furthermore, Choi and cols. showed that CB1r mRNA levels were higher in the PFC of major depression patients (224). However, in a recent study a lack of CB1r protein expression differences was found between depressive subjects and paired control patients (225).

In the last years, an increasing effort has been made to elucidate the alterations of ECS components (mainly the endocannabinoids AEA and 2-AG) in blood samples of patients with depression, to identify possible trait, prognosis or monitoring biomarkers that could improve the therapeutic approach. In a cohort of 28 women with diagnostic criteria for clinical depression and without medication, serum 2-AG content was significantly decreased, and this decrease was negatively correlated with duration of the depressive episode (226). Similarly, basal serum concentrations of AEA and 2-AG were significantly lower in women with nontreated major depression, and the exposure to a stressful situation significantly increased 2-AG concentrations without modifying AEA (227). However, another study described increased plasma concentrations of both AEA and 2-AG in depressed patients, and the elevation of 2-AG was significantly associated with SSRI antidepressant therapy (228). Interestingly, the antidepressant-related effects or physical exercise on eCBs levels were also analyzed. Intense exercise in control healthy patients induced a significant increase in AEA serum levels that was correlated with higher BDNF levels, whereas 2-AG concentrations remained stable (229). On the contrary, moderate exercise in women with MDD produced significant elevations in AEA but not in 2-AG, although both eCBs presented significant moderate negative associations between serum changes and mood states (230). Finally, ECT significantly elevated AEA and 2-AG levels in the cerebrospinal fluid (CSF) of patients with major depression (231).

The ECS-related polymorphic gene variant study results are relevant because of the potential diagnostic and therapeutic implications. Regarding the CNR1 and the single nucleotide polymorphism (SNP) rs1049353 (G1359A) that may contribute to the susceptibility to mood disorders (232), G-allele has been associated with higher depressive-related symptomatology (233) and increased risk of antidepressant treatment resistance in women with comorbid anxiety disorder (234). However, it provides a better response to citalopram in male depressive patients (235), whereas A-allele decreased risk to develop depression because of childhood physical abuse (236).

Furthermore, several CNR1 polymorphisms appeared to be related with high neuroticism and low agreeableness personality traits, increasing the risk to develop depression (237). On the other hand, the presence of 2 long alleles of the polymorphic triplet (AAT)n of CNR1 gene was associated with reduced prevalence of depression in Parkinson's disease patients (238). In addition, the minor C allele of the CNR1 rs2023239 polymorphism may confer a protective effect against lifetime development of MDD in methadone-maintained patients (239). Despite the previous findings, a recent meta-analysis points out that CNR1 rs1049353 or AAT triplet repeat polymorphism had no association with susceptibility to depression (240).

Other relevant gene polymorphisms of the ECS are those related with FAAH and CB2r. First, variants of the FAAH gene may be related with susceptibility to mood disorders such as major depression (232). In fact, genetically reduced FAAH activity in A allele carriers of FAAH rs324420 (C385A) polymorphism constitutes a risk factor to develop anxiety and depression in patients exposed to repetitive childhood trauma. Interestingly, the authors noted that this genotype could entail pharmacogenomic consequences, namely ineffectiveness or adverse effects of FAAH inhibitors in this subpopulation (175). Second, polymorphisms of CNR2 were first studied by Onaivi and cols. in Japanese depressed patients, revealing a high incidence of Q63R but not H316Y polymorphism (204, 241). Recently, the R allele of Q63R CNR2 polymorphism, together with the A allele of FAAH C385A polymorphism were associated with increased sensitivity for childhood trauma and subsequent expression of anxious and depressive phenotypes (176). Finally, according to the previously mentioned recent meta-analysis performed by Kong and cols., CNR2 rs2501432 polymorphism might be closely associated with depression (240).

Schizophrenia

According to the DMS-5 schizophrenia is a psychotic disorder associated with a myriad of signs including positive symptoms (delusion, hallucinations, disorganized speech or grossly disorganized or catatonic behavior), negative symptoms (lack of motivation and social withdrawal), and cognitive symptoms (reduced attention and altered speech) (63, 242, 243). An extensive body of literature supports the role of ECS in schizophrenia neuropathology, a fact that is mainly sustained by the psychotic effects derived from cannabis consumption and attributed to the exogenous cannabinoid THC (244). Therefore, a great interest has been posed in the identification of specific biomarkers related with ECS functioning for preventive, diagnostic, or therapeutic purposes.

Animal Models

Preclinical research that focused on the role of ECS in schizophrenia relies on the evaluation of sensorimotor gating deficits by the prepulse inhibition (PPI) paradigm (245, 246). Among all the components of the ECS, CB1r is critically involved in schizophrenia. In fact, results of studies using pharmacological approaches showed that CB1r activation induces psychotic-like effects, while blockade of CB1r presents opposite actions. Decreases in startle responses together with PPI disruption were achieved by CP-55,940 administration, and rimonabant completely reversed these effects (247). A similar experiment was carried out in which CP-55,940 decreased startle response and impaired PPI, and rimonabant significantly reversed CP-55,940-induced deficits in PPI only at the lower prepulse intensity (248). This CB1r-mediated auditory gating disruption was further confirmed by measuring neuronal network oscillations in the Hipp and entorhinal cortex of Sprague-Dawley rats. CP-55,940 significantly impaired sensory gating and neuronal oscillation, an effect that was reversed by AM251 (249).

After learning that the modulation of the CB1r produced sensorimotor alterations, different animal models of schizophrenia were used to find out if CB1r blockade could be a strategy with therapeutic potential. Blockade of N-methyl-D-aspartate (NMDA) receptors (NMDAr) was used to simulate schizophrenia-like symptoms in rodents (250). Interestingly, the administration of AM251 significantly abolished phencyclidine-induced disruption of PPI in a similar way to clozapine (251), as well as impairments in recognition memory or increased behavioral despair in the FST (252). Another NMDAr antagonist used to model schizophrenia-like behavior is MK-801. The administration of the CB1r antagonist AVE1625 reversed MK801-induced cognitive impairments and decreased catalepsy and weight gain induced by clinically used antipsychotic drugs (haloperidol, olanzapine) (253). Furthermore, AM251 attenuated amnesic effects and hyperactivity induced by MK-801 (254). Therefore, it appears that blockade of CB1r may have relevant therapeutic applications for the treatment of schizophrenia.

In the so-called 'three-hit' animal model of schizophrenia, CB1r binding and cannabinoid agonist-mediated G-protein activation decreases in the cortical, subcortical, and cerebellar brain regions (255). In a neurodevelopmental animal model of schizophrenia induced by the gestational administration of methylazoxymethanol (MAM), CB1r mRNA levels were lower in the PFC and higher in the dorsolateral striatum of adult MAM-treated Sprague-Dawley rats relative to the control group (256). Moreover, in the spontaneously hypertensive rat (SHR) strain, partially reproducing some schizophrenia-like behavioral aspects, CB1r immunoreactivity was significantly increased in the PL, cingulate cortex, and CA3 region of the Hipp (257). Recently, two studies found decreased methylation of the cannabinoid receptor interacting protein (CNR1P1) DNA promoter in the ventral Hipp (vHipp) of rats exposed to the MAM model (258, 259). CNR1P1 is an intracellular protein that interacts with the C-terminal tail of CB1r and regulates its intrinsic activity. Interestingly, a lentivirus-mediated overexpression of CNR1P1 in the vHipp of Sprague-Dawley rats induced significant schizophrenia-like cognitive and social interaction impairments, together with an increase of dopamine neuron population activity in the VTA (260).

Apart from the many preclinical studies supporting the pivotal role of CB1r, animal models of schizophrenia provided interesting results about eCBs brain level alterations. In this regard, a significant increase in 2-AG levels in the PFC of PCP-treated Lister-Hooded rats was reversed by treatment with THC, which in turn induces a large reduction of AEA in the same

region (261). Furthermore, in Sprague-Dawley rats exposed to a bilateral olfactory bulbectomy, considered as an animal model of depression and schizophrenia, a significant decrease of AEA and 2-AG levels was found in the ventral striatum (262). In addition, mice with a heterozygous deletion of neuregulin 1 (Nrg 1 HET mice), a well-accepted and characterized animal model of schizophrenia (263), displayed relevant alterations in eCBs levels (264).

Finally, CB2r has also been recently involved in schizophrenia. Ishiguro and cols. studied the effects of the pharmacological blockade of CB2r in two animal models of schizophrenia induced by the administration of MK-801 or metamphetamine. The CB2r antagonist AM630 significantly exacerbated the MK-801- or metamphetamine-induced hyperlocomotion and PPI disruption, suggesting that CB2r was mediating these actions (265). Our group analyzed exhaustively the behavioral profile of $CB2^{-/-}$ mice to evaluate the implication of CB2r in schizophrenia-like behavior. The phenotype showed by $CB2^{-/-}$ mice resembled some relevant features of schizophrenia such as increased sensitivity to motor effects of cocaine, anxiety- and depressive-like behavior, disrupted short- and long-term memory consolidation and impaired PPI. These behavioral alterations were accompanied by gene expression changes in different targets from dopaminergic, noradrenergic, and serotonergic systems. Interestingly, the atypical antipsychotic risperidone significantly improved PPI disruption induced by CB2r deletion and differentially modulated some of the neurochemical disturbances compared with WT mice (266). In addition, the activation of CB2r by the agonist JWH015 reversed PPI disruptions of the MK-801-induced animal model of schizophrenia, and this effect was specifically mediated by CB2r since only AM630 but not AM251 abolished PPI improvement (267). Furthermore, activation of CB2r (JWH133) and blockade (AM630) increased MK-801-induced hyperlocomotion, although this effect was much more evident and pronounced with AM630 (268). Therefore, these results strongly suggest that CB2r functional regulation is significantly involved in schizophrenia-like behavior.

Human Studies

To date, an extensive and great effort has been made to elucidate the role that CB1r plays in schizophrenia. Accumulated clinical data clearly shows significant alterations of CB1r protein and gene expression levels, as well as certain CNR1 polymorphisms correlations, especially in the brain but also in the peripheral blood cells from schizophrenic patients in comparison with healthy control subjects. The information reviewed and detailed below provides important clues to further investigate the application of CB1r-related measures as potential trait, state, prognostic or even therapeutic biomarkers.

Several published studies analyzed CB1r protein and gene expression levels in different *post-mortem* brain regions from schizophrenic patients. Several studies examined quantitative autoradiography to evaluate CB1r availability through the binding of different radioligands. A significant increase in CB1r availability was shown in the dorsolateral prefrontal cortex (DLPFC) (269–271), although this increase was only present in paranoid schizophrenic patients (272). Interestingly, a recent study failed to show differences in CB1r-mediated functional coupling to G-proteins in the PFC of schizophrenic and control patients (273). Furthermore, higher CB1r binding levels were shown in the left ACC (274) and in superficial layers of the posterior cingulate cortex (PCC) (275), whereas no changes were found in the superior temporal gyrus (STG) (276) from schizophrenic patients. In contrast, some authors reported lower CB1r protein levels measured by immunocytochemistry (277) or Western blot (278) and decreased CB1r gene expression analyzed by *in situ* hybridization (277) or quantitative real time polymerase chain reaction (qRT-PCR) (273) in the PFC from schizophrenic patients compared with control subjects. Volk and cols. specifically addressed this apparent discrepancy between CB1r binding and protein or gene expression levels. In a cohort of 21 schizophrenic patients presenting lower levels of both CB1r mRNA and protein in the PFC, relative to matched healthy comparison subjects, they obtained an increased CB1r binding (271).

Neuroimaging experiments were recently carried out to obtain an *in vivo* approximation of the disturbances related with CB1r in schizophrenia. In this regard, PET studies yielded dissimilar results. Wong and cols. studied CB1r binding employing the novel PET tracer [^{11}C]-OMAR (JHU 75528) in schizophrenic patients and matched controls. CB1r binding was higher in several brain regions of patients with schizophrenia, only reaching statistical significance in the pons. Interestingly, a significant correlation was found between CB1r binding and schizophrenia-related symptomatology (279). In addition, Ceccarini and cols. also showed a significant increase of CB1r binding in the NAc, insula, cingulate cortex, inferior frontal cortex, parietal and mediotemporal lobes in schizophrenic patients compared with controls measured with [^{18}F]-MK-9470 PET. It is relevant to highlight that in the nontreated schizophrenia patients, CB1r binding was negatively correlated to negative symptoms and to depression scores, especially in the NAc (280). On the contrary, Rangathan and cols. obtained an opposite result with lower CB1r availability levels ([^{11}C]-OMAR PET) in the Amy, caudate, PCC, Hipp, Hyp, and insula of schizophrenic patients (281). An interesting commentary on these discrepancies was published, in which several confounding factors such as symptom severity, sex, age, PET tracer, statistical analysis method or comorbid nicotine use are discussed. Overall, it could be concluded that CB1r has an important but yet complex and poorly understood role in schizophrenia (282). Finally, a very recent study examined CB1r availability by [^{18}F]-FMPEP-d2 or [^{11}C]-MePPEP PET, in first episode psychosis (FEP). Significant lower CB1r availability was found in patients with schizophrenia, independently of antipsychotic medication treatment. Greater reduction in CB1r availability was associated with greater symptom severity and poorer cognitive functioning (283).

The possible association between CNR1 polymorphisms and schizophrenia has been explored. In this sense, negative results were obtained with a single-base polymorphism within the first

exon of the CNR1 (284), the polymorphism rs1049353 1359G/A at codon 453 in the coding region of CNR1 (285–288), or other CNR1 polymorphisms such as rs6454674 (287), AL136096 (287), rs806368 (288, 289), rs806379 (288), rs806380 (288), rs806376 (289), and rs806366 (289). However, significant associations of CNR1 polymorphisms rs7766029, rs806366, and rs1049353 were described (290). Regarding (AAT)n triplet repeat in the promoter region of the CNR1 gene, discrepant results were reported since Tsai and cols. suggested that this polymorphism was not directly involved in the pathogenesis of schizophrenia in a Chinese population (291), whereas it was significantly associated with the hebephrenic or disorganized subtype of schizophrenia (285). Interestingly, some relevant associations were identified between specific CNR1 polymorphisms and therapeutic response. Hamdani and cols. described increase G-allele frequency of the rs1049353 polymorphism in responsive schizophrenic patients, with a dose effect of the G allele. Thus, the authors proposed that the G allele of CNR1 rs1049353 polymorphism could represent a "psychopharmacogenetic" biomarker to take into consideration for the treatment of schizophrenia (288). In addition, in 65 FEP patients, TT genotype of the CNR1 rs2023239 polymorphism was associated with a better improvement of negative and positive symptoms (292). Similarly, in another group of patients with FEP, carriers of rs7766029 CC genotype presented significantly higher improvement in verbal memory and attention while carriers of rs12720071 AG genotype showed a better improvement in executive functions (293). Furthermore, minor alleles of CNR1 polymorphisms rs6928499, rs1535255, and rs2023239 might be associated with a lower risk to develop antipsychotic-induced metabolic syndrome. These relevant data could result in potential pharmacogenetic applications to optimize drug management of schizophrenic patients (294). On the contrary, one study showed that G allele carriers of the CNR1 rs1049353 (G1359A) polymorphism might be associated with a poorer therapeutic response (233).

Gene and protein analysis of CB1r in peripheral blood cells from schizophrenic patients attracted much attention. Peripheral cell (*e.g.* lymphocytes) changes could be mirroring, at least in part, some of the neuropathological hallmarks of the disorder. In this regard, the first published study did not detect changes in the CB1r mRNA levels in peripheral blood mononuclear cells (PBMCs) between schizophrenia and control patients (295). Similarly, no differences were observed in CB1r levels of peripheral immune cells by flow cytometry between control and schizophrenic patients, although a positive correlation between CB1r expression on monocytes and cognitive impairment was detected (296). However, an opposite result revealed an increase of CB1r in PBMCs of schizophrenic patients also evaluated by flow cytometry (297). Furthermore, there is an increase of CB1r mRNA levels in PBMCs of schizophrenic patients (298, 299) that may correlate with a reduced DNA methylation of CNR1 promoter region (299). Moreover, CB1r gene expression was correlated positively with positive and negative syndrome scale (PANSS) total symptom severity and negatively with cognitive functioning measures (298).

Besides the extensive literature evaluating the role of CB1r in schizophrenia, some efforts were done to complete the picture regarding the involvement of eCBs and its degrading and synthesizing enzymes as biomarkers. Leweke and cols reported a significant increase of AEA levels in the CSF of schizophrenic patients (300). In antipsychotic naïve first-episode paranoid schizophrenic patients, there was an eightfold increase in AEA levels in the CSF, whereas no alteration was present in patients treated with typical but not atypical antipsychotics. Furthermore, AEA levels were negatively correlated with psychotic symptoms in nonmedicated acute schizophrenics (301). Similarly, blood AEA levels were higher in patients with acute schizophrenia and were normalized with the clinical remission (295). Increased AEA levels were also detected in the CSF of schizophrenic patients who used cannabis. Interestingly, the increase of AEA was more than 10-fold higher in low-frequency compared with high-frequency cannabis users (302). In addition, higher AEA serum levels were obtained in twin-pairs discordant for schizophrenia (303), or in schizophrenic patients with substance use disorder (SUD) comorbidity, considering that baseline AEA predicted endpoint SUD scores (304). However, other studies showed different results such as no changes in serum AEA levels (305), increased 2-AG and decreased AEA in the cerebellum, Hipp, and PFC of schizophrenic patients (306).

With regard to degrading or synthesizing eCBs enzymes, the relationship of some FAAH or NAPDE-PLD polymorphisms with schizophrenia was studied, but no significant associations were obtained (290, 307). In addition, FAAH and MAGL mRNA levels were similar while FAAH activity was higher in the PFC of schizophrenic patients compared to controls (273). Interestingly, a reduction of FAAH mRNA levels correlated with clinical remission in schizophrenic patients (295). Furthermore, in FEP patients, some interesting correlations were detected between peripheral FAAH and DAGL expression and short-term verbal memory, NAPE-PLD expression and working memory, and MAGL expression and attention. Accordingly, the authors suggested the use of these ECS elements as biomarkers or pharmacological targets for FEP (308). Finally, mRNA levels of the 2-AG metabolizing enzyme, α-β-hydrolase domain 6 (ABHD6), were significantly increased in patients with schizophrenia (309).

Notwithstanding the scarce literature exploring the role of CB2r in schizophrenia, some important findings suggest its involvement and draw attention to research on its therapeutic potential. Perhaps, de Marchi and cols. published the first evidence measuring CB2r mRNA levels by semi-quantitative RT-PCR in PBMCs from schizophrenic patients in their acute phase, and when clinical remission was achieved after antipsychotic treatment with olanzapine. CB2r gene expression significantly decreased in PBMCs from patients in clinical remission (295). In FEP patients, CB2r protein expression was significantly down-regulated together with reduced levels of eCBs synthesizing enzymes (NAPE-PLD and DAGL) (310). Interestingly, increased CB2r gene expression was found in schizophrenic patients' PBMCs (298), correlating with PANSS and cognitive performance severity (296, 298), and in cells of the

innate immune system (297). On the other hand, Ishiguro and cols. evaluated the implication of specific CNR2 polymorphisms in schizophrenia. R63 allele of rs2501432 (R63Q), C allele of rs12744386, and the haplotype of the R63-C allele were significantly increased in patients with schizophrenia in comparison with control subjects. Apparently, these polymorphic alterations of CNR2 are associated with loss of function. A lower response to CB2r ligands was found in cultured CHO cells transfected with the R63 allele. Reduced CB2r mRNA and protein expression levels were found in the DLPFC of schizophrenic patients independently of the diagnosis (265). In addition, the association between three CNR2 polymorphisms (rs2501432C/T, rs2229579C/T, rs2501401G/A), and schizophrenia was explored (311). However, other CNR2 polymorphisms (rs6689530 and rs34570472) were not associated with schizophrenia in a Korean population (289).

Bipolar Disorder

Bipolar disorder (BD) is a debilitating, lifelong neuropsychiatric illness characterized by unsteady mood states alternating from (hypo)mania to depression. According to the DSM-5, for a diagnosis of BD it is necessary to meet specific criteria for a manic episode that may be followed by hypomanic or major depressive episodes (63). Despite the availability of effective pharmacological agents, BD is inadequately treated in a subset of patients, so the identification of new therapeutic targets is necessary. In this sense, the close implication of ECS in mood regulation suggested its involvement in BD (312). This assumption is supported by the observation of the effects of high doses of cannabis and THC in healthy patients, producing psychosis, sometimes with marked hypomanic features (313). In addition, THC and cannabidiol (CBD), the main components of *Cannabis sativa* plant, may present mood stabilizing properties. Therefore, there is an increasing interest to evaluate ECS implication in BD.

First studies were focused on the evaluation of polymorphisms of CNR1 gene in BD pathophysiology. One study carried out in patients with BD within a Turkish population investigated the implication of three types of polymorphisms of CNR1 in this disease, demonstrating that only one of them (rs6454674) could be correlated with BD. In addition, the mean of the yearly maniac attacks was statistically higher in patients presenting heterozygote rs6454674 T/G polymorphisms compared to those with homozygote polymorphism (314). In addition, the association of CNR1 rs1049353 (1359 G/A) and FAAH rs324420 SNP (cDNA 385C to A) polymorphisms with BD was assessed in a Caucasian population. Here, the authors concluded that the distribution of CNR1 1359 G/A genotypes and alleles did not differ between BD and healthy patients, whereas the frequency of the AC genotype of FAAH (cDNA 385C to A) polymorphism was slightly higher in BD patients (232).

Nevertheless, other studies did not identify differences in ECS components between BD and healthy controls. Indeed, no differences were obtained between BD patients and healthy controls in DNA methylation of the CNR1 gene promotor region (299). Furthermore, a polymorphism of CNR1 promotor region was evaluated in another study, and no changes were observed in BD patients, concluding that it was not likely to relate with BD (315). Koethe and cols. carried out a study with *post-mortem* brain samples from BD patients and controls, evaluating numerical density of neurons and immunopositive glial cells for CB1r. No changes were found in these patients (223). Furthermore, in another study evaluating polymorphisms of CNR1 and FAAH, no significant differences or association were observed in BD patients (316).

Because of these contradictory results, some authors shifted their attention to the implication of CB2r in BD, with limited but promising findings. A genetic association was observed in patients with BD and CNR2 rs41311993 (524C/A) polymorphism, but not SNPs of rs2229572 (1073C/T) or rs2501432 (315A/G), suggesting that CB2r may play a role in BD (317). In addition, a genome-wide association study carried out in a population from the UK biobank, identified the association of a locus in CNR2 with distressing psychotic experiences, providing support for a shared genetic liability with BD and other neuropsychiatric disorders (318).

In summary, there is limited information about the implication of ECS in the pathophysiology of BD. Thus, more preclinical and clinical studies are needed to explore further its role in the development of this neuropsychiatric disorder and its usefulness as a therapeutic target to improve BD management.

Post-Traumatic Stress Disorder

Post-traumatic stress disorder (PTSD) is a chronic and disabling mental disease caused by the exposure to stressful, frightening or distressing events, and is included in the category of trauma- and stressor-related disorders in the DSM-5 (63). PTSD patients experience intrusion symptoms, persistent avoidance of any stimuli associated with the traumatic event, negative alterations in cognition and mood, and disturbances in arousal and reactivity, that must last more than 1 month and produce distress or functional impairment (63). The neurobiological mechanisms underlying PTSD-related symptomatology are not completely understood, being a limiting factor to identify new therapeutic targets. In this regard, a relevant association between ECS and PTSD was suggested providing interesting results about the potential development of new pharmacological approaches. Indeed, preclinical and clinical findings point out the involvement of certain ECS components in PTSD symptomatology, such as CB1r or FAAH, suggesting its potential role as biomarkers for PTSD (319, 320).

Animal Studies

The involvement of CB1r in PTSD is supported by the presence of this receptor in brain areas regulating the response to stress and to changes observed in different animal models of PTSD. For instance, using a shock and reminder model of PTSD, higher mRNA levels of CB1r were detected in the BLA (133), and increased CB1r protein expression was found in the BLA and the CA1 region of the Hipp (321) of exposed mice as well. On the other hand, in a predator exposure-based PTSD model, anxiety-like behavior was negatively correlated with CB1r gene expression in the PFC and the amygdaloid complex, whereas

no changes were observed in the Hipp (322). In addition, Xing and cols. reported in young Sprague-Dawley rats, that the exposure to an unpredictable electric shock model of PTSD induced a down-regulation of CB1r gene expression in comparison with nonstressed rats. Interestingly, the authors showed sex differences in the stress-related regulation of CB1r, showing that females presented higher mRNA levels of CB1r, as well as greater CB1r inactivation by phosphorylation. The authors concluded that these sex-related differences could lead to increased susceptibility to stress-related anxiety disorders, including PTSD, in females (323). A genetic approach was used to evaluate further the involvement of CB1r in the regulation of stress response. Repeated exposure to an acoustic stressor (high intensity bell sound) did not produce changes in adrenocorticotropin hormone (ACTH) or corticosterone (CS) levels in $CB1^{-/-}$ mice. These results suggested that the presence of CB1r is essential in the regulation of the stress response, and that $CB1^{-/-}$ mice may result appropriate to model some forms of PTSD (324).

Pharmacological manipulation approaches of the CB1r were also explored in several rodent models of PTSD and its potential usefulness as a therapeutic biomarker. The blockade of CB1r with rimonabant increased freezing behavior in a PTSD model of shock and reminder during cued expression/extinction training (325). On the other hand, several authors evaluated the effects of cannabinoid activation by WIN-55,212-2 administration into hippocampal CA1 region. The results showed a normalization of shock-induced upregulation of CB1r in the PFC and CA1 region of the Hipp (321, 326) and facilitation of inhibitory avoidance extinction in a fear-related inhibitory avoidance paradigm (327). All these effects were blocked by AM251 administration. Furthermore, WIN-55,212 administration into the BLA normalized stress-induced effects on inhibitory avoidance and acoustic startle response and facilitated fear extinction in a single prolonged stress (SPS) model of PTSD. These effects were blocked by AM251 (321, 328, 329). Similarly, the injection of WIN-55,212 in the NAc of rats exposed to a shock and reminder model of PTSD significantly facilitated the fear extinction process (330). Interestingly, Goodman and Packard demonstrated that systemic or intradorsolateral striatum (DLS) administration of WIN-55,212 could impair the consolidation of stimulus–response memory, suggesting relevant consequences for neuropsychiatric disorders such as PTSD (331). However, the intra-PFC administration of WIN-55,212 did not modulate fear extinction disturbances induced by the exposure to the SPS model (329). Finally, according to the results obtained with WIN-55,212, Reich and cols. studied the effects of a CB1r selective agonist, ACEA, in rats exposed to 3 weeks of a chronic-mild-unpredictable protocol followed by fear conditioning evaluation. In this study, ACEA administration significantly reduced freezing behavior in stressed rats, enhancing long-term extinction of fear-related memories (332).

In order to validate that cannabinoid activation improves disturbances induced by stress- or trauma-related stimuli, the effects of pharmacological endocannabinoid signaling facilitation were analyzed. In a fear conditioning paradigm, the administration of AM404 led to a dose-dependent enhancement in fear extinction, as well as a decreased shock-induced reinstatement of fear. Interestingly, the administration of rimonabant together with AM404 reversed the improvement of extinction, suggesting that AM404 effects were related to an increase in CB1r activation during extinction training (333). In addition, the injection of the FAAH inhibitor, URB597, normalized the upregulation of CB1r in the CA1 of Hipp and BLA of rats exposed to a shock and reminder model of PTSD (334) and attenuated startle response and anxiety-like behavior in a predator exposure animal model of PTSD (335). Interestingly, these effects were abolished by CB1r blockade, suggesting the implication of CB1r on URB597 effects (335, 335). Similar results were obtained with the administration of URB597 into CA1 (Hipp) and BLA brain regions, showing a facilitation of extinction processes and attenuation of startle response, anxiety- and depression-like behaviors mediated by CB1r activation (133, 336, 337). Furthermore, URB597 administration additionally prevented the increase of CB1r levels in CA1 and BLA after rodent exposure to shock and reminder model of PTSD (321).

FAAH inhibition significantly facilitates CB1r-mediated signaling of AEA and can produce a greater beneficial spectrum of biological effects than those caused by direct CB1r activation. Interestingly, the role of FAAH in learning and memory was evaluated by using another FAAH inhibitor, OL-135. The administration of this drug increased acquisition and extinction rates in mice exposed to fixed platform water maze test. Rimonabant blocked OL-135-induced effects on both acquisition and extinction levels (338). In the same study, the authors revealed that $FAAH^{-/-}$ mice phenotype was similar to that obtained after OL-135 administration, suggesting that the increase in AEA levels facilitates extinction processes, and that CB1r would be critically involved (338). FAAH inhibition and the consequent increase of AEA in the brain regions involved in the regulation of stress and anxiety seem to restore dysfunctional homeostasis of AEA signaling because of stress exposure. Thus, FAAH must be strongly considered as a target for PTSD management (139).

Human Studies

According to the involvement of ECS components in several behavioral traits of PTSD in animal models, various studies explored alterations in different biological samples (*post-mortem* brain tissue, blood, hair) collected from PTSD patients and adequately paired controls. At the peripheral level, some authors studied the possible correlation between CNR1 polymorphisms and PTSD symptoms. The rs1049353 polymorphism of CNR1 was studied in PTSD patients to correlate specific alleles or genotypes with fear and/or dysphoric symptoms of PTSD. This study suggested that rs1049353 polymorphism interacts with childhood physical abuse to increase fear but not dysphoric symptoms in PTSD (339). In another study carried out in a Caucasian population, the association between variants of CNR1 gene haplotypes and diagnosis of PTSD was studied. The authors reported that the

variant C-A was more common in PTSD cases compared to non-PTSD controls, and the variant C-G was less common in PTSD compared to non-PTSD patients (340).

A different approach was the measurement of plasmatic eCBs in a selected cohort of patients that suffer the terroristic attacks of the World Trade Center in 2001 and met the diagnostic criteria for PTSD. 2-AG, AEA, and cortisol concentrations were measured. Only 2-AG was significantly reduced in PTSD patients, while no significant differences were found in AEA or cortisol concentrations (341). Another study showed reduced AEA and cortisol concentrations in PTSD patients compared to healthy controls with lifetime clinical histories of trauma (342). Despite these contradictory results, the fluctuations in plasmatic concentrations of eCBs may affect the reproducibility of the evaluation. Thus, the assessment of eCBs alterations in hair samples provides a more stable measurement. Hair concentrations of PEA (palmitoylethanolamide), OEA (oleoylethanolamide) and SEA (steraoylethanolamide) were measured in war survivors with and without PTSD. A regression analysis revealed a strong negative relationship between these endocannabinoids and the severity of PTSD symptoms. OEA concentrations were significantly reduced in hair samples from PTSD patients (343).

Only one human study analyzed CB1r binding using the CB1r-selective radioligand [11C]OMAR by PET. Results showed elevated CB1r binding values, especially in women, together with lower AEA and cortisol in PTSD patients. The authors suggested that abnormal CB1r-mediated AEA signaling is involved in the etiology of PTSD (342). In addition, fMRI was also used to evaluate FAAH implication in PTSD symptomatology. A common SNP (C385A) in the human FAAH gene was correlated with the quicker habituation of amygdala reactivity to threat and lower score on stress-reactivity. This variant reduced FAAH activity and possibly increased AEA-induced endocannabinoid signaling (344–346). Furthermore, Rabinak and cols. conducted an fMRI study with healthy volunteers and patients receiving acute dronabinol (synthetic THC) oral administration in a standard Pavlovian fear extinction paradigm. Interestingly, dronabinol enhanced extinction learning, providing the first evidence about the feasibility of pharmacological enhancement of extinction learning in humans using cannabinoid system modulators (347, 348). Some clinical trials with PTSD patients suggested the usefulness of dronabinol for improving the global PTSD symptom severity, sleep quality, frequency of nightmares, and PTSD hyperarousal symptoms (349). Similar results were obtained with nabilone, since its administration to PTSD patients improved insomnia, PTSD symptoms, and global assessment of functioning, reducing the frequency and intensity of nightmares (350, 351). Nevertheless, more randomized and controlled clinical trials are needed to confirm dronabinol or nabilone potential therapeutic application in the management of PTSD.

Attention-Deficit/Hyperactivity Disorder

ADHD is a neuropsychiatric disorder characterized by persistent pattern of inattention and/or hyperactivity-impulsivity that interferes or reduces the quality of social, academic, or occupational functioning in accordance with DSM-5 (63). In the last years, the identification of different components of the ECS that are potentially involved in ADHD pathophysiological mechanisms has attracted much attention as shown below.

Animal Studies

An experiment carried out in SHR rats (an animal model reproducing some features of ADHD) evaluated the modulating effects of the cannabinoid system on impulsivity, using a delay reinforcement task and the administration of WIN55212-2 or AM251 (352). This study concluded that treatment with WIN55212-2 decreased whereas AM251 increased the choices of the large reward, suggesting that CB1r plays a relevant role in impulsive behavior. Furthermore, basal gene and protein expression of CB1r in the brainstem of SHR rats was significantly lower in comparison with their normotensive counterpart, Wistar rats (353). Moreover, the overexpression of four genes, between them CNR1, was strongly associated with overall poor performance on mice during their gestational growth because of a malnutrition *via* high-fat or low-protein diets on the dam (354). These abnormal disturbances on diet in the gestational period are linked to the etiology of multiple neurodevelopmental disorders, including ADHD (355).

The psychostimulant drug amphetamine is often prescribed to treat ADHD. The administration of amphetamine increases monoamine neurotransmission in the brain regions as NAc and medial PFC. Accumulating reports supported the role of CB1r in the regulation of monoamine release, suggesting its possible involvement in ADHD. The administration of rimonabant did not affect monoamine release whereas dose-dependently abolished amphetamine-induced dopamine release in the NAc. This result suggested that CB1r is essential to reach the therapeutic effect of amphetamine, mediated at least in part, by the enhancement of dopaminergic signaling in the mesolimbic system in the NAc (356).

Human Studies

As previously stated, there is large available evidence regarding the role of different variants of CNR1 gene in psychiatry. In relation to ADHD, SNP variants at the CNR1 gene were tested on a family-based sample of trios (an ADHD child and their parents) and on an unselected adolescent sample from Northern Finland. The study detected a significant association of a SNP haplotype (C-G) with ADHD suggesting a greater risk in males than females (340). Another study reported the interaction of the two most studied *CNR1* polymorphisms, rs806379 and rs1049353, that are involved with early psychosocial adversity (357). These polymorphisms of the CB1 receptor are highly associated with impulsivity representing an usual phenotype involved in ADHD (358).

Eating Disorders

The most common eating disorders are anorexia nervosa (AN) and bulimia nervosa (BN). According to DSM-5, AN is characterized by distorted body image and excessive dieting leading to severe weight loss with a pathological fear of becoming fat, whereas BN is characterized by recurrent episodes of binge eating alternated with recurrent

inappropriate compensatory behavior to prevent weight gain (63). Importantly, ECS plays a major regulatory role on feeding behaviors and energy balance (359) that has drawn attention to its relationship with ADHD neurobiology. This can be confirmed by rodents with diet-induced or genetic obesity promoting an increase of endocannabinoid hypothalamic levels (360).

Animal Studies

The participation of CB1r in the regulation of feeding behavior is well established. The acute administration of rimonabant decreased food intake and body weight gain and reduced CB1r gene expression in the PVN of male Wistar rats. However, a chronic treatment led to tolerance to the hypophagic effects of CB1r blockade without changes in food intake, body weight, or hypothalamic mRNA gene expression (361). Another study on rimonabant-treated male Sprague-Dawley rats showed that the colocalized Fos labeling of hypothalamic regions with anorexigenic and orexigenic peptides had decreased neuropeptide Y (NPY) levels (362). More recently, another study was performed in CB1r conditional and $CB2^{-/-}$ mice. The hypothalamic neuropeptide expression pattern displayed a marked decrease of proopiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) expression in the arcuate nucleus of the hypothalamus (ARC), both neuropeptides involved on anorexigenic and behavioral changes in food intake (363).

C57BL/6J mice were treated with naltrexone (opioid receptor antagonist), rimonabant, and BD-1063 (sigma-1 receptor antagonist) on an intermittent maladaptive feeding animal model. All the treatments reduced overconsumption of a palatable food (364). In addition, the administration of other cannabinoid compounds such as CBD or CB1r antagonists significantly reduced food intake and body weight gain (365–368). Recently, a study based on the activity-based anorexia (ABA) model reproducing key aspects of human AN, measured levels of AEA, 2-AG, and the CB1r in different brain regions of female ABA Sprague-Dawley rats. 2-AG significantly decreased in various brain areas but not in the caudate putamen, whereas no changes were observed in AEA. Density of CB1r was reduced in the dentate gyrus of Hipp and in the lateral Hyp (369). These results suggested that ECS is involved in the contribution and maintenance of some aspects in the pathophysiology of AN.

Human Studies

Although there is some progress in the understanding of the mechanisms underlying eating disorders and body weight regulation, there is still lack of information to suggest cannabinoid related treatment for patients with AN and BN. However, some studies suggest that the ECS, primarily CB1r, plays a key role in the reward areas and metabolic patterns involved in food intake and weight gain. In this regard, a PET study on 54 patients with food intake disorders (FID, including AN and BN) revealed an inverse association between regional CB1r availability and body mass index (BMI) in the Hyp and brainstem areas in both patients with FID and healthy individuals. However, FID patients negatively correlated with BMI throughout the mesolimbic reward system (370). Also, global CB1r availability is significantly increased in the cortical and subcortical brain areas in AN patients compared with healthy controls, maybe due to a compensatory mechanism of an underactive ECS in these patients (371). Finally, eating disorder female patients presented lower CB1r mRNA levels in PBMCs (372).

CONCLUSIONS

The close involvement of the ECS in the etiology and neuropathology of neuropsychiatric disorders is undeniable. Considering the urgent need to identify new and better biomarkers in psychiatry, the evidence included in this review provides an overview of the opportunities that cannabinoid receptors (**Table 1**), endogenous cannabinoid ligands (**Table 2**), or their metabolizing enzymes (**Table 3**) offer as potential biomarkers in the clinical setting. The large number of pharmacological studies with various cannabinoid compounds, mainly conducted in animal models, reported interesting and promising information to design new therapeutic strategies that, alone or in combination with the drugs currently used in psychiatry, may improve the efficacy and safety of psychiatric disorders treatment.

According to the information gathered in the present review from pharmacological and genetic approaches mainly performed in rodents, some general conclusions can be drawn regarding the usefulness of ECS components as therapeutic biomarkers (**Figure 2**). The blockade or genetic deletion of CB1r is closely associated with the worsening of emotional behavioral traits, as revealed principally in animal models of anxiety, depression or PTSD, whereas CB1r pharmacological activation induces an improvement effect. On the other hand, CB1r activation induces psychotic symptoms, while CB1r blockade presents antipsychotic effects. Regarding CB2r, its pharmacological activation or its overexpression by means of genetic manipulation or chronic treatment induced upregulation improving anxiety- and depressive-like behaviors, as well as schizophrenia-like traits. In contrast, all these behaviors worsen by CB2r blockade or gene deletion. Interestingly, the strengthening of the endocannabinoid tone, by the inhibition of enzymatic degradation or the blockade of reuptake mechanisms, is closely related with an improvement, particularly in emotional regulation as explored in animal models of anxiety, depression, and PTSD.

Therefore, the available evidence points out that the functional manipulation of the ECS components presents a great therapeutic potential. However, the close interaction of the ECS with other neurotransmitter or neurohormonal systems, as well as the specific and differential neuroanatomical distribution of the ECS components, provides a complex scenario not only from a therapeutic point of view but also considering the occurrence of side effects. In this sense, some aspects should be critically addressed, especially from a pharmacological perspective. The dosing, duration, and mechanism of action involved in the manipulation of the ECS are crucial to reach an improvement and limit adverse reactions.

TABLE 1 | Main findings from human studies supporting the role of CB1r and CB2r as biomarkers in psychiatric disorders.

CB1r					
Subjects/Diagnosis	**Sample/Intervention**	**Method**	**Measurement**	**Results**	**References**
Healthy controls	THC (10 mg) p.o.	[^{11}C]MePPEP PET	CB1r availability in amygdala	↑ CB1r	Bhattacharyya et al. (166)
Healthy controls	Rimonabant (90 mg) p.o.	Visual Analogue Mood Scale	Anxiety level	↑ anxiety	Bergamaschi et al. (168)
AD/DD	Buccal mucosa cells	DNA Genotyping	CNR1 rs7766029 polymorphism	↑ frequency financial-related anxiety and depression	Gonda et al. (170)
DD	PMBT – anterior cingulate cortex	Immunohistochemistry	Density of CB1r immunopositive glial cells	↓ CB1r	Koethe et al. (223)
DD	PMBT – dorsolateral prefrontal cortex	Quantitative polymerase chain reaction (qPCR)	CB1r relative gene expression	↑ CB1r	Choi et al. (224)
DD	Blood	DNA Genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↑ frequency	Monteleone et al. (232)
DD/SCZ	Blood	DNA Genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↑ depressive symptoms in G-allele carriers	Schennach et al. (233)
DD	Blood	DNA Genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↑ treatment resistance in G-allele carriers	Domschke et al. (234)
DD	Blood	DNA Genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↑ citalopram response in GG genotype male carriers	Mitjans et al. (235)
Missouri Adolescent Female Twin Study (MOAFTS) participants	Blood	DNA Genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↓ risk for anhedonia/DD in A-allele carriers with childhood trauma	Agrawal et al. (236)
DD in Parkinson's disease	Blood	DNA Genotyping	CNR1 (AAT)n triplet polymorphism	↓ risk for DD in 2 long alleles carriers	Barrero et al. (238)
DD in methadone-maintained patients	Blood	DNA Genotyping	CNR1 rs2023239 polymorphism	↓ risk for DD in C-allele carriers	Icick et al. (239)
SCZ	PMBT – dorsolateral prefrontal cortex	In situ [^{3}H]CP-55940 radioligand binding	CB1r binding	↑ CB1r	Dean et al. (269)
SCZ	PMBT – dorsolateral prefrontal cortex	In situ [^{3}H]MePPEP radioligand binding	CB1r binding	↑ CB1r	Jenko et al. (270)
SCZ	PMBT – prefrontal cortex	In situ [^{3}H]OMAR radioligand binding	CB1r binding	↑ CB1r	Volk et al. (271)
SCZ	PMBT – anterior cingulate cortex	In situ [^{3}H]SR141716A radioligand binding	CB1r binding	↑ CB1r	Zavitsanou et al. (274)
SCZ	PMBT – posterior cingulate cortex	In situ [^{3}H]CP-55,940 radioligand binding	CB1r binding	↑ CB1r	Newell et al. (275)
SCZ	PMBT – posterior cingulate cortex	In situ hybridization Immunohistochemistry	CB1r mRNA CB1r protein	↓ CB1r ↓ CB1r	Eggan et al. (277)
SCZ	PMBT – prefrontal cortex	Western Blot	CB1r protein	↓ CB1r ↓ CB1r in antipsychotic-treated patients	Urigüen et al. (278)
SCZ	PMBT – prefrontal cortex	Quantitative polymerase chain reaction (qPCR)	CB1r relative gene expression	↓ CB1r	Muguruzaf et al. (273)
SCZ	*In vivo* neuroimaging (several brain regions)	[^{11}C]-OMAR PET	CB1r binding	↑ CB1r (only in the pons)	Wong et al. (279)
SCZ	*In vivo* neuroimaging (several brain regions)	[^{18}F]-MK-9470 PET	CB1r binding	↑ CB1r	Ceccarini et al. (280)
SCZ	*In vivo* neuroimaging (several brain regions)	[^{11}C]-OMAR PET	CB1r binding	↓ CB1r	Ranganathan et al. (281)
FEP	*In vivo* neuroimaging (several brain regions)	[^{18}F]-FMPEP-d2 or [^{11}C]-MePPEP PET	CB1r binding	↓ CB1r ↔ severity	Borgan et al. (283)
SCZ	Blood	DNA genotyping	CNR1 rs1049353, rs7766029, rs806366 polymorphisms	Nominal association	Costa et al. (290)
SCZ	Blood	DNA genotyping	CNR1 (AAT)n triplet polymorphism	9 and 17 repeat alleles ↔ ↑ susceptibility disorganized SCZ	Ujike et al. (285)
SCZ	Blood	DNA genotyping	CNR1 rs1049353 (1359 G/A) polymorphism	↑ treatment response in G-allele carriers	Hamdani et al. (290)
SCZ	Blood	DNA genotyping	CNR1 rs2023239 polymorphism	↑ better improvement in TT genotype carriers	Suárez-Pinilla et al. (292)

(Continued)

TABLE 1 | Continued

CB1r					
Subjects/Diagnosis	**Sample/Intervention**	**Method**	**Measurement**	**Results**	**References**
FEP	Blood	DNA genotyping	CNR1 rs7766029, rs12720071 polymorphisms	↑ better improvement in rs7766029 CC genotype or rs12720071 AG genotype	Kuzman, R. et al. (293)
SCZ	Blood	DNA genotyping	CNR1 rs6928499, rs1535255, rs2023239 polymorphisms	↓ risk metabolic syndrome in minor alleles carriers	Yu et al. (294)
SCZ	Blood	DNA genotyping	CB1r relative gene expression	↑ treatment response in G-allele carriers	Schennach et al. (233)
SCZ	Blood – PBMCs	Flow cytometry	CNR1 rs1049353 (1359 G/A) polymorphism	↑ CB1r	De Campos-Carli et al. (297)
SCZ	Blood - PBMCs	Quantitative polymerase chain reaction (qPCR)	CB1r expression	↑ CB1r	Chase et al. (298)
BD	Blood	DNA genotyping	CNR1 rs6454674 polymorphism	↑ severity in T/G heterozygotes	Alpak et al. (314)
Detroit Neighborhood Health Study (DNHS) participants	Blood	DNA genotyping	CNR1 rs1049353 polymorphism	↑ risk for PTSD-related symptoms in A-allele carriers with childhood abuse	Mota et al. (339)
PTSD	*In vivo* neuroimaging (several brain regions)	[^{11}C]-OMAR PET	CB1r binding	↑ CB1r	Neumeister, A. et al. (342)
ADHD	Blood	DNA genotyping	SNP variants at the CNR1 gene	↑ frequency SNP haplotype (C-G)	Lu et al. (340)
ADHD (alcoholic patients)	Blood	DNA genotyping	CNR1 (AAT)n triplet polymorphism	↑ frequency longer form of alleles	Ponce et al. (358)
AN/BN	*In vivo* neuroimaging (several brain regions)	[^{18}F]-MK-9470 PET	CB1r binding	↑ CB1r	Gérard et al. (371)
AN/BN	Blood	Quantitative polymerase chain reaction (qPCR)	CB1r relative gene expression	↓ CB1r in AN/BN women with self-injurious behavior	Schroeder et al. (372)
CB2r					
Subjects/Diagnosis	**Sample**	**Method**	**Measurement**	**Results**	**References**
General population	Buccal mucosa	DNA genotyping	CNR2 rs2501432 (R63Q) polymorphism	↑ risk for AD/DD in rs2501432 R-allele carriers with childhood trauma	Lazary et al. (176)
DD	Blood	DNA genotyping	CNR2 rs2501431 polymorphism	↑ depressive symptoms in G-allele carriers	Mitjans et al. (235)
DD	Blood	DNA genotyping	CNR2 rs2501432 (R63Q) polymorphism	↑ frequency R63Q polymorphism	Onaivi et al. (249, 241)
SCZ	Blood – PBMCs	Quantitative polymerase chain reaction (qPCR)	CB2r relative gene expression	↓ CB2r with clinical remission	De Marchi et al. (295)
SCZ	Blood – PBMCs	Quantitative polymerase chain reaction (qPCR)	CB2r relative gene expression	↑ CB2r	Chase et al. (298)
SCZ	Blood – PBMCs	Flow cytometry	CB2r expression	↑ CB2r	De Campos-Carli et al. (297)
SCZ	Blood – PBMCs	Western Blot	CB2r expression	↓ CB2r	Bioque et al. (310)
SCZ	Blood	DNA genotyping	CNR2 rs2501432 (R63Q), rs12744386 polymorphism	↑ frequency in rs2501432 R63 and rs12744386 C alleles	Ishiguro et al. (265)
SCZ	Blood	DNA genotyping	CNR2 rs2501432C/T polymorphism	↑ risk for SCZ in T-allele carriers	Tong et al. (311)
BD	Blood	DNA genotyping	CNR2 rs41311993 (524C/A) polymorphism	↑ frequency 524C/A polymorphism	Minocci et al. (317)

AD, anxiety disorder; DD, depressive disorder; SCZ, schizophrenia; FEP, first episode psychosis; BD, bipolar disorder; PTSD, post-traumatic stress disorder; ADHD, attention-deficit hyperactivity disorder; AN, anorexia nervosa; BN, bulimia nervosa; PMBT, post-mortem brain tissue; PET, positron emission tomography; PBMCs, peripheral blood mononuclear cells. ↓: decrease and ↑: increase.

TABLE 2 | Main findings from human studies supporting the role of AEA and 2-AG as biomarkers in psychiatric disorders.

AEA & 2-AG					
Subjects/ Diagnosis	**Sample**	**Method**	**Measurement**	**Results**	**References**
DD	Serum	Chemical ionization liquid chromatography-mass spectrometry (LC-APCI-MS)	AEA and 2-AG quantification	↑ AEA in minor depression ↓ 2-AG in major depression	Hill et al. (226)
DD	Serum	Chemical ionization liquid chromatography-mass spectrometry (LC-APCI-MS)	AEA and 2-AG quantification	↓ AEA ↓ 2-AG	Hill et al. (227)
DD	Plasma	Chromatography-coupled tandem mass spectrometry system	AEA and 2-AG quantification	↑ AEA	Romero-Sanchiz et al. (228)
DD	Serum	Electrospray ionization liquid chromatography-mass spectrometry (LC-ESI-MS-MS)	AEA and 2-AG quantification	↑ AEA = 2-AG after moderate exercise	Meyer et al. (230)
DD	CSF	Liquid chromatography-multiple reaction monitoring (LC/MRM)	AEA and 2-AG quantification	↑ AEA ↑ 2-AG after ECT	Kranaster et al. (231)
SCZ	CSF	High pressure liquid chromatography-gas chromatography/ mass spectrometry (HPLC-GC/MS)	AEA quantification	↑ AEA	Leweke et al. (300)
SCZ	CSF	High pressure liquid chromatography-mass spectrometry (HPLC-MS)	AEA quantification	↑ AEA in antipsychotics-naïve patients = AEA in typical antipsychotics-treated patients	Giuffrida et al. (301)
SCZ	Blood	Chemical ionization liquid chromatography-mass spectrometry (LC-APCI-MS)	AEA quantification	↑ AEA ↓ AEA with clinical remission	De Marchi et al. (295)
SCZ	Plasma	Liquid chromatography-mass spectrometry (LC-MS)	AEA quantification	↑ AEA	Koethe, D. et al. (303)
SCZ	PMBT (several regions)	Liquid chromatography coupled with triple quadrupole mass spectrometry (LC/MS/MS)	AEA and 2-AG quantification	↓ AEA ↑ 2-AG	Muguruza et al. (306)
PTSD	Plasma	Chemical ionization liquid chromatography-mass spectrometry (LC-APCI-MS)	AEA and 2-AG quantification	= AEA ↓ 2-AG	Hill et al. (341)
AN	Plasma	Chemical ionization liquid chromatography-mass spectrometry (LC-APCI-MS)	AEA and 2-AG quantification	↑ AEA =2-AG	Monteleone et al. (370)

DD, depressive disorder; SCZ, schizophrenia; PTSD, post-traumatic stress disorder; AN, anorexia nervosa; CSF, cerebrospinal fluid; PMBT, post-mortem brain tissue; AEA, anandamide; 2-AG, 2-araquinoylglycerol.
↓: decrease and ↑: increase.

TABLE 3 | Main findings from human studies supporting the role of FAAH as a biomarker in psychiatric disorders.

FAAH					
Subjects/Diagnosis	**Sample**	**Method**	**Measurement**	**Results**	**References**
Healthy controls	Blood	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	= anxiety-related self-reports in A-allele and C/C genotypes	Gärtner et al. (171)
PING study participants	Saliva	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	↓ anxiety level in A-allele adolescent carriers	Gee et al. (172)
Project FRONTIER participants	Blood	DNA Genotyping	FAAH rs324420 (C385A) & CRFR1 minor alleles polymorphisms	↑ anxiety level in FAAH A-allele and CRFR1 non-minors alleles carriers	Harris et al. (173)
Duke Neurogenetics Study (DNS) participants	Saliva	DNA Genotyping	FAAH rs324420 (C385A) and CRHR1 rs110402 polymorphisms	↑ risk for AD in FAAH A-allele and CRHR1 A-allele carriers	Demers et al. (174)
General population	Buccal mucosa	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	↑ risk for AD/DD in A-allele carriers with childhood trauma	Lazary et al. (175)
General population	Buccal mucosa	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	↑ risk for AD/DD in A-allele carriers with childhood trauma	Lazary et al. (176)
DD/BD	Blood	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	↑ frequency AC genotype carriers	Monteleone et al. (232)
SCZ	PMBT – prefrontal cortex	Enzymatic assay – scintillation counting	FAAH activity	↑ FAAH activity	Muguruza et al. (273)
SCZ	Blood	Quantitative polymerase chain reaction (qPCR)	FAAH relative gene expression	↓ FAAH with clinical remission	De Marchi et al. (295)
BD	Blood	DNA Genotyping	FAAH rs324420 (C385A) polymorphism	↑ frequency AC genotype carriers	Monteleone et al. (232)

AD, anxiety disorder; DD, depressive disorder; SCZ, schizophrenia; BD, bipolar disorder; PMBT, post-mortem brain tissue; FAAH, fatty acid amido hydrolase.
↓: decrease and ↑: increase.

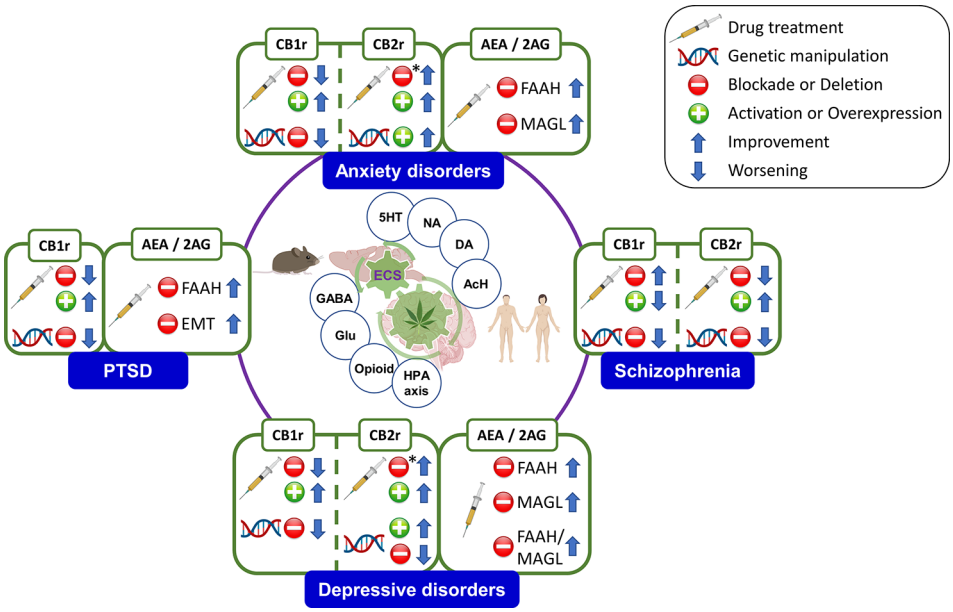

FIGURE 2 | Main findings regarding the therapeutic potential of the functional manipulation of the endocannabinoid system (ECS) components by pharmacological and genetic approaches in anxiety, depression, schizophrenia, and post-traumatic stress disorder (PTSD). CB1r, cannabinoid receptor 1; CB2r, cannabinoid receptor 2; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; 5-HT, serotonin; NA, noradrenaline; DA, dopamine; AcH: acetylcholine; GABA, gamma-aminobutyric acid; Glu, glutamate; HPA axis, Hypothalamus–Pituitary–Adrenal axis; *, chronic treatment.

Apart from the widely explored role of CB1r, it should be noted that in recent years an increasing emphasis is being placed on the design of strategies to regulate endogenous cannabinoid tone, through inhibitors of the degradation or reuptake of eCBs. Since this approach provided negative results, particularly regarding the inhibition of FAAH (373), current trends focused on the combination of different mechanisms of action to enhance the endocannabinoid tone (374). Moreover, the pharmacological modulation of CB2r has also attracted much attention given its safety profile and the wide range of properties attributed to it, including mood and cognitive regulation. Thus, future priorities for both human and animal research would be the potentiation of both endocannabinoid tone and CB2r-mediated actions.

The trials carried out on patients show alterations of the ECS components at different levels, which in certain cases are related to risk or predictive factors regarding the evolution of the disease, or the degree of response to drug treatment. It is relevant to highlight the underlying sex-dependent effects in terms of sexual dimorphism of the ECS (375) and sex differences in prevalence rates and presentation of the psychiatric disorders (376). These could be involved not only in the changes of the ECS components to provide sex-related diagnostic or prognostic biomarkers, but also in the pharmacological actions derived from the treatment with cannabinoid compounds (377). Finally, it is important to note that more *in vivo* clinical studies are recently being carried out employing blood samples (PBMCs, plasma) or neuroimaging techniques (PET, fMRI) to identify ECS-related alterations, providing very relevant data. In this sense, a higher effort is required to design and perform more clinical studies, especially increasing the sample sizes to achieve greater significance, representativeness and reproducibility, finally making possible to identify some of the ECS components as useful biomarkers applicable to clinical practice in psychiatry.

AUTHOR CONTRIBUTIONS

FN and JM designed the sections and contents of the review manuscript. FN oversaw the organization to distribute the writing tasks among the authors and participated in manuscript writing. MG-G, RJ-B, GR, AG, and AA-O perform the literature searches and participated in the manuscript writing. All the authors critically reviewed and approved the final version of the manuscript.

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Diagnostic and Predictive Applications of Functional Near-Infrared Spectroscopy for Major Depressive Disorder: A Systematic Review

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Introduction: Major depressive disorder (MDD) is a global psychiatric disorder with no established biomarker. There is growing evidence that functional near-infrared spectroscopy (fNIRS) has the ability to aid in the diagnosis and prediction of the treatment response of MDD. The aim of this review was to systematically review, and gather the evidence from existing studies that used fNIRS signals in the diagnosis of MDD, correlations with depression symptomatology, and the monitoring of treatment response.

Methods: PubMed, EMBASE, ScienceDirect, and Cochrane Library databases were searched for published English articles from 1980 to June 2019 that focused on the application of fNIRS for (i) differentiating depressed versus nondepressed individuals, (ii) correlating with depression symptomatology, and in turn (iii) monitoring treatment responses in depression. Studies were included if they utilized fNIRS to evaluate cerebral hemodynamic variations in patients with MDD of any age group. The quality of the evidence was assessed using the Newcastle–Ottawa quality assessment scale.

Results: A total of 64 studies were included in this review, with 12 studies being longitudinal, while the rest were cross-sectional. More than two-thirds of the studies (n = 49) had acceptable quality. fNIRS consistently demonstrated attenuated cerebral hemodynamic changes in depressed compared to healthy individuals. fNIRS signals have also shown promise in correlating with individual symptoms of depression and monitoring various treatment responses.

Conclusions: This review provides comprehensive updated evidence of the diagnostic and predictive applications of fNIRS in patients with MDD. Future studies involving larger

sample sizes, standardized methodology, examination of more brain regions in an integrative approach, and longitudinal follow-ups are needed.

Keywords: diagnostic, prediction, functional near-infrared spectroscopy, major depressive disorder, systematic review

INTRODUCTION

Major depressive disorder (MDD) is a global mental illness which is increasingly prevalent in modern societies. As per the World Health Organization, MDD affected approximately 322 million people of all ages globally. The total number of people likely to have depression increased by 18.4% between 2005 and 2015, and this number is expected to increase exponentially over time (1). Symptoms of depression include low mood, decreased energy, poor attention, memory problems, disturbed appetite and sleep, anhedonia, feelings of guilt, and worthlessness (2). In severe cases, depression may present with psychotic symptoms, suicidal thoughts and increase the possibility of unnatural death (3). MDD can also cause significant disability. Depression is one of the leading cause of world disability in the year 2020, and in 10 years' time, it is anticipated to be the biggest cause of global disease burden overtaking cardiovascular diseases (1). Despite its gaining prevalence, MDD is still considerably undertreated and under diagnosed, especially in primary care settings (4).

Depressive disorders have been correlated with problems in the limbic, thalamic and cortical areas (5). MDD has also been correlated with neuropsychological deficiencies in numerous cognitive areas, involving attention, language, memory, and executive function (6). To diagnose patients with MDD, clinicians conventionally refer to the International Statistical Classification of Diseases and Related Health Problems—10th revision (ICD-10) or the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) classifications as guides to confirm the diagnosis. However, the accuracy of reaching a diagnosis of MDD relying on history taking remains debated. Diagnosis is often based on the subjective assessment and clinical experience of the clinicians. Some patients may also not be forthcoming about their symptoms, especially suicidal ideation. Furthermore, many of the psychiatric symptoms are polymorphous and may overlap in various psychiatric disorders, thereby making diagnosis all the more challenging.

MDD is a multifaceted and varied illness in which up to two thirds of patients could experience treatment resistance that protracts and worsens the episodes (7). Only approximately 33% of patients with MDD attain remission, despite being treated with optimal medications based on measurement-based care and consensus guideline. Furthermore, the probability of treatment response seems to decrease with each new treatment option (8). Treatment-resistant depression (TRD) is related to increased morbidity and mortality, with recurring and chronic periods in the long run (9). Hence, it would be useful if there are ways to ascertain improvements in treatment at any stage of the illness, as it would offer wider benefits for global management of depression.

Biomarkers offer a conceivable target for assisting in the diagnosis and identifying predictors of response to various interventions (10). Biomarkers may come in various forms, such as inflammatory markers, endogenously produced hormones and brain imaging. In recent years, brain-imaging techniques such as electroencephalography (EEG), functional magnetic resonance imaging (fMRI), positron emission tomography (PET), and magnetoencephalography (MEG) existed to be used as adjuncts to help clinicians diagnose MDD. According to some neuroimaging findings using PET and fMRI to investigate brain function in patients with depression (11–13), they found blood flow reduction in the prefrontal cortex to be associated with a decline in activity within the cingulate cortex. There had been reports using neuroimaging techniques recording hemodynamic response relating to brain activity during cognitive stimulation on depressed patients. One such study using fMRI performed by Okada et al. revealed decreased left prefrontal activation and reduced task performance in depressed patients utilizing the verbal fluency task (VFT) (11). However, these tests are expensive to conduct. Additionally, the patients were required to place themselves in an awkward posture, i.e., lying supine in a narrow space with the head fixed during the investigation (14).

In 2009, functional near-infrared spectroscopy (fNIRS) was sanctioned in Japan. It was classified to be an advanced medical technology for differentiation of psychiatric illness (15). Additionally, in 2013, fNIRS obtained medical insurance coverage for being an adjunct diagnostic tool. fNIRS examinations have since been utilized for psychophysiological assessment of cognitive function.

fNIRS is a form of spectroscopy that utilizes light sources between a spectral window of 650 to 1000 nm which penetrates organic tissues. Oxygenated hemoglobin (oxy-Hb) variations are then computed using the variance in absorbance using the modified Beer–Lambert law. This is a noninvasive technique that can detect cortical oxygenation levels of hemoglobin (16), and low cortical oxygenation levels have been correlated with depressive illness. The advantages of using fNIRS are that it is a relatively inexpensive procedure, portable, and easy to set up, and it does not involve nonionizing radiation. Hence, it may be repeated multiple times on an as-needed and when-needed basis for patients. In fact, fNIRS has been applied in many other areas of the medical field, such as cognition and preoperative functional assessment (17). With regard to psychiatric illness, in addition to MDD, there are studies pertaining to its use for patients with schizophrenia and bipolar disorder among other disorders (18) (**Table 1**). Many fNIRS studies involve tasks that help activate brain activity in the subject, such as VFT or passively viewing photographs to trigger an emotional response.

TABLE 1 | Studies using fNIRS to assess different psychiatric disorders.

Study	Psychiatric disorder	Key finding
Noda T. et al. (19)	Schizophrenia	Prefrontal and temporal region oxy-Hb uptake during the post-verbal fluency task (VFT) period was associated with working memory deficits in patients with schizophrenia.
Hirose T. et al. (20)	Bipolar disorder	Suicide risk in patients with bipolar disorder was correlated with delayed activation timing of NIRS signal during the VFT in the prefrontal region.
Katzorke A. et al. (21)	Dementia/ cognitive impairment	Patients with mild cognitive impairment had decreased hemodynamic response in the inferior frontotemporal cortex as compared to healthy controls using VFT.
Ueda S. et al. (22)	Attention-deficit hyperactivity disorder (ADHD)	Adult ADHD patients had reduced prefrontal hemodynamic response during the Stroop Color–Word Task compared to healthy controls, and this response was similar to pediatric studies.

Oxy-Hb, oxygenated hemoglobin; fNIRS, functional near-infrared spectroscopy.

To date, most of the studies on the diagnostic and predictive applications of fNIRS for MDD have been conducted in Japan. Furthermore, most studies have been conducted with a relatively small number of participants. To date, there is only one meta-analysis, published in 2015, looking at fNIRS for differentiating patients with depression from healthy subjects (23). Since then, more studies have been conducted on the matter as the use of fNIRS on depressed individuals to help in diagnosis and monitoring treatment response has gained acceptance over the last few years. Through a meta-analysis, Zhang et al. found that MDD patients had considerably decreased prefrontal cortex activation when undertaking cognitive tasks relative to controls. Patients with MDD, as opposed to controls, were associated with reduced rise in oxy-Hb in prefrontal regions during cognitive stimulation. This distinctive pattern of blood oxygen variations in the prefrontal cortex in MDD patients may be used as an objective diagnostic instrument for MDD. However, with such a low quantity of studies in the first meta-analysis, we decided to evaluate the use of fNIRS as a diagnostic biomarker for the diagnosis of MDD and its distinction between different stages of the illness, and to discuss its usefulness as a monitoring biomarker for treatment response in MDD patients, as based on a systematic review of the latest available literature. This paper aims to cover (i) the use of fNIRS to differentiate depressed from healthy individuals, (ii) correlation of fNIRS signals with depression symptomatology, and (iii) how it can be applied to monitor treatment response.

METHODS

Data Sources and Search Strategy

This study was conducted on the basis of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA). A systematic review was completed with published English-language literature from 1980 to June 2019, focusing on the utility of fNIRS for (i) differentiating depressed versus nondepressed individuals, (ii) correlating with depression symptomatology, and (iii) monitoring treatment responses in depression. The four electronic databases searched were PubMed, EMBASE, ScienceDirect, and Cochrane Library. The search terms used were "Spectroscopy" or "Near-infrared" or "near-infrared spectroscopy" or "fNIRS" or "optical topography" and "Depression" or "depressed" or "depressive disorder" or "mood disorder" or "affective disorder." Terms were searched as both text words and subject headings. The team scanned for related publications, conference proceedings and bibliographies of papers gathered manually. This systematic review was funded by the National University of Singapore iHeathtech Other Operating Expenses (R-722-000-004-731).

Eligibility Criteria and Data Collection

Studies were short-listed if the authors utilized fNIRS to measure cerebral hemodynamic variations in patients with MDD of any age group. All titles and abstracts retrieved from the databases were independently reviewed by two reviewers (LL and NC). Where appropriate, full-text papers were extracted for further inspection. Studies which were selected by either reviewer and fulfilled the inclusion criteria proceeded on to full-text review, whereby the study characteristics and results were extracted. Any discrepancies in the study selection were brought to the attention of the third reviewer (CH) and resolved by discussion. Studies were divided into the three categories that were most appropriate for the three questions set out in the review. The data review form consisted of the subsequent data: authors and publication year, country, sample characteristics, diagnostic criteria used, type of NIRS device, paradigm used, and main findings of the study. As much data as possible were obtained from the articles, and efforts were made to contact the authors if supplementary data were needed.

Quality Assessment of the Articles

The articles included in the full-text review were evaluated by utilizing the Newcastle–Ottawa quality assessment scale (NOS) (24), with the subsequent generation of a table with star scores for each study. There is no overall score that determines whether a study is "good" or "bad," but a star is awarded for meeting each criterion involving selection and outcome, with the exception of compatibility where two stars can be awarded. Two reviewers (LL and NC) independently assessed the papers, and discrepancies were brought to the third reviewer (CH) to resolve by discussion.

RESULTS

Study Selection

A total of 4868 citations were identified from our database search, with 342 from PubMed, 1798 from EMBASE, 1625 from ScienceDirect, and 1103 from Cochrane Library. After reviewing the titles, abstracts, and removing duplicated publications, 128 articles were selected. Of these, 64 studies met the inclusion criteria and were encompassed in this

analysis. The selection process is displayed in **Figure 1**, constructed according to the PRISMA statement. Forty-seven studies were from Japan, eight studies from China, six studies from Germany, one study from the UK, one study from the USA, and one study from Uzbekistan. These studies were further divided into categories based on which of the three questions they addressed: (i) differentiation of depressed from healthy individuals, (ii) correlation with depression symptomatology, and (iii) assessment of treatment response. Fifty-one studies addressed the first question, 31 studies addressed the second question, and 16 studies addressed the third question. Twelve studies were longitudinal, while the rest were cross-sectional studies. Seven studies were extracted from conference proceedings, while the remaining were full-text articles.

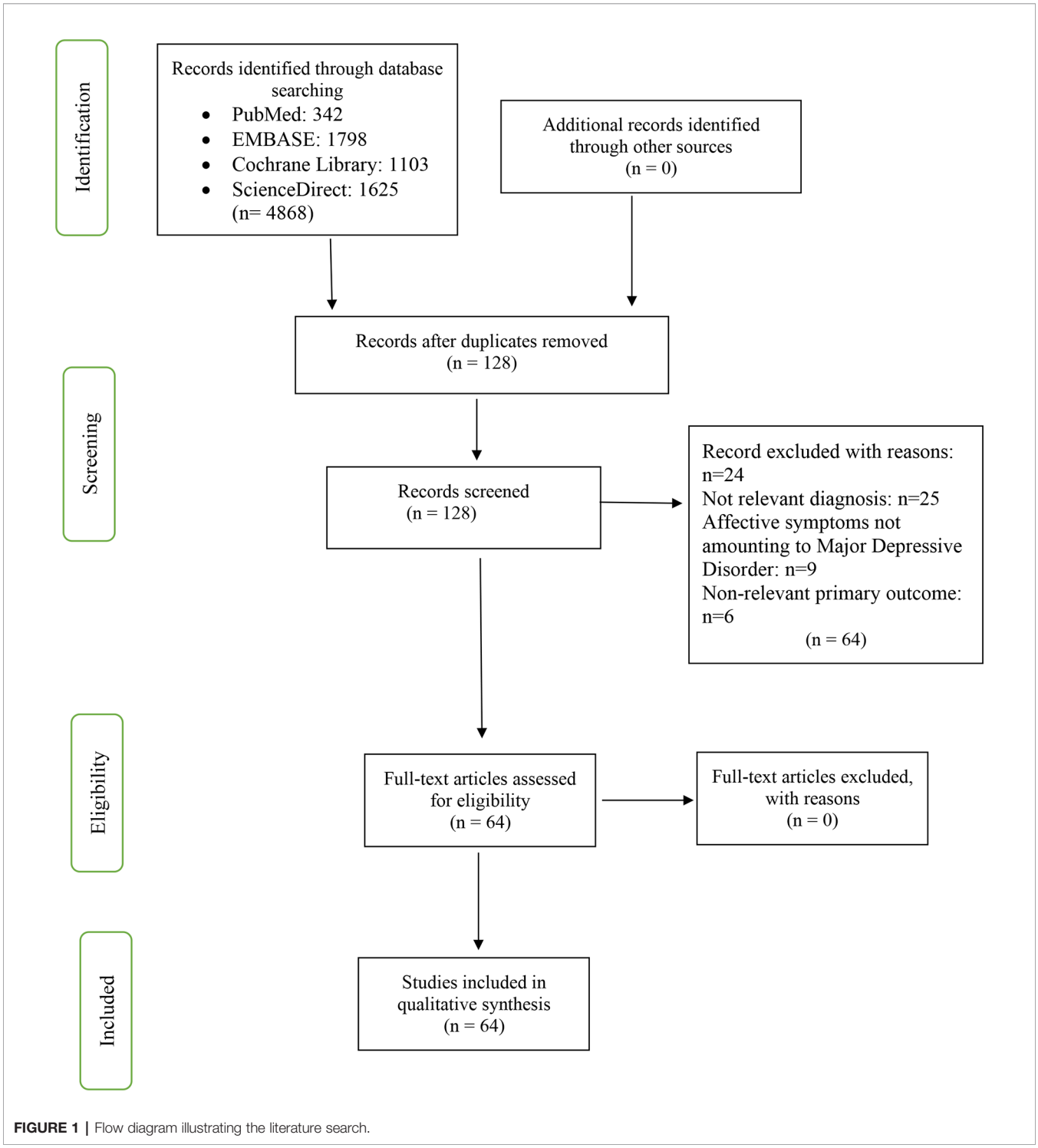

FIGURE 1 | Flow diagram illustrating the literature search.

Use of fNIRS to Differentiate Depressed From Healthy Individuals

We identified 51 papers that used fNIRS to distinguish depressed patients from healthy controls (HCs) (**Table 2**). The bulk of the studies were conducted in Japan (n = 38), followed by China (n = 7), Germany (n = 4), and the United Kingdom (n = 1). The pooled sample across the studies comprised a total of 2094 unipolar depressed patients and 2457 HCs. All the depressed patients in the respective studies fulfilled the ICD-10 or DSM-IV diagnostic criteria for MDD, with the exception of two studies that used the DSM-5 and one study that used the DSM-III as the diagnostic criteria instrument. The psychopathology measure used for the studies was mostly the Hamilton Depression Rating Scale (HAMD). With the exception of six studies that did not include an indication of the medications that were consumed by the depressed patients, three studies that included medication-naive patients and two studies that included antidepressant-naive patients, the depressed patients generally received antidepressants of different types and dosages with or without other types of medication. The majority of studies utilized the VFT as the active paradigm, while others adopted the verbal repetition task, hyperventilation and paper-bag breathing, visuospatial task, Stroop task, Sternberg's task, Tower of Hanoi, CO_2 inhalation task, working memory (WM) task, word generation tasks (WGT), emotional Stroop task, facial emotion recognition task, image-recall task, mirror drawing task (MDT), trail-making test (TMT), or a combination of tasks [e.g., word fluency task (WFT) with right-finger-tapping task]. The fNIRS instruments that were used and the number of channels utilized, with a large proportion using the 52-channel ETG-4000, varied across studies. Most of the studies measured the concentration of oxy-Hb and deoxygenated hemoglobin (deoxy-Hb) during various tasks for distinguishing the depressed from the healthy. The probe is normally positioned at the frontal (usually prefrontal) and/or temporal areas, with the exception of two papers that measured the parietal area as well and one paper that measured only the left and right hemispheres in general. For the papers that included the assessment of the parietal brain areas, one of them included subjects suffering from Alzheimer's disease, and the parietal dysfunctions were linked to the initial phase of dementia (30). Other studies such as Rosenbaum et al. (72) examined the frontoparietal networks of patients with early- and late-life depression (LLD), which is associated with cognitive control. The study discovered that the frontoparietal networks appeared to be essential in LLD as patients suffering from LLD and memory impairment are shown to be at elevated risk for having dementia (75).

The overall results showed that the depressed patients showed a smaller oxy-Hb increase than the controls, with a smaller increase in the frontal (especially the prefrontal) and temporal activation (appreciably lower activation in the depressed compared to HCs) while performing the WM task, VFT, WGT, and TMT. Kito et al. (30) measured both the frontal and parietal brain areas, and the results showed decreased cortical activation throughout the VFT in patients with depression and the HCs. With the same brain areas being measured, the depressed patients and HCs showed noteworthy dissimilarities in functional connectivity (FC) both in the resting state and during TMT performance, with depressed patients showing a decrease, and HCs showing a surge, in FC from the resting state to TMT performance (72). When subjects were asked to hyperventilate, there was a significant decrease in oxy-Hb levels, and deoxy-Hb was considerably elevated in the depressed and HCs, with the depressed patients showing a lesser reduction in oxy-Hb than healthy individuals (26, 31). During paper-bag breathing, Matsuo et al. (31) revealed that oxy-Hb significantly increased in both groups, whereas deoxy-Hb significantly decreased in the depressed juxtaposed to the healthy. Matsuo et al. (26) established no noteworthy alterations in oxy-Hb and deoxy-Hb in depressed patients or in HCs. Using the emotional Stroop task, Matsubara et al. found that the HCs compared to the depressed patients showed notably increased oxy-Hb during the happy-word trials, but the decreases in oxy-Hb during the threat-word trials were comparable in both depressed patients and HCs (32). However, Matsubara et al. and Nishizawa et al. concluded that the depressed when comparing with HCs showed notable increased in oxy-Hb within the left middle frontal region while performing the threat task, whereas depressed patients compared to HCs showed no noteworthy variation in oxy-Hb while performing the happy task (42, 60). For the CO_2 inhalation task, the vasomotor reactivity was significantly decreased in the depressed patients than controls (35). When subjects were performing right-finger-tapping tasks, the increase in oxy-Hb was higher in the depressed patients when comparing with HCs (18). In the image-recall task, the change in oxy-Hb in the HCs was considerably more than in the depressed while experiencing unpleasant conditions (53).

Almost all of the studies did not indicate the specificity and sensitivity of differentiating between depressed patients and HCs, with the exception of one that indicated an 80% sensitivity but with its specificity being unspecified (29). One study had indicated a sensitivity of 71.5% and a specificity of 70% for differentiating depressed patients from those with Alzheimer's disease (30), and another study mentioned a sensitivity of 0.71 and a specificity of 0.46 to distinguish between the euthymic, unipolar and bipolar depressive patients (37). Last, there was one study that indicated different sensitivities and specificities, which were dependent on the integral values of the two regions of interest (region 1 consisted of frontopolar and dorsolateral prefrontal cortical regions, while region 2 consisted of the middle and superior temporal cortical regions and the ventrolateral prefrontal cortex) (39).

Correlation of fNIRS Signals With Depression Symptomatology

We identified 31 eligible papers reporting on fNIRS studies in which cerebral hemodynamic changes were correlated with depression symptomatology in a total of 1424 patients (**Table 3**). These studies were generally in those with MDD, but some were inclusive of patients with other mental health illness, for example: bipolar disorder, affective disorder, and

TABLE 2 | Summary of fNIRS studies differentiating depressed patients from healthy controls.

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
Pu et al., (25)	Japan	MDD: 24 (12/12) HC: 26 (8/18)	MDD: 47.9 ± 13.9 HC: 42.4 ± 9.3	DSM-IV-TR (MINI)	BDI HAMD	All on antidepressants	52-Channel NIRS (ETG-4000)	2-Back task with blocked periodic baseline, activation	PF T	– MDD group had ↓ response sensitivity and accuracy than HC.
Matsuo et al. (26)	Japan	UNI: 8 (1/7) BP: 1 (1/0) HC: 10 (0/10)	UNI+BP: 65.6 ± 6.4 HC: 59.5 ± 5.9	DSM-IV (unspecified)	HAMD	All on medication	HEO-200	Verbal repetition task, VFT, Hyperventilation, Paper-bag breathing	F	– VFT: Oxy-Hb ↑ and deoxy-Hb ↓ in HC group but no noteworthy changes in depressed group. – Hyperventilation: oxy-Hb ↓ while deoxy-Hb ↑
Herrmann et al. (27)	Germany	MDD: 9 (5/4) HC: 9 (5/4)	MDD: 37.3 ± 13.8 HC: 35.1 ± 5.5	ICD-10	BDI	All on medication	2-Channel NIRO-300 monitor	VFT	PF	– Oxy-Hb ↑ in HC. – MDD had significantly ↓ activation.
Shoji et al. (28)	Japan	MDD: 26 HC: 32	NA	ICD-10	HAMD	NA	44-channel ETG-4000 (Hitachi)	Word generation tasks	PF	– Oxy-Hb variations in MDD were appreciably lesser than HC in all word tasks.
Kinoshita et al. (29)	Japan	MDD: 17	44.2 ± 12.2	DSM-IV (SCID)	HAMD	Majority on antidepressants	22-Channel ETG-4000 (Hitachi)	DEX/CRH test VFT	F	– Results did not fit well with the diagnostic criteria (DSM or ICD).
Kito et al. (30)	Japan	MDD: 30 (9/21) HC: 33 (11/22)	MDD: 71.1 ± 6.8 HC: 69.6 ± 5.5	DSM-IV	HAMD	All on medication	FOIRE-3000 (Shimadzu)	VFT	F, P	– Cortical activation in the VFT in MDD ↓ compared to HC.
Matsuo et al. (31)	Japan	MDD: 14 (4/10) HC: 21 (3/18)	MDD: 56.1 ± 17.3 HC: 50.3 ± 12.6	DSM-IV	HAMD	All on medication	Single channel HEO-200 (Omron)	VFT Hyperventilation Paper-bag breathing	F	– VFT: ↑ in oxy-Hb was lower in MDD compared to HC. – Hyperventilation: MDD demonstrated an appreciably smaller reduction in oxy-Hb than HC. – Paper-bag breathing: oxy-Hb ↑ in both groups while deoxy-Hb ↓ in MDD.
Matsubara et al. (32)	Japan	MDD: 10 HC: 10	NA	NA	NA	NA	52-Channel ETG-4000 (Hitachi)	Emotional Stroop task	T	– HC showed ↑ in oxy-Hb in the fronto-temporal regions as opposed to MDD.
Koike et al. (33)	Japan	MDD: 405 HC: 369	NA	NA	NA	NA	fNIRS	VFT	PF	– The intensity of signals was smaller in MDD. – The duration of time taken to complete the task was later in MDD.
Azechi et al. (34)	Japan	MDD: 30 HC: 30	NA	NA	NA	NA	2-Channel NIRS	VFT, TOH, SBT Stroop Task	F	– Task performances of the VFT and TOH were ↓ in MDD than in HC.
Matsuo et al. (35)	Japan	MDD: 10 (5/5) HC: 10 (6/4)	MDD: 62.2 ± 4.8 HC: 58.7 ± 5.8	DSM-IV (MINI)	HAMD	Only 4 patients had medication	24-Channel ETG-100 (Hitachi)	VFT WRT CO_2 inhalation	PF	– During cognitive task, there was ↓ activation of PF cortex in MDD. – Negative association between ↓ PF activation while performing the cognitive task and degree of hyperintensity in the periventricular region or left F cortex in MDD. – CO_2 inhalation causing vasomotor reactivity was ↓ in MDD than HC.
Ohta et al. (36)	Japan	MDD: 17 (5/12) HC: 24 (12/12)	MDD: 42.8 ± 18.2 HC: 36.2 ± 16.5	DSM-IV (MINI)	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	WFT	F	– Improvement of oxy-Hb values in the bilateral F cortices, – MDD showed attenuated ↑ in oxy-Hb while doing WFT in the bilateral F regions. – Hypofrontality in MDD is most notable in the left medial inferior F lobe.

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
										– Enhancement of oxy-Hb was not as notable on the right-sided channels in MDD.
Suto et al. (18)	Japan	MDD: 10 (9/1) HC: 16 (12/4)	MDD: 47.9 ± 12.8 HC: 42.9 ± 4.6	DSM-IV	HAMD	All on medication	24-Channel ETG-100 (Hitachi)	WFT Right-finger-tapping task	F, T	– During first half of task period, MDD had ↓ oxy-Hb increase than HC. ↑were seen in the anterior lower T and lower F channels. – Finger tapping tasks in MDD patients caused ↑ in oxy-Hb compared to HC.
Shimodera et al. (37)	Japan	UNI: 39 (19/20) BP: 14 (7/7) HC: 24 (13/11)	UNI: 56.9 ± 12.6 BP: 51.4 ± 14.0 HC: 40.9 ± 10.6	DSM-IV-TR	HAMD	All on medication	52-Channel OMM-3000/16 (Shimadzu)	VFT	PF	– Both UNI and BP showed ↓ area under curves (AUCs) than HC. – MDD showed significantly ↓ weighted center (WC) than BP or HC.
Ma et al. (38)	China	Menopausal MD: 30 (0/30) MDD: 30 (0/30) HC: 30 (0/30)	MD: 51.17 ± 6.06 MDD: 37.50 ± 10.60 HC: 34.83 ± 8.77	DSM-IV	HAMD	Only 20 patients took medication	45-Channel FOIRE-3000 (Shimadzu)	VFT	PF	– MD and MDD both showed ↓ oxy-Hb activation in bilateral DLPFC than HC, but involving different channels. – Atypical hemodynamics of the left DLPFC can discriminate HC from MDD using NIRS.
Takizawa et al. (39)	Japan	UNI: 153 (76/77) BP: 134 (65/69) HC: 590 (276/314)	UNI: 43.8 ± 12.7 BP: 44.0 ± 14.9 HC: 43.9 ± 15.7	DSM-IV SCID	HAMD	10 drug-free patients with UNI	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– NIRS can differentiate HC from patients, and differentiate UNI from BP.
Liske et al. (40)	Germany	MDD: 41 HC: 46	NA	NA	SIMS	NA	NA	Motor task–pressing button	P	– In HC, a noteworthy contrast in the NIRS signal resulting from the left P brain regions but this was weaker in MDD.
Zhu et al. (41)	China	UNI: 35 (11/24) BP: 39 (19/20) HC: 36 (18/18)	UNI: 35.9 ± 13.2 BP: 37.0 ± 12.9 HC: 33.6 ± 10.3	DSM-IV-TR (MINI)	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	1-Back version of the n-back WMT	PF, T	– Compared to HCs, UNI and BP ↓ activation of oxy-Hb in the inferior PF region during WMT. – Distinct prefrontal activation patterns in the Broca's area and left frontopolar region, underline BD and UD.
Matsubara et al. (42)	Japan	UNI: 16 (8/8) BP: 16 (8/8) HC: 20 (10/10)	UNI: 45.4 ± 12.2 BP: 44.1 ± 17.5 HC: 41.4 ± 8.5	DSM-IV-TR (MINI)	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	Emotional Stroop task	PF	-While doing the threat task, depressed patients revealed ↑ oxy-Hb in the left middle frontal region. – When performing happy task, depressed patients, did not display any meaningful changes in oxy-Hb.
Akashi et al. (43)	Japan	MDD: 52 (32/20) Subdivided into with/without discrepancy HC: 48 (21/27)	MDD: 41.8 ± 12.7 HC: 38.9 ± 9.5	DSM-IV (MINI)	Structured interview guide for HAMD (SIGH-D)	Most of the patients taking medications	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– In the FT regions, upsurge in mean oxy-Hb were ↓ in MDD than in HC.
Gao et al. (44)	China	MDD: 27 (7/20) HC: 24 (11/13)	MDD: 40.78 ± 13.42 HC: 43.13 ± 11.28	DSM-IV	HAMD	All were medication free for at least 4 weeks	CW-NIRS	Facial emotion recognition	PF	– Hemodynamic variations between the bilateral PF cortex and left PF cortex may provide dependable predictors for diagnosing depression

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
Schecklmann et al. (45)	Germany	UNI: 16 (9/7) BP: 14 (3/11) HC: 15 (7/8)	UNI: 43.4 ± 9.8 BP: 40.8 ± 10.2 HC: 40.9 ± 8.0	ICD-10	BDI-II	All on medication	52-Channel ETG-4000 (Hitachi)	WMT	PF, F	Results discovered unspecific deficits that inhibited discrimination between bipolar and unipolar depression in domains of working memory.
Tomioka et al. [3,4] (46)	Japan	MDD: 25 (3/22) HC: 62 (14/48)	MDD: 51.9 ± 16.6 HC: 51.7 ± 17.2	DSM-IV	HAMD	Medication-naive	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– MDD showed ↓ oxy-Hb values when comparing to with HC in the bilateral F and T cortices at baseline. – Hypofrontality response to VFT may represent a potential trait marker for depression
Ohtani et al. [3,4] (47)	Japan	UNI: 10 (4/6) BP: 18 (9/9) HC: 14 (7/7)	UNI: 39.2 ± 12.1 BP: 39.7 ± 9 HC: 33.6 ± 8.3	DSM-IV-TR	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– UNI and BP showed ↓ activation than HCs in the bilateral ventrolateral PF cortex and the anterior part of the T cortex.
Masuda et al. [3,4] (48)	Japan	MDD: 47 Response group to SSRIs: 28 (15/13) Nonresponse group: 19 (6/13) HC: 63 (35/28)	Response group: 48.9 ± 2.9) Nonresponse group: 43.2 ± 3.3 HC: 41.7 ± 1.4	DSM-IV-TR	HAMD	Medication-naive	47-Channel ETG-7100 (Hitachi)	VFT	PF, T	– In FT region, hemodynamic responses were ↓ in patients with response and nonresponse than in HC prior to treatment. – In the medial F region, hemodynamic responses were ↑ in patients with response to prior treatment.
Feng et al. [4] (49)	China	MDD: 15 (7/8) HC: 15 (6/9)	MDD: 30.93 ± 13.47 HC: 30.87 ± 10.11	DSM-5 SCI	HAMD	NA	45-Channel FOIRE-3000 (Shimadzu)	VFT	PF	– After the music treatment, average active oxy-Hb values of some channels were ↑ in both HC and MDD. – After music therapy, patients with MDD demonstrated substantial activation in the OFC, DLPFC, and VMPFC.
Hirano et al. [3,4] (50)	Japan	MDD: 30 (11/19) HC: 108 (45/63)	MDD: 59.4 ± 14.2 HC: 58.9 ± 13	ICD-10	MADRS QIDS-SR	All on medication	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– MDD exhibited ↓ oxy-Hb values in the bilateral F cortex while doing VFT than HC.
Downey et al. [4] (51)	UK	MDD: 18 HC: 51	NA	NA	NA	NA	MiniNTS 4 detectors/24 sources (UCL)	VFT, N-back working memory tasks	PF	– MDD had ↓ bilateral PF cortex oxy-Hb responses to VFT unlike HC, and this was additionally ↓ after 4 ECT sessions. – On WM task, MDD showed PF cortex inhibition at baseline and a different time course of oxy– and deoxy-Hb following 4 ECT.
Rosenbaum et al. [3] (52)	Germany	MDD: 60 HC: 24	MDD: 40 ± 14.79 HC: 33 ± 11.45	DSM-IV (SCI)	PHQ-9 MADRS	32% of patients treated with antidepressant medication	52-Channel ETG-4000 (Hitachi)	7-min resting phase, VAS, rumination response scale	P	– MDD as compared to HC, showed ↓ functional connectivity in parts of the DMN.

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
Kondo et al. [3] (53)	Japan	MDD: 25 (17/8) HC: 25 (18/7)	MDD: 36 ± 8.91 HC: 34.1 ± 10.1	DSM-IV-TR SCI	HAMD	All medicated with antidepressants	44-Channel ETG-4000 (Hitachi)	Image-recall task	F, T	– The oxy-Hb in HC was ↑ compared to MDD in bilateral F region. – The severity of depression was related to ↓ in oxy-Hb in left F lobe.
Zhu et al. [3] (54)	China	Affective disorder (AD): 28 (8/20) HC: 30 (21/9)	AD: 23.32 ± 5.01 HC: 23.60 ± 2.03	DSM-IV	SDS	13 patients were medicated. 15 free of any medicine	42-Channel FOIRE-3000 (Shimadzu)	Resting state measurements	F	– Relative to HC, AD demonstrated ↓ in intraregional and symmetrical interhemispheric connectivity in the PFC, revealed ↓ locally functional connectivity in the right IFG, and ↓ long-distance connectivity concerning the bilateral IFG.
Okada et al. [3] (55)	Japan	MDD: 36 (24/12) HC: 36 (21/12)	MDD (male): 23.3 ± 2.5 MDD (female): 21.3 ± 1.1 HC (male): 23.9 ± 2.4 HC (female): 23.6 ± 2.1	DSM-III-R	HAMD	13 patients received antidepressant medication. 23 were medication free for a minimum of 3 months	Multichannel near-IR spectrophotometry (NIRS)	MDT	LT, RT brain	– Nearly half of patients revealed a "nondominant hemisphere response pattern" – The supposedly "nondominant" hemisphere may convert to being dominant during the depression
Uemura et al. [3] (56)	Japan	MDD: 13 (6/7) HC: 67 (28/39)	MDD: 74.5 ± 5.8 HC: 73.8 ± 5.3	NA	GDS	All subjects were medicated.	8-Channel FOIRE–3000; (Shimadzu)	Trail-making test, part B (TMT-B; tablet version)	PF	– Oxy-Hb activation when performing the TMT-B was ↓ in MDD in both the right and left PF cortex. – ↓ PF activation in the elderly with depressive symptomology may cause deterioration in executive function.
Kinou et al.[3] (57)	Japan	MDD: 32 (15/17) HC: 32 (15/17)	MDD: 44.8 ± 9.8 HC: 45.7 ± 13.5	DSM-IV	H	All, except 3 patients	52-Channel ETG-4000 (Hitachi)	VFT	PF	– MDD revealed ↓ oxy-Hb activation during the task.
Yamagata et al.[3] (58)	Japan	Early-onset depression (EOD): 11 (2/9) Late-onset (LOD): 12 (3/9) HC: 13 (8/5)	EOD: 68.4 ± 5.6 LOD: 70.2 ± 1.9 HC: 70.3 ± 4.4	DSM-IV	HAMD	All patients were taking one prescribed antidepressant.	52-Channel ETG-4000 (Hitachi)	WFT	F, T	– HC demonstrated ↑ in oxy-Hb vs. surges in oxy-Hb being minimally ↓ in EOD and extremely ↓ in LOD, bilaterally throughout the F cortices and T areas. – Attenuated activation in the left lateral PF and T areas may help to differentiate LOD and EOD.
Noda et al.[3] (59)	Japan	MDD: 30 (14/16) HC: 30 (14/16)	MDD: 36.7 ± 11.6 HC: 35.1 ± 9.4	DSM-IV (SCI)	GRID-HAMD	All patients medicated with antidepressants.	52-Channels ETG-4000 (Hitachi)	VFT	F. T	– Oxy-Hb increases while performing task was meaningfully ↓ in MDD. – ↓ Right F-T activation on NIRS during VFT is related to the MDD severity.
Nishizawa et al. (60)	Japan	MDD: 14 (7/7) HC: 20 (13/7)	MDD: 38.2 ± 12.9 HC: 29.0 ± 5.7	DSM-IV-TR (SCID)	HAMD	All taking antidepressants except for four patients.	22-Channel ETG-4000 (Hitachi)	Emotional Stroop task	F, T	– Hyperactivated oxy-Hb was witnessed in the left F cortex on contact to unfavorable stimuli, but no noteworthy dissimilarity was established between MDD and HC on exposure to favorable stimuli.

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
Akiyama et al. [3] (61)	Japan	MDD: 177 (73/104) HC: 50 (40/10)	MDD: 47.2 ± 15.1 HC: 32.7 ± 7.5	DSM-IV-TR	HAMD	All patients were on antidepressants	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Significant hypoactivation in bilateral F-T regions was observed in MDD compared with HC.
Ohi et al.[3] (62)	Japan	UNI: 26 (17/9) BP: 22 (13/9) HC: 51 (33/18)	UNI: 41.1 ± 12.7 BP: 39.9 ± 12.5 HC: 35.7 ± 11.9	DSM-V	HAMD	Chlorpromazine equivalents of total antipsychotics	52-Channel ETG-4000 (Hitachi)	VFT	PF	– UNI and BP groups had ↓ PF activity than HC. – Patients with and without family history had ↓ PF activity than HC subjects. – Demonstrate connection of more serious PF dysfunction with higher genetic loading for disease.
Takei et al.[3] (63)	Japan	UNI: 29 (14/15) BP: 31 (14/17) HC: 31 (11/20)	UNI: 34.5 ± 9.0 BP: 34.9 ± 6.6 HC: 33.6 ± 10.0	DSM-IV	HAMD	Nearly all patients were on medication.	52-Channel ETG-4000 (Hitachi)	Conversation task and control task	F, T	– Both UNI and BP showed ↓ of continuous activation in the left DLPFC and left FPC, and decreased rapid change in bilateral FPC activation. – F activation while conversing ↓ in equally in UNI and BP. – Pathophysiological character of UNI and BP are reflected in continuous activation and rapid change change Impaired adaptive ability in UNI may be related to ↓ amount of rapid change in right FPC.
Pu et al.[3] (64)	Japan	Late-onset depression (LOD): 24 (6/18) HC: 30 (14/16)	LOD: 72.3 ± 5.5 HC: 72.0 ± 4.7	DSM-IV (MINI)	BDI HAMD	Antidepressant-naive	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– LOD had ↓ activation in both PF and superior T cortices than HC. – ↓ frontopolar cortical activation was linked with social functioning impairment in LOD.
Tsujii et al.[3] (65)	Japan	MDD: Suicide attempters (SAs): 30 (8/22) Nonattempters (NAs): 38 (16/22) HC: 40 (15/25)	MDD (SAs): 37.6 ± 10.0 MDD (NAs): 38.8 ± 9.7 HC: 38.2 ± 10.5	DSM-IV (MINI)	HAMD	All on medication.	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– MDD had significantly ↓ activation in the bilateral FT regions compared to HCs. – SAs demonstrated ↓ hemodynamic response in the left precentral gyrus than HCs and NAs. – Aggression and hopelessness were negatively associated with hemodynamic responses in the right middle F gyrus in SAs but not in HCs and NAs.
Pu et al.[3] (66)	Japan	Late-onset depression (LOD): 36 (9/27) HC: 35 (11/24)	LOD: 71.8 ± 5.1 HC: 70.9 ± 4.3	DSM-IV (MINI)	BDI HAMD	Antidepressant-naive	52-Channel ETG-4000 (Hitachi)	Working memory (WM) task	PF, T	– LOD was correlated with ↓ PF and T activation when comparing with HC. – Hemodynamic response in PF and T regions when performing WM task could correlate with social functioning in LOD.
Tsujii et al.[3] (67)	Japan	MDD with melancholia (MDD-MF): 30 (15/15)	MDD-MF: 42.2 ± 11.8 MDD-NMF: 40.6 ±	DSM-IV (MINI)	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– Both MDD groups demonstrated ↓ hemodynamic responses in the F-T regions.

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
		MDD without -melancholia (MDD-NMF): 52 (18/34) HC: 68 (32/36)	11.7 HC: 40.5 ± 10.6							
Liu et al.[3] (68)	China	MDD: 30 (12/18) HC: 30 (16/14)	MDD: 38.38 ± 12.8 HC: 33.2 ± 10.5	DSM-IV-TR	HAMD	Free of medication	52-Channel FOIRE-3000 (Shimadzu)	VFT	PF	– ↓ Activation in lateral and lower PFC in MDD. – Antero-medial PFC and bilateral PFC were correlated with the degree of depressive symptoms. – MDD patients with obsession–compulsion symptoms and anxiety exhibited a PFC ↓ activation state in NIRS.
Wang et al.[3] (69)	China	First-episode MDD (fMDD): 36 (15/21) Recurrent MDD: 34 (11/23) HC: 37 (22/15)	fMDD: 38.75 ± 13.86 Recurrent MDD: 43.26 ± 13.85 HC: 35.70 ± 11.39	DSM-IV	HAMD	All were on antidepressants	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– In comparison with HC and fMDD, chronic MDD had significantly ↓ brain activation over right PF and superior T cortices. – Variation in activations in bilateral F and T regions. HC: more channels in left than right hemisphere; fMDD: more channels in right than left hemisphere; recurrent MDD: only channels in left hemisphere.
Tsujii et al.[3] (70)	Japan	MDD with melancholia (MDD-MF): 32 (16/16) MDD without melancholia (MDD-NMF): 28 (15/13) HC: 24 (11/13)	MDD-MF: 40.8 ± 15.3 MDD-NMF: 38.9 ± 11.8 HC: 38.6 ± 9.2	DSM-IV (MINI)	SIGH-D BDI-II	All on medication	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– Noteworthy disparities were witnessed in mean oxy-Hb fluctuations of MDD-MF in 25 channels and in those with MDD-NMF in 12 channels compared to HC.
Nishida et al.[3] (71)	Japan	MDD: 14 (7/7) HC: 15 (8/7)	MDD: 46.2 ± 11.9 HC: 45.5 ± 10.9	DSM-IV-TR (MINI)	HAMD	All on medication	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– MDD revealed a ↓ oxy-Hb activation than HC, predominantly in ventrolateral PF and T cortex regions.
Rosenbaum et al.[3] (72)	Germany	Depressed: 49 Nondepressed: 51 2 participants diagnosed with bipolar disorder and eating disorder.	Depressed: 64.08 ± 7.06 Nondepressed: 64.16 ± 6.14	NA	BDI GDS	54% took medication.	38-Channel ETG-4000 (Hitachi)	Adapted trail-making test (TMT-A, TMT-B, and TMT-C)	F, P	– Depressed and nondepressed revealed substantial differences in functional connectivity (FC) while doing the task performance and at rest. – During task performance, depressed patients exhibited ↓ FC in a left frontopolar cortical network, and ↑ FC in a left frontoparietal cortical network at the resting state and altered FC and network organization during different mental states.

(Continued)

TABLE 2 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria (instrument)	Psychopathology measure	Medication	NIRS device	Paradigm	Brain area	Main findings
Pu et al.[3] (73)	Japan	MDD: 67 (29/38) MDD with suicidal ideation: 31 (11/20) MDD without suicidal ideation: 36 (18/18) HC: 67 (29/38)	MDD: 58.1 ± 16.0 MDD with suicidal ideation: 57.3 ± 15.7 MDD without suicidal ideation: 58.7 ± 16.5 HC: 58.1 ± 17.8	DSM-IV (MINI)	HAMD	All on antidepressants	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Regional hemodynamic changes were considerably ↓ in MDD than in HCs in PF and T regions.
Pu et al.[3] (74)	Japan	MDD: 26 (11/15) HC: 30 (12/18)	MDD: 47.9 ± 19.2 HC: 50.5 ± 19.7	DSM-IV-TR (MINI)	BDI HAMD	All on antidepressants	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Regional hemodynamic changes were appreciably ↓ in MDD than in HC in PF and T regions, and was correlated positively with task-oriented coping (adaptive coping) in the bilateral ventrolateral and dorsolateral prefrontal cortex, and the midline frontopolar and bilateral orbitofrontal cortex regions.

[3]*This article can also be found in the summary in* ***Table 3****.*

[4]*This article can also be found in the summary in* ***Table 4****.*

MDD, major depressive disorder; HC, healthy control; BDI, Beck depression inventory; HAMD, Hamilton depression rating scale; UNI, unipolar depression; BP, bipolar disorder; VFT, verbal fluency task; PF, prefrontal; T, temporal, F, frontal; NA, not available; TOH, Tower of Hanoi; SBT, Sternberg's task; WRT, word repetition task; MINI, Mini-international neuropsychiatric interview; DSM, Diagnostic and Statistical Manual; ICD, International Classification of Disease; SCID, structured clinical interview for DSM-IV; SIMS, structured inventory of malingered symptomatology; WMT, working memory task; IFG, inferior frontal gyrus; MDT, mirror drawing task; DLPFC, dorsolateral prefrontal cortex; PFC, prefrontal cortex; SIGH-D, structured interview guide for the Hamilton depression rating scale.

TABLE 3 | Summary of fNIRS studies correlating cerebral hemodynamic changes with depression symptomatology.

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for symptomatology	Specific symptomatology	NIRS device/no. of channels	Paradigm	Brain area	Main finding
Kawano et al. (76)	Japan	N: 25 *included other disorders	44.1 ± 9.3	DSM-IV	HAMD	Depressive symptoms	22-Channel ETG-4000 (Hitachi)	VFT	F, T	– The integral value of blood flow in frontal lobe was negatively correlated with the degree of depression
Onishi et al.[4] (77)	Japan	N: 10 (5/5)	71.0 ± 6.0	DSM-IV	HAMD MMSE	Depressive symptoms Cognitive functioning	48-Channel ETG-4000 (Hitachi)	Rock, paper, scissors (RPS)	PF	– Negative correlation between ratio of HAMD and oxy-Hb was observed.
Hirano et al.[2,4] (50)	Japan	N: 30 (11/19) *inclusive of bipolar patients	59.4 ± 14.2	ICD-10	MADRS QIDS-SR MMSE	Depressive symptoms Cognitive functioning	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– After ECT, the reduction of degree of depression was associated with increase in oxy-Hb values in the right ventrolateral PF cortex. – Changes in oxy-Hb is significantly interrelated with MADRS score ↓ but not significantly correlated with ↓ in total QIDS-SR total scores.
Masuda et al.[2,4] (48)	Japan	N: 47 Response group to SSRIs: 28 (15/13) Nonresponse group: 19 (6/13)	Response group: 48.9 ± 2.9 Nonresponse group: 43.2 ± 3.3	DSM-IV	POMS STAI DACS	Anxiety symptoms Depressive symptoms	47-Channel ETG-7100 (Hitachi)	VFT	PF, T	– Hemodynamic responses in medial F region were significantly greater before treatment in patients with a response to SSRIs than those with no response.
Kondo et al.[2] (53)	Japan	N: 25 (17/8)	36 ± 8.91	DSM-IV	HAMD	Depressive symptoms	44-Channel ETG-4000 (Hitachi)	Image-recall task	F, T	– A noteworthy negative correlation between oxy-Hb and HAMD score was seen in left F region during the unpleasant condition. – ↓ in oxy-Hb in left F lobe was associated to degree of depression.
Koseki et al. (78)	Japan	MDD: 75 (39/36)	39.23 ± 12.49	DSM-IV	HAMD ATQ-R NART STAI	Depressive symptoms Automatic thoughts State/trait anxiety	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Activation in right superior temporal gyrus was associated to deviation to negative of the proportion of negative and positive thought.
Zhu et al.[2] (54)	China	Affective disorder (AD): 28 (8/20)	23.32 ± 5.01	DSM-IV	SDS	Depressive symptoms	42-Channel FOIRE-3000 (Shimadzu)	Resting state	F	– Degree of self-reported symptoms of depression was negatively associated with strength of intraregional and symmetrically interhemispheric connectivity in the PFC.
Uemura et al.[2] (56)	Japan	N: 13 (6/7)	74.5 ± 5.8	NA	GDS MMSE	Depressive symptoms	8-Channel FOIRE-3000 (Shimadzu)	TMT-B	PF	– Both oxy-Hb activation in left and right hemisphere were significantly negatively associated with GDS.
Noda et al.[2] (59)	Japan	N: 30 (14/16)	36.7 ± 11.6	DSM-IV (SCID-I)	GRID-HAMD	Depressive symptoms	52-Channels ETG-4000 (Hitachi)	VFT	F, T	– Average increase in oxy-Hb during the task revealed a significant negative association with the HAMD total scores.

(Continued)

TABLE 3 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for symptomatology	Specific symptomatology	NIRS device/no. of channels	Paradigm	Brain area	Main finding
Akiyama et al.[2] (61)	Japan	N: 177 (73/104)	47.2 ± 15.1	DSM-IV-TR	HAMD PHQ-9	Depressive symptoms	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Left lateral F-T activation was significantly ↓ in the group with depressed mood or anhedonia.
Tomioka et al.[2,4] (46)	Japan	N: 25 (3/22)	51.9 ± 16.6	DSM-IV	HAMD	Depressive symptoms	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	-Patients with MDD who revealed ↑ baseline oxy-Hb activation while performing VFT in the inferior F and middle T regions showed more improvements in depressive symptoms after being treated.
Rosenbaum et al.[2] (72)	Germany	N: 49 *inclusive of other disorders	64.08 ± 7.06	NA	BDI GDS	Depressive symptoms Cognitive functioning	38-Channel ETG-4000 (Hitachi)	TMT-A TMT-B TMT-C	F, P	– Depressed patients revealed ↓ FC in a left frontopolar cortical network while doing task performance ↑ FC in a left frontoparietal cortical network at the resting state.
Yamagata et al.[2] (58)	Japan	Early-onset depression (EOD): 11 (2/9) Late-onset depression (LOD): 12 (3/9)	EOD: 68.4 ± 5.6 LOD: 70.2 ± 1.9	DSM-IV	HAMD MMSE	Depressive symptoms	52-Channel ETG-4000, NIRS system (Hitachi)	WFT	F, T	– HAMD score exhibited negative correlations with oxy-Hb for two channels.
Akashi et al. (79)	Japan	n = 48	NA	NA	NA	Inhibitory deficit	47-Channel NIRS	Stop-signal task	F	– MDD showed the variations of brain dysfunctions correlated in the inhibitory controls.
Satomura et al. (80)	Japan	Initial (T0)–N: 65 After 1.5 years (T1.5)–N: 45	39.8 ± 11.8	DSM-IV (SCID-I)	HAMD GAF	Depressive symptoms Global functioning	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Brain activation in the bilateral MFG and right IFG as calculated by NIRS may differentially denote clinical severity and trait-related anomalies in MDD.
Ohtani et al.[2,4] (47)	Japan	N: 10 (4/6)	39.2 ± 12.1	DSM-IV	SASS HAMD	Social adaptation Depressive symptoms	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Longitudinal changes in SASS scores were positively related with magnitude of change in the right VLPFC/aTC activation in MDD group.
Pu et al.[2] (64)	Japan	Late-onset MDD (LOD): N: 24 (6/18)	72.3 ± 5.5	DSM-IV (MINI)	BDI HAMD SASS MMSE	Depressive symptoms Social functioning	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Average oxy-Hb changes in LOD patients had a significantly positive association with SASS scores.
Pu et al.[2] (66)	Japan	Late-onset depression (LOD): 36 (9/27)	71.8 ± 5.1	DSM-IV (MINI)	BDI HAMD SASS MMSE	Depressive symptoms Social functioning	52-Channel ETG-4000 (Hitachi)	Working memory (WM) task	PF, T	– Reduced activation in PF and T regions was significantly associated to ↓ scores on the SASS in patient group and might act as a biological marker of social functioning in LOD patients.
Pu et al.[2] (74)	Japan	N: 26 (11/15)	47.9 ± 19.2	DSM-IV-TR (MINI)	HAMD BDI CISS	Depressive symptoms Coping styles	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Regional hemodynamic changes were ↓ in MDD group compared to the control group in PF and T areas, and positively interrelated with task-oriented coping (adaptive coping) in the various PF regions.

(Continued)

TABLE 3 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for symptomatology	Specific symptomatology	NIRS device/no. of channels	Paradigm	Brain area	Main finding
Pu et al.[2] (81)	Japan	N: 67, 31 with suicidal ideation and 36 without	58.1 ± 16.0	DSM-IV (MINI)	HAMD	Suicidal ideation	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Hemodynamic variations correlated negatively with the degree of suicidal thinking in OFC, FPC, and DLPFC.
Nishida et al.[2] (71)	Japan	N: 14 (7/7)	46.2 ± 11.9	DSM-IV-TR (MINI)	HAMD PSQI ESS	Depressive symptoms Sleep quality	52-Channel ETG-4000 (Hitachi)	VFT	PF, T	– Significant negative correlations between average oxy-Hb variations during VFT and PSQI scores. – Mean oxy-Hb changes showed no significant correlations with ESS scores. – No significant association between average oxy-Hb variations during the task and sleep variables.
Tsujii et al.[2] (70)	Japan	MDD with melancholia (MDD-M): 32 (16/16) MDD without melancholia (MDD-NM): 28 (15/13)	MDD-MF: 40.8 ± 15.3 MDD-NMF: 38.9 ± 11.8	DSM-IV (MINI)	BDI-II SIGH-D GAF	Depressive symptoms Psychomotor retardation	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– No meaningful associations between average oxy-Hb changes and GAF/HAMD/BDI-II total scores. – Psychomotor retardation on HAMD indicated noteworthy positive correlation with average oxy-Hb changes in right T areas in MDD-MF but revealed significant negative association with average oxy-Hb changes in the middle to left T region in MDD-NMF.
Tsujii et al.[2] (67)	Japan	MDD with melancholia (MDD-M): 30 (15/15) MDD without melancholia (MDD-NM): 52 (18/34)	MDD-M: 42.2 ± 11.8 MDD-NM: 40.6 ± 11.7	DSM-IV (MINI)	HAMD SF-36	Depressive symptoms Quality of life	52-Channel ETG-4000 (Hitachi)	VFT	F, T	MDD-M patients reveal qualitatively dissimilar prefrontal dysfunction patterns correlated with emotional role functioning as compared to MDD-NM patients.
Wang et al.[2] (69)	China	First-episode MDD (fMDD): 36 (15/21) Recurrent MDD (rMDD): 34 (11/23)	fMDD: 38.75 ± 13.86 rMDD: 43.26 ± 13.85	DSM-IV	HAMD	Depressive symptoms	52-Channel ETG-4000 (Hitachi)	VFT	F, T	-rMDD group had ↓ increases in oxy-H comparing to the fMDD group.
Liu et al.[2] (68)	China	N: 30 (12/18)	38.38 ± 12.8	DSM-IV-TR	HAMD HAMA Y-BOCS	Depressive, anxiety, obsessive–compulsive symptoms	52-Channel FOIRE-3000 (Shimadzu)	VFT	PF	– Average oxy-Hb changes revealed significant positive correlation with HAMD scores. -No statistical relationship was witnessed on the degree of obsessive–compulsive symptoms.
Rosenbaum et al.[2] (52)	Germany	N: 60	40 ± 14.79	DSM-IV (SCI)	PHQ-9 MADRS	Depressive symptom State and trait	52-Channel ETG-4000 (Hitachi)	RRS VAS Self-report on	P	– Subjects who are depressed revealed ↓ functional connectivity in parts of the DMN compared to HCs. – mind-wandering revealed positive

(Continued)

TABLE 3 | Continued

Source	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for symptomatology	Specific symptomatology	NIRS device/no. of channels	Paradigm	Brain area	Main finding
						aspect of rumination		inner experience		associations, whereas rumination was negatively associated with FC in the cortical parts of the DMN
Okada et al.[2] (55)	Japan	N: 36 (24/12)	Male: 23.3 ± 2.5 Female: 21.3 ± 1.1	DSM-III-R	HAMD	Depressive symptoms	Multichannel near-IR spectrophotometry	Mirror drawing task (MDT)	Left and right hemispheres	– Nearly half of the patients revealed a "nondominant hemisphere response pattern," which was not witnessed in normal subjects during the MDT. – During the course of depression, the supposedly "nondominant" hemisphere may become dominant.
Kinou et al.[2] (57)	Japan	N: 32 (15/17) *included HC, MDD, schizophrenia	44.8 ± 9.8	DSM-IV	HAMD Global assessment of functioning	Depressive symptoms	52-Channel ETG-4000 (Hitachi)	VFT	PF	– Lower Global Functioning scores were correlated with ↓ hemodynamic responses.
Takei et al.[2] (63)	Japan	N: 29 (14/15)	34.5 ± 9.0	DSM-IV	HAMD GAF	Depressive symptoms Cognitive functioning	52-Channel ETG-4000 (Hitachi)	Conversation task and control task	F, T	-Prompt change in activation was positively associated with GAF scores in the MDD patients.
Ohi et al.[2] (62)	Japan	N: 26 (17/9)	41.1 ± 12.7	DSM-5	HAMD Clinical interview for family history	Depressive symptoms Family history/ familial loadings	52-Channel ETG-4000 (Hitachi)	VFT	PF	– Illustration of the association of significantly more severe PF dysfunction with higher genetic loading in major mental illnesses.
Tsujii et al.[2] (65)	Japan	MDD: suicide attempters (SAs): 30 (8/22) nonattempters (NAs): 38 (16/22)	MDD (SAs): 37.6 ± 10.0 MDD (NAs): 38.8 ± 9.7	DSM-IV (MINI)	HAMD Barratt impulsiveness scale Buss– Perry aggression questionnaire Beck hopelessness scale	Depressive symptoms Impulsivity Aggression Hopelessness	52-Channel ETG-4000 (Hitachi)	VFT	F, T	– SAs revealed smaller hemodynamic response in the left precentral gyrus than NAs and HCs. – Hemodynamic responses in the right middle F gyrus were negatively correlated with aggression and hopelessness in SAs.

[2]*This article can also be found in the summary in* ***Table 2****.*

[4]*This article can also be found in the summary in* ***Table 4****.*

MDD, major depressive disorder; HC, healthy control; HAMD, Hamilton depression rating scale; VFT, verbal fluency task; PF, prefrontal; T, temporal, F, frontal; NA, not available; MINI, Mini-international neuropsychiatric interview; DSM, Diagnostic and Statistical Manual; ICD, International Classification of Disease; MMSE, Mini-Mental State Examination; SASS, school and staffing survey; POMS, profile of mood states; STAI, state-trait anxiety inventory; MADRS, Montgomery–Asberg depression rating scale; QIDS-SR, quick inventory of depressive symptomatology-self report; DSRS, dementia severity rating scale; HAMA, Hamilton anxiety rating scale; GDS, geriatric depression scale; PHQ, patient health questionnaire; BDI, Beck depression inventory; TMT, trail-making test; SCID, structured clinical interview for DSM-IV; IFG, inferior frontal gyrus; MFG, medial frontal gyrus; DLPFC, dorsolateral prefrontal cortex; OFC, orbitofrontal cortex; FPC, frontopolar cortex; PSQI, Pittsburgh sleep quality index; ESS, Epworth sleepiness scale; SIGH-D, structured interview guide for the Hamilton depression rating scale; Y-BOCS, Yale–Brown obsessive–compulsive scale; RRS, rumination response scale; VAS, visual analog scale; DMN, default mode network; MDT, mirror drawing task; GAF, global assessment of functioning; ATQR, Automatic thoughts questionnaire-revised.

TABLE 4 | Summary of fNIRS studies assessing antidepressant/treatment response.

Source.	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for treatment response	Treatment outcome	Medication (mg)/ treatment	NIRS device/no. of channels	Duration/no. of follow-up	Paradigm	Brain area	Main finding
Tomioka et al.[2,3] (46)	Japan	MDD: 25 (3/22)	51.9 ± 16.6	DSM-IV	HAMD	Depressive symptoms	Imipramine (118.7 ± 67.3)	52-Channel ETG-4000 (Hitachi)	12 weeks	VFT	PF, T	– NIRS signals before initiation of treatment could foretell patients' clinical response upon being treated
Onishi et al.[3] (77).	Japan	MDD: 10 (5/5)	71.0 ± 6.0	DSM-IV	HAMD MMSE	Depressive symptoms Cognitive functioning	Mianserin (2), Sodium valproate (2), Paroxetine (2), Lithium (2), Amoxapine (1), Olanzapine (3), Milnacipran (5), Maprotiline (1)	48-Channel ETG-4000 (Hitachi)	2 follow-ups (1 day following improvement in depressive symptoms after treatment, and another day >4 weeks later)	Rock, paper, scissors (RPS)	PF	– The more left PF cortical activity tended to ↑, symptoms of depression ↓
Ohtani et al.[2,3] (47)	Japan	MDD: 10 (4/6)	39.2 ± 12.1	DSM-IV	HAMD SASS	Social functioning	Chlorpromazine (1), Imipramine (3), Diazepam (4)	52-Channel ETG-4000 (Hitachi)	6 months	VFT	PF, T	– Longitudinal variations in SASS results were correlated positively with degree of change in the right ventrolateral PF cortex and the anterior part of the T cortex activation in MDD.
Yamagata et al. (82)	Japan	MDD: 11 (5/6)	36.3 ± 11.2	DSM-IV	HAMD	Depressive symptoms	Sertraline; week 4: (29.5 ± 10.1), week 8: (61.4 ± 20.4), week 12: (65.9 ± 23.1)	52-Channel ETG-4000 (Hitachi)	12 weeks, 3 follow-ups (weeks 4, 8, 12)	VFT	PF, T	– NIRS may be a biological marker in MDD patients for predicting clinical response to Sertraline.
Masuda et al.[2,3] (48)	Japan	MDD: 47 Response group to SSRIs: 28 (15/13) Nonresponse group: 19 (6/13)	Response (48.9 ± 2.9) Nonresponse (43.2 ± 3.3)	DSM-IV	POMS STAI DACS	Overall functioning for response Group	Escitalopram (33), Paroxetine (7), Sertraline (5), Fluvoxamine (2)	47-Channel ETG-7100 (Hitachi)	12 weeks, weekly or biweekly follow-up	VFT	PF, T	Response to SSRI in MDD is predicted by different hemodynamic activities in the frontotempral cortex.
Feng et al.[2] (49)	China	MDD: 15 (7/8)	30.9 ± 13.5	DSM-V	HAMD	Depressive symptoms	Music therapy, either "creative" (composing music) or "receptive" (listening to music)	45-Channel FOIRE-3000 (Shimadzu)	10 days, one session (60 min) a day	VFT,	PF	Music therapy could activate frontal cortex areas to improve mood and cognitive abilities
Aoki et al. (83)	Japan	MDD: 2 (1/1)	24.0 ± 2.0	ICD-10	NA	NA	Animal-assisted therapy (AAT) with medication	42-Channel FOIRE-3000 (Shimadzu)	Pretest and posttest design	VFT	PF	AAT helps to stimulate prefrontal activity in MDD and the effects of AAT can be evaluated by NIRS
Hirano et al.[2,3] (50)	Japan	MDD = 30 (11/19)	59.4 ± 14.2	ICD-10	MADRS QIDS-SR MMSE	Reduction in MADRS and QIDS-	Electroconvulsive therapy (ECT)	52-Channel ETG-4000 (Hitachi)	3x per week, till stable response	VFT	PF, T	Acute therapeutic effects of ECT on MDD patients is correlated to

(Continued)

TABLE 4 | Continued

Source.	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for treatment response	Treatment outcome	Medication (mg)/ treatment	NIRS device/no. of channels	Duration/no. of follow-up	Paradigm	Brain area	Main finding
						SR scores (improved functioning)						recovery from abnormal functional responses to cognitive tasks in frontal brain regions.
Takamiya et al. (84)	Japan	MDD: 33 (17/16)	46.4 ± 11.7	DSM-IV	HAMD	Differences between low-dose/ high-dose groups in HAMD scores	High-dose group (> 1 defined daily dose, N =10), low-dose group (< 1 defined daily dose, N = 23)	52-Channel ETG-4000 (Hitachi)	Cross-sectional study	VFT	PF, T	The dose-dependent influence of antidepressants on NIRS signals should be considered while deciphering NIRS data.
Shinba et al. (85)	Japan	MDD: 15 (11/4)	45.4 ± 10.8	DSM-IV	MADRS	Improved functioning	Transcranial magnetic stimulation (TMS), Fluvoxamine (89.6 ± 85.8)	NIRO-3000 (Hamamatsu)	6 weeks, 5 sessions a week	NA	PF	Maintenance of frontal activation [measured by frontal hemoglobin concentration (fHbC)] during TMS stimulation is related to effectiveness of treating MDD patients.
Usami et al. (86)	Japan	MDD: 10 (1/9)	12.9 ± 0.9	DSM-IV	DSRS	Depressive symptoms Global functioning	NA	2-Channel Spectratech	6 weeks	VFT	PF	Concentration of oxy-Hb could be utilized as a state marker for changes in depressed children.
Payzieva & Maxmudova (87)	Uzbekistan	MDD: 5	NA	NA	NA	NA	NA	OxyPrem (BORL, Switzerland)	Pretest and posttest design	Mental arithmetic task	PF	Computerized cognitive exercises may help in improve cognition of MDD patients and NIRS can be used to monitor cognitive functions.
Pu et al. (81)	Japan	MDD: 29 (7/22)	72.4 ± 5.7	DSM-IV	HAMD SASS	Depression symptoms Social functioning	Paroxetine (10–40 mg, N = 15), Milnacipran (50–150 mg, N = 14)	52-Channel ETG-4000 (Hitachi)	8 weeks	VFT	PF, T	Social functioning improvements were superior in late-onset depression with initial ↓NIRS activation in the right ventrolateral PF area.
Downey et al.[2] (51)	UK	MDD: 18	NA	NA	NA	NA	Ketamine	4 detectors and 24 sources MiniNTS (UCL)	NA	VFT	PF	PF cortical responses appear to be ↓in the severely depressed and additionally suppressed by ECT treatment
Schiffer et al. (88)	US	MDD: 10 (5/5)	35.1 ± 7.1	DSM-IV	HAMD HAMA	Depression and anxiety symptoms	4-min near-infrared (NIR) light photobiomodulation	INVOS system (Somanetics)	4 weeks, 2 follow-ups (weeks 2, 4)	Lateral visual field stimulation	PF	NIR-PBM may have uses for depression treatment

(Continued)

TABLE 4 | Continued

Source.	Country	Sample size (male/female)	Age (mean ± standard deviation)	Diagnostic criteria instrument	Psychopathology measure for treatment response	Treatment outcome	Medication (mg)/ treatment	NIRS device/no. of channels	Duration/no. of follow-up	Paradigm	Brain area	Main finding
Eschweiler et al. (89)	Germany	MDD: 12 (4/8)	57.0 ± 8.0	DSM-IV	HAMD BDI	Depressive symptoms	(PBM) treatment to left/right forehead Repetitive transcranial magnetic stimulation (rTMS)	Four-site NIRS	4 weeks, 4 follow-ups (weeks 1, 2, 3, 4)	Arithmetic and mirror-tracing tasks	PF	Low local hemodynamic responses predict clinical benefits of rTMS.

[2]*This article can also be found in the summary in* ***Table 2***.

[3]*This article can also be found in the summary in* ***Table 3***.

MDD: major depressive disorder; HC, healthy control; HAMD, Hamilton depression rating scale; VFT, verbal fluency task; PF, prefrontal; T, temporal, F, frontal; NA, not available; MINI, Mini-international neuropsychiatric interview; DSM, Diagnostic and Statistical Manual; ICD, International Classification of Diseases; MMSE, Mini-Mental State Examination; SASS, school and staffing survey; POMS, profile of mood states; STAI, state-trait anxiety inventory; MADRS, Montgomery–Asberg depression rating scale; QIDS-SR, quick inventory of depressive symptomatology-self report; DSRS, dementia severity rating scale; HAMA, Hamilton anxiety rating scale.

schizophrenia. The majority of the studies adopted the VFT, except for 12 studies that utilized paradigms such as WFT, RPS task, image-recall task, resting state measurements, TMT, stop-signal task, WM task, MDT, conversation task, emotional Stroop task, and the use of self-report measures such as the rumination response scale (RRS) and visual analog scales (VAS). The NIRS instruments utilized markedly differed from study to study.

According to our assessment of the authors' conclusions, almost every study discovered that oxy-Hb concentration values were negatively correlated with the degree of total depressive symptoms [HAMD, geriatric depression scale (GDS), Montgomery–Asberg depression rating scale (MADRS), quick inventory of depressive symptomatology-self rated (QIDS-SR), etc.] and Uemura et al. even demonstrated that such a significant negative correlation was still present after correcting for confounding factors such as gender, age, and educational history (56). Such a negative correlation was further supported by Wang et al. who illustrated that the recurrent MDD group had notably diminished increases in oxy-Hb compared to first-episode MDD patients (69). Additionally, Nishizawa et al. utilized the emotional Stroop task to evaluate brain regions associated with the severity of depression. They found that hyperactivated oxy-Hb was seen in the left frontal cortex upon contact to adverse stimuli, but no meaningful differences were established between the depressed compared with the healthy upon exposure to favorable stimuli (60). Additionally, the degree of depression was contrariwise associated with an evoked wave in the left upper frontal cortex following exposure to favorable stimuli. Akiyama et al. also found that there was significant decrease in left lateral frontotemporal activation in active depressed patients as compared to those who still had residual depressive symptoms despite MDD remission (61), further suggesting the possibility of NIRS's ability to differentiate depression of varying severity.

While such a noteworthy negative correlation was established in many papers, there were some studies that observed no significant correlations (70) and even a significant positive association (68) between HAMD scores and oxy-Hb concentrations. Such results could have been due to methodological limitations that were faced by these studies. For example, in the study by Tsujii et al. (70), the sample size was small. Thus, such slight difference in the hemodynamic response in the left temporal region among the two groups was not statistically different. A type II error might be a possibility if the size of the sample was enlarged, and the dissimilarity between the healthy and depressed in the left channels might be meaningful. Additionally, Liu et al. (68) argued that such inconsistency in results could have been due to the dynamic process of psychiatric disease development; therefore, there are some findings that may be momentary markers and not persistent markers.

Apart from the general negative association between the oxy-Hb concentration values and the degree of total depressive symptoms, many of the studies looked into a variety of individual depressive symptoms, such as suicidal thoughts, sleep quality, psychomotor retardation, obsessive–compulsive

symptoms, cognitive functioning [assessed by the Mini-Mental State Examination (MMSE), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) test battery, etc.], and many others.

Suicidality is often observed in MDD patients. Suicidal ideation usually precedes a suicide attempt. Pu et al. investigated individuals prior to any attempt (73), while Tsujii et al. explored responses after an attempt (65). Pu et al. (73) found that the hemodynamic variations in the OFC, FPC, and DLPFC negatively correlated to the degree of suicidal thinking in depressed individuals; Tsujii et al. (65) revealed that the suicide attempters (SAs) revealed a reduced hemodynamic response in the left precentral gyrus compared to the nonattempters (NAs) and HCs, and the hemodynamic responses in the right middle frontal gyrus were negatively correlated with aggression and hopelessness in the SAs but not in the HCs and NAs.

Nishida et al. examined the relationship between sleep quality and oxy-Hb concentrations (71). They detected a negative relationship between average oxy-Hb concentration variations while performing the task and Pittsburgh sleep quality index (PSQI) scores. This illustrated that self-rated sleep disturbances were related to a reduced left prefrontal reactivity when performing VFT in MDD patients, thus indicating that the reactivity of the prefrontal region was vulnerable to sleep disorders.

Psychomotor retardation is another classic symptom of MDD. In Tsujii et al., psychomotor retardation on the HAMD showed noteworthy affirmative association with average oxy-Hb changes in the right temporal region in the MDD patients with melancholic features (MDD-MF) but displayed a suggestive negative association with average oxy-Hb changes in the middle to left temporal region in the MDD patients with non-melancholic features (MDD-NMF) (70). These findings were consistent with earlier functional neuroimaging studies that determined that MDD with psychomotor retardation is related to reduced blood flow in patients with MDD-NMF, unlike those suffering from MDD-MF. Thus, this suggested that the pathophysiology of non-melancholic MDD is distinctive from melancholia. Specifically, the pathophysiology of melancholia could be linked to the right temporal region. MDD patients often showed impaired inhibitory control. In the study by Akashi et al. fluctuations in oxy-Hb concentrations in MDD patients in the superior frontal probes were associated with impairments in performance in inhibitory controls (79).

With regard to patients' functioning, while no statistical significance was established with the degree of obsessive–compulsive symptoms (68), average oxy-Hb concentrations were significantly positively interrelated with the global assessment of functioning (GAF) scores (57, 63) and SASS scores (47, 64, 66). For SASS in particular, observational changes in bilateral ventrolateral prefrontal cortex activity and the anterior portion of the temporal cortex (VLPFC/aTC) were associated with gains in social adaptability in MDD patients, although the MDD groups revealed less activation than the HCs in the VLPFC/aTC. Additionally, when Tsujii et al. assessed quality of life (QOL) using the Medical Outcomes Study 36-item short-form health survey (SF-36), hemodynamic responses in the prefrontal region were positively associated with the role emotional domain scores for the MDD-MF patients. Comparatively, MDD-NMF patients showed no noteworthy correlations (67). This indicated that while lower functioning was associated with lower oxy-Hb concentrations, patients with MDD-MF had qualitatively distinctive prefrontal impairment features associated with emotional role functioning unlike patients with MDD-NMF.

Pu et al. assessed coping styles with the coping inventory for stressful situations (CISS) and discovered that MDD patients were more possibly using an emotion-oriented coping style as compared to using task-orientated and avoidance-orientated coping. Emotion-oriented coping style was also positively associated with subjective assessments of the degree of depression (74). Task-orientated coping was positive correlated with regional hemodynamic changes in various prefrontal regions. The findings suggested that the hemodynamic responses in the various prefrontal regions when performing VFT imply patients who suffer from MDD utilize task-orientated coping when depressed.

Apart from the use of oxy-Hb concentration measures, three papers also assessed FC (52, 54, 72). First, in patients with affective disorders, Zhu et al. (54) showed altered patterns of FC, representing far greater disorganized patterns of correlation maps, by outlining and examining four FC types: the intraregional connectivity (SC-I), the intrahemispheric connectivity (SC-II), the symmetrically interhemispheric connectivity (LC-I), and the asymmetrical interhemispheric connectivity (LC-II). This showed that patients suffering from affective disorders indicated considerably weaker intraregional connectivity and symmetrical interhemispheric connectivity in the IFG as compared to HCs and that such disruptions in the right IFG are correlated with problems in emotional reactions and reducing negative thinking in affective disorder patients. Second, similar to previous results found by many others, Rosenbaum et al. (72) also found that the subjects who were depressed revealed increased FC in a left frontoparietal cortical network at the resting state and decreased FC in a left frontopolar cortical network while doing a performance task. However, when these relations were calculated on behalf of the separate diagnostic groups, these correlations were congruent only in the non-depressed group and varied in the depressed group, thus implying that these results might be more related to depression status than to the degree of symptoms per se. In comparison to controls, the depressed individuals indicated an exceedingly connected network with a left hemispheric focus during the resting-state condition, and this result was inferred to be related to rumination. This relationship was further examined by Rosenbaum et al. (52). Though ruminative thoughts were negatively associated with FC in the cortical areas of the default mode network (DMN), mind-wandering displayed positive correlations with FC.

Moreover, Okada et al. illustrated hemodynamic changes using brain response patterns (55). The response patterns were cerebral responses to the MDT. During the MDT, the overall response areas were analyzed by using a planimeter as the areas

demarcated by the total Hb response curves and the baseline. The area deviation ratio was calculated by dividing the difference between the blood volume response areas of the right and left hemispheres by the hemisphere blood volume response area showing a lower response. When the ratios were higher (range 4–44), response patterns were classified as dominant hemispheric response patterns in normal subjects or non-dominant hemispheric response patterns in MDD patients. They demonstrated that about 50% of patients (12 out of 24 males and 4 out of 12 females) showed a "non-dominant hemisphere response pattern," which was not seen in healthy subjects, and this "non-dominant" hemisphere could become dominant during depression. Furthermore, significant negative associations were found between the oxy-Hb and the age of onset in six channels but there were no noteworthy associations between age and oxy-Hb at the period of assessment.

Use of fNIRS to Monitor Treatment Response

All 16 studies included in **Table 4** were case–control studies. Eleven studies were performed in Japan, one study in China, one study in Uzbekistan, one study in United Kingdom, one study in the United States, and one study in Germany. The combined sample throughout the 16 studies comprised of 282 MDD patients. Every patient met the MDD criteria of DSM-IV, DSM-5 or ICD-10. The majority of the studies examined the correlation of cerebral hemodynamic changes with improvements in the depressive symptoms of patients, while five studies (47, 48, 50, 77, 81) looked at either social and/or cognitive functioning of the patients as the treatment outcome.

Almost all patients responded favorably to treatment, as the articles reported significant improvement in social functioning or a decrease in depressive symptoms. However, the patients in the study by Takamiya et al. showed no meaningful differences in depressive scores following treatment in both the high- and low-dose groups (84). In group comparisons, there were significant outcomes on NIRS signals for high doses of antidepressants compared with low doses. This highlighted that the dose-dependent influence of antidepressants on the NIRS signal ought to be considered while understanding NIRS data. The most commonly used paradigm to stimulate cognitive activity was the VFT. One study (77) used a rock, paper, scissors (RPS) task. Two studies (87, 89) used mental arithmetic tasks, and another study (88) utilized a lateral visual field simulation. The prefrontal brain regions were examined in all of these studies.

Measuring NIRS signals before starting treatment has been shown to predict treatment progress in medication-naive patients suffering from MDD (46). In the paper by Tomioka et al. (46), the average oxy-Hb concentration values in the prefrontal and temporal regions during the VFT were significantly correlated with improvements in HAMD scores after treatment with antidepressants. The group comparisons revealed that the mean oxy-Hb concentration variations in the temporal and prefrontal cortices while performing VFT were considerably reduced in the depression group compared to HC group. This result concurred among other studies (18) that explored the uses of NIRS for the treatment of MDD.

Similar to the study by Tomioka et al. (46), Yamagata et al. also conducted a study on the application of NIRS in medication-naive participants with MDD (82). The latter study highlighted the noninvasive nature of NIRS as they conducted repeated measurements at short intervals (three follow-ups at 4-week intervals). According to their findings, they found a substantial adverse association between mean oxy-Hb concentration values in the significant cluster (at week 4) and differences in HAMD scores from weeks 4 to 8 ($r = -.73$) and from weeks 8 to 12 ($r = -.63$). As such, they also concluded that NIRS could be used in predicting MDD patients' response to antidepressant treatment.

Ohtani et al. looked at social adaptation in their study, and in their 6-month follow-up, they found that NIRS signals could be a predictor for the longitudinal assessment of social adaptation (47). Longitudinal increases in temporal and prefrontal regions were shown to be correlated with improvements in social adaptation for patients, although there was no significant change in the severity of their clinical symptoms.

On the other hand, Pu et al. investigated patients' social functioning in their study (81). The pretreatment NIRS activation in the prefrontal region was found to be positively associated with pretreatment social adaptation self-evaluation scale (SASS) scores and negatively correlated with increase in SASS scores after 8 weeks. Their discoveries suggested that NIRS signals would be useful in evaluating social functioning in patients afflicted by late-onset depression (prior to treatment) and predicting the improvement in their social functioning after treatment. Furthermore, fNIRS can be used to predict the treatment response of not only antidepressants but also other novel treatments, such as neurostimulation (50, 85, 89), music therapy (49), and animal-assisted therapy (83).

Quality Assessment of the Included Studies

The NOS was used to assess the risk of bias in the included studies, and the results are shown in **Table 5**. No study had a complete score of eight stars. More than two-thirds of the studies had acceptable quality, of which 49 studies were awarded four or more stars, with 11 studies given seven stars, 14 studies given six stars, 19 studies given five stars, and 5 studies given four stars. Fifteen studies fared more poorly in quality with less than four stars, of which 3 studies were given three stars, 11 studies were given two stars, and 1 study was given one star.

DISCUSSION

Currently, there is no specific test or biomarker for diagnosing and monitoring the progression of depression. MRI studies have shown reduced size in the medial and lateral prefrontal cortex, amygdala, hippocampus, and striatum; increased functional activity in the medial prefrontal cortex, amygdala, and hippocampus; and decreased activity in the lateral prefrontal cortex and striatum in depressed subjects compared with HCs (90). Nevertheless, the applicability and utility of MRI in clinical practice is limited due to the high cost, need to stay immobile, presence of noise while scanning, long scanning duration, and

TABLE 5 | Risks of bias within studies.

	Selection				Comparability	Exposure		
Study sources	**Is the case definition adequate?**	**Representative of cases**	**Selection of controls**	**Definition of controls**		**Determination of exposure**	**Same method for determining cases and controls**	**Nonresponse rates**
Suto et al. (18)	★			★	★	★	★	
Usami et al. (86)	★					★		
Takei et al. (63)	★		★	★	★★	★	★	
Shimodera et al. (37)	★		★	★	★	★		
Ma et al. (38)	★		★	★	★★	★	★	
Takizawa et al. (39)	★		★	★	★★	★	★	
Zhu et al. (54)	★		★	★	★★	★	★	
Matsubara et al. (42)	★		★	★	★★	★	★	
Pu et al. (81)	★				★	★	★	
Akashi et al. (43)	★			★	★	★	★	
Downey et al. (51)	★				★	★	★	
Gao et al. (44)	★		★	★	★	★	★	
Pu et al. (64)	★		★	★	★★	★	★	
Tsujii et al. (65)	★		★	★	★★	★	★	
Pu et al. (74)	★		★	★	★	★	★	
Schecklmann et al. (45)	★			★	★	★	★	
Tsujii et al. (67)	★			★	★	★	★	
Liu et al. (68)	★		★	★	★★	★	★	
Wang et al. (69)	★			★	★	★	★	
Tsujii et al. (70)	★		★	★	★	★	★	
Satomura et al. (80)	★				★	★		★
Nishida et al. (71)	★			★	★	★	★	
Rosenbaum et al. (72)	★		★	★	★	★	★	
Pu et al. (73)	★		★	★	★	★	★	
Koseiki et al. (78)	★					★		
Pu et al. (74)	★		★	★	★	★	★	
Schiffer et al. (88)	★				★	★		
Eschweiler et al. (89)	★		★	★	★	★	★	
Tomioka et al. (46)	★		★	★	★			
Pu et al. (25)	★	★		★	★★	★	★	
Matsuo et al. (26)	★			★	★★	★	★	★
Onishi et al. (77)	★					★		
Rosenbaum et al. (52)	★			★	★	★	★	★
Ohtani et al. (47)	★			★	★★	★	★	★

(Continued)

TABLE 5 | Continued

	Selection				Comparability	Exposure		
Study sources	**Is the case definition adequate?**	**Representative of cases**	**Selection of controls**	**Definition of controls**		**Determination of exposure**	**Same method for determining cases and controls**	**Nonresponse rates**
Herrmann et al. (27)	★	★		★	★	★	★	
Yamagata et al. (82)	★					★		
Kondo et al. (53)	★			★	★	★	★	★
Shoji et al. (28)	★			★	★	★	★	
Kinoshita et al. (29)	★					★		
Kito et al. (30)	★			★	★	★	★	
Kawano et al. (76)	★	★				★		
Matsuo et al. (31)	★	★				★		
Zhu et al. (54)	★		★	★	★	★	★	
Okada et al. (55)	★			★	★	★	★	
Uemura et al. (56)	★					★		
Matsubara et al. (32)	★			★	★	★	★	
Masuda et al. (48)	★					★		
Kinou et al. (57)	★			★	★	★	★	
Yamagata et al. (58)	★			★	★	★	★	
Koike et al. (33)	★				★			
Feng et al. (49)	★		★	★	★	★	★	
Aoki et al. (83)	★					★		
Noda et al. (59)	★			★	★	★	★	
Hirano et al. (50)	★			★	★	★	★	
Azechi et al. (34)	★			★	★	★	★	
Nishizawa et al. (60)	★			★	★	★	★	
Takamiya et al. (84)	★					★		
Matsuo et al. (35)	★			★	★★	★	★	
Ohta et al. (36)	★			★	★	★	★	
Akiyama et al. (61)	★		★	★	★	★	★	
Ohi et al. (62)	★			★	★	★	★	
Shinba et al. (85)	★					★		
Akashi et al. (79)	★							
Liske et al. (40)	★		★	★		★		★

risk of inducing claustrophobia. These considerations may be especially relevant for depressed patients who may be emotionally sensitive and thus find it harder to tolerate being confined to a tight space with recurrent noise for a prolonged period. Compared to fMRI, fNIRS is less expensive, fast to operate, quiet, and more portable and, thus, able to perform the scan in the clinic or ward directly and allows body movement in a naturalistic environment (91). However, some of its inherent limitations may influence the accuracy and eventual applicability in clinical practice. For instance, its spatial resolution and penetration depth are limited, which reduces access to the subcortical regions that have a pertinent role in psychiatric disorders. As fNIRS is established on the principles of neurovascular coupling, it is affected by blood pressure, hemoglobin level, blood circulation, vasculature, and carbon dioxide concentration (92, 93). Nevertheless, a meticulous study design, enhanced fNIRS techniques, and statistical processing could potentially mitigate these shortfalls.

This is the first systematic review on the application of fNIRS to depression that intended to provide an overview of the up-to-date information of 3 pertinent clinical questions: (i) the usefulness of fNIRS in differentiating depressed from healthy individuals, (ii) the correlation of fNIRS signals with depression symptomatology, and in turn (iii) monitoring treatment response. Answers to these questions are essential in ascertaining the applicability and utility of fNIRS as a tool to assist the diagnosing of depression, predicting clinical symptoms of patients, monitoring the treatment response and disease progression, and for prognosticating the disease. Based on the combined findings of the reviewed papers, most of the studies were conducted in Japan, although there has been an increasing trend for studies being conducted in China, Germany and other countries. The number of fNIRS studies has also exponentially increased year on year, suggesting an increased recognition of this technology in mainstream science. The most commonly used active paradigm is the VFT. This is not surprising as the VFT is quite a common bedside neuropsychological test that has been extensively utilized to ascertain executive function and language content (94) and is easy to perform within a short period, though it is imperative to realize that the performance on the VFT can be influenced by culture. Participants are obliged to state as many unique words that begin with a particular letter or are from a semantic category in a restricted time (up to a minute usually). Cognitive dysfunction occurs in patients who are depressed, of which impairments in executive ability is one of the core domains, in addition to learning/memory, attention/concentration, and processing speed (95). As a validated test to ascertain executive function, the VFT is able to elicit distinct differences in performance and neuroimaging responses between depressed and healthy people (23).

Based on the findings of this systematic review, the fNIRS signals show promise as an ideal biomarker in addressing our review's three fundamental questions: do fNIRS signals differentiate depressed from nondepressed individuals, correlate with specific depressive symptomatology, and in turn aid the monitoring of treatment responses (regardless of whether the treatment is a medication or another treatment modality)? The findings of our systematic review demonstrated a consistent pattern of attenuated pre-frontal stimulation in patients with depression while conducting cognitive tasks using the NIRS method, and cerebro-hemodynamic changes are associated with particular symptoms encountered by patients and treatment responses. However, most studies did not include sensitivity and specificity data that compared depressed patients from HCs, which would be important to elucidate the validity of fNIRS as a diagnostic tool. Thus, future studies may need to consider standardizing their analysis such that comparisons across studies can be made. The correlation of brain signals with clinical symptoms such as suicidal ideation, sleep quality, and psychomotor retardation may facilitate more in-depth personalized profiling of the individual's depression and may be able to better identify risk issues in management (e.g., self-harm, suicide, neglect). Symptom identification can be especially helpful when patients are not forthcoming about their symptoms, which makes it difficult to ascertain their risks. However, the majority of the selected studies had small sample populations, thereby reducing the power of the study, and there were variations in study methodologies, including the device and paradigm used, which limited the ability to effectively combine or compare results. One important aspect of fNIRS as a biomarker would be its potential to prognosticate the disease, i.e., whether the patient will improve or worsen with time. Nevertheless, the existing studies were either cross-sectional or had short longitudinal follow-ups, which made it difficult to address this issue. Longer longitudinal studies of at least 6 months to a year would be beneficial, considering that most depressive episodes last for at least a few months. Furthermore, longitudinal studies can help to elucidate whether the hemodynamic responses recorded by NIRS are a state- or trait-dependent marker of depression, as the current evidence is conflicting and confounded by several reasons.

Most studies scanned their subjects 2 weeks to 1 month after starting antidepressants, and then scans were performed monthly. Having more frequent fNIRS measurements in longitudinal studies (e.g., weekly) can provide us with a better understanding of brain dynamics and minimize the influence of confounding factors. The majority of studies to date have examined only the temporal and frontal regions. It may thus be worthwhile to expand the assessment to other brain regions, such as the occipital and parietal regions, especially because these two regions have also been implicated in depression (96, 97). Furthermore, most studies examined hemodynamic changes in discrete brain regions, with only a few studies investigating FC aberrations and even fewer ascertaining effective connectivity across brain regions (97). Connectivity studies allow a more holistic approach to how various brain areas interactively function and may represent another means of examining the disease state. Most of the studies involved patients on medication, and those with medication-naive patients had small sample sizes. Thus, we are unable to exclude the possibility that antidepressants interfered with the signal results. Studies have highlighted that antidepressants can affect

the NIRS signals (84). Therefore, it may be worthwhile to have more studies involving scans of medication-naive patients, though this can be practically challenging at the ground level.

An encouraging application is combining different neuroimaging modalities to allow a more accurate and comprehensive surveillance of the neurophysiological changes. This can include combining fNIRS with EEG, fMRI, PET, or diffusion tensor imaging (DTI) to complement fNIRS. This can in turn improve the spatial and temporal resolution and thereby increase sensitivity and specificity. Nevertheless, the applicability of combining modalities such as fMRI and PET is limited due to the immobile and bulky apparatus, and although EEG is portable, use of EEG increases the setup time, and the increased noise-signal ratio with artifacts may reduce its accuracy. On the other hand, integrative approaches that combine various modalities of biomarkers such as genetic, neuroimaging, and neuropsychological data that are analyzed using high-dimensional multivariate statistical methods are gaining prominence and may more comprehensively facilitate disease diagnostics and prediction (98).

Our review has a number of limitations. First, we selected observational studies that had small sample sizes, thereby increasing the risk of selective and confounding biases and reduced power due to small effect sizes. However, based on our search, there were no randomized controlled trials of patients with depression using fNIRS. Second, we only included studies in English and searched in four databases. We recognize that there are an increasing number of fNIRS studies conducted in China and other countries that may not be published in English and indexed in these four databases. Thus, this potentially led to publication bias. Third, we also included conference proceedings, and several details of these datasets were unavailable from the source. However, it was essential to include them to reflect the spectrum of studies being performed in the current fNIRS research landscape. Regardless, this review has strengths in being the first to combine three pertinent questions pertaining to fNIRS as a biomarker, and there has not been an updated systematic review of fNIRS in depression since 2015. This study is a timely update of the current landscape of using fNIRS on depressed patients, given the increasing availability of this technology worldwide.

In conclusion, there is a good amount of evidence in the current literature to suggest fNIRS as a diagnostic and predictive tool for MDD, and it has shown consistent hemodynamic patterns in depressed patients compared to healthy individuals. There is also an increasing amount of studies on fNIRS in depression, indicating the increased recognition of this technique in the study of depression. Future studies involving larger sample sizes, standardized methodology, examination of more brain regions in an integrative approach, and longitudinal follow-ups are needed to advance the use of fNIRS in psychiatric clinical practice and research.

AUTHOR CONTRIBUTIONS

Conceptualization: CH. Data extraction and review: LL, AL, NC, RT, SL. Writing: CH. Review and manuscript amendment: LL, AL, NC, RT, SL, RH.

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REVIEW
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Circular RNA in Schizophrenia and Depression

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Schizophrenia (SZ) and depression (DEP) are two common major psychiatric disorders that are associated with high risk of suicide. These disorders affect not only physical and mental health, but they also affect the social function of the individual. However, diagnoses of SZ and DEP are mainly based on symptomatic changes and the clinical experience of psychiatrists. These rather subjective measures can induce misdiagnoses and missed diagnoses. Therefore, it is necessary to further explore objective indexes for improving the early diagnoses and prognoses of SZ and DEP. Current research indicates that non-coding RNA (ncRNA) may play a role in the occurrence and development of SZ and DEP. Circular RNA (circRNA), as an important component of ncRNA, is associated with many biological functions, especially post-transcriptional regulation. Since circRNA is easily detected in peripheral blood and has a high degree of spatiotemporal tissue specificity and stability, these attributes provide us with a new idea to further explore the potential value for the diagnosis and treatment of SZ and DEP. Here, we summarize the classification, characteristics, and biological functions of circRNA and the most significant results of experimental studies, aiming to highlight the involvement of circRNA in SZ and DEP.

Keywords: schizophrenia (SZ), depression (DEP), circular RNA (circRNA), biological function, expression, epigenetic characteristics

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INTRODUCTION

Among the great quantity molecular regulatory factors affecting gene expression, non-coding RNA (ncRNA) is known to be a class of important regulatory factors. NcRNA refers to a kind of RNAs that do not encode proteins, but can participate in genetic regulatory processes at multiple levels, including interactions with DNA, RNA, and proteins through a variety of mechanisms (1). Gene regulation of ncRNAs *via* epigenetics plays an important role in many major diseases, such as schizophrenia (SZ) and depression (DEP) (2–4). At present, SZ is considered to represent an umbrella of genetic diseases that are mainly characterized by a certain number of genetic-interaction networks and their differential clinical symptoms (5). For DEP, studies have proposed a pathogenic model that involves interactions among the environment, genetics, and epigenetics (6). Moreover,

the high rate of comorbidity observed in psychiatric disorders indicates a large genetic correlation between each other, and the existence of shared molecular mechanisms involved in the occurrence and development of diseases (7). Sha Liu et al.'s genome-wide association study (GWAS) of seven psychiatric disorders (8), including SZ, DEP, bipolar disorder (BD), autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), anxiety disorder (ANX), and neuroticism, also confirmed this. And they further found that chromosomes 5q14.3, 11q23.2, and 7p22.3 are the three genomic regions with the highest pleiotropic effects, suggesting that genetic factors have significant influence on SZ and DEP. Although few in-depth studies have been published on specific signaling pathways and networks regulated by ncRNAs in SZ and DEP, especially in terms of circular RNAs (circRNAs), it is possible that further elucidation of ncRNAs may be useful for better understanding the pathogenesis of SZ and DEP.

CircRNA, as an important type of ncRNA, forms an annular closed structure by undergoing reverse splicing, which was first discovered in plants in the 1970s (9). Since it was originally found only in a few transcriptional genes, circRNAs were considered to be a product of splicing errors from low-abundance transcriptional processes. However, this hypothesis has since been refuted. CircRNAs are widely present in human cells, some of which are expressed at as much as 10-fold higher levels compared to those of their linear isomer counterparts (10–12). CircRNA differs from linear RNA in that it is directly joined together to form a covalently closed structure without a 3′ tail and 5′ cap element (13–15). Due to the circular structure of circRNA, it is not easily degraded by RNA enzymes and also exhibits a high stability within cells (16, 17), enabling it to stably perform cellular functions over a longer period of time.

At present, the diagnostic criteria for SZ and DEP are still based solely on symptomology. Although diagnostic criteria—such as from the Diagnostic and Statistical Manual of Mental Disorders (Fifth Edition) (DSM-5) of American Psychiatric Association and the International Classification of Diseases (ICD-11)—are currently available, diagnoses and evaluations of treatment efficacies for SZ and DEP still primarily rely on clinical meetings with psychiatrists and lack objective physiological, biochemical, and pathological indicators. Hence, this more subjective diagnostic system can easily cause misdiagnoses and missed diagnoses. The above characteristics of circRNAs provide us with a novel approach to explore the biological basis for the occurrence and development of SZ and DEP. Therefore, the focus of this review is on circRNAs and their possible involvement in both SZ and DEP.

METHODS

We searched PubMed and GeenMedical for publications over the period of 1976 to 2019 and included the following the key phrases during searching: "circular RNA and neuropsychiatric disorders" "circular RNA and schizophrenia" "circular RNA and depression" and "circular RNA and biological function". Only papers in English were used in the preparation of this review. The references obtained were screened by the authors to determine which ones would be included for discussion in the present review.

OVERVIEW OF CIRCRNAS

Classifications and Looping Mechanisms of circRNAs

CircRNAs are divided into the following four categories: (1) whole-exonic-type circRNAs (EcircRNA); (2) circRNAs with introns and exons (EIcirRNA) (18); (3) lasso-type circRNAs composed of introns (ciRNA); and (4) circRNAs produced by cyclization of viral RNA genomes, ribosomal RNAs, small-nuclear RNAs, and transfer RNAs (tricRNA) (**Figure 1**). Based on their multiple biological mechanisms, circRNAs can retain the ability to either stay inside the nucleus or undergo nuclear export. This selective retention is important for determining the biological roles of different circRNAs and further provides us with research ideas for exploring the potential of circRNAs in the occurrence and development of SZ, DEP, and many other diseases. CircRNA is generally considered to be the result of reverse splicing. This indicates that the downstream 5′ donor site is covalently cyclized with the upstream 3′ receptor site (19). At present, there are three main looping mechanisms of circRNAs.

Cable-Tail-Insertion Cyclization Dependent on a Shearing Body

In eukaryotic cells, the formation of circRNAs by exon cable-tail-insertion cyclization (20, 21) is the earliest and most common way of looping, and more than 80% of circRNAs contain exons. Exon cable-tail-insertion cyclization relies on a typical shearing-body mechanism. On the mRNA precursor, the downstream 5′ donor site of the exon is catalyzed to link to the upstream 3′ receptor site by successively assembling small-nuclear ribosomal proteins to form cable-tail-insertion cyclization. Eventually, RNA is formed by further shearing. However, the specific mechanism underlying this process remains unclear.

Promotion of circRNA Formation by Cyclic-Acting Elements

Partial introns on both sides of circRNA exons contain reverse complementary sequences that can form RNA double chains side by side at shear sites (22). Finally, two kinds of different circRNAs with or without introns are formed by variable shearing. In general, a nucleotide sequence of 30–40 nt in length can promote the cyclization of circRNAs. Furthermore, exons inside can also compete for RNA pairing and ultimately form different types of circRNAs *via* variable shearing.

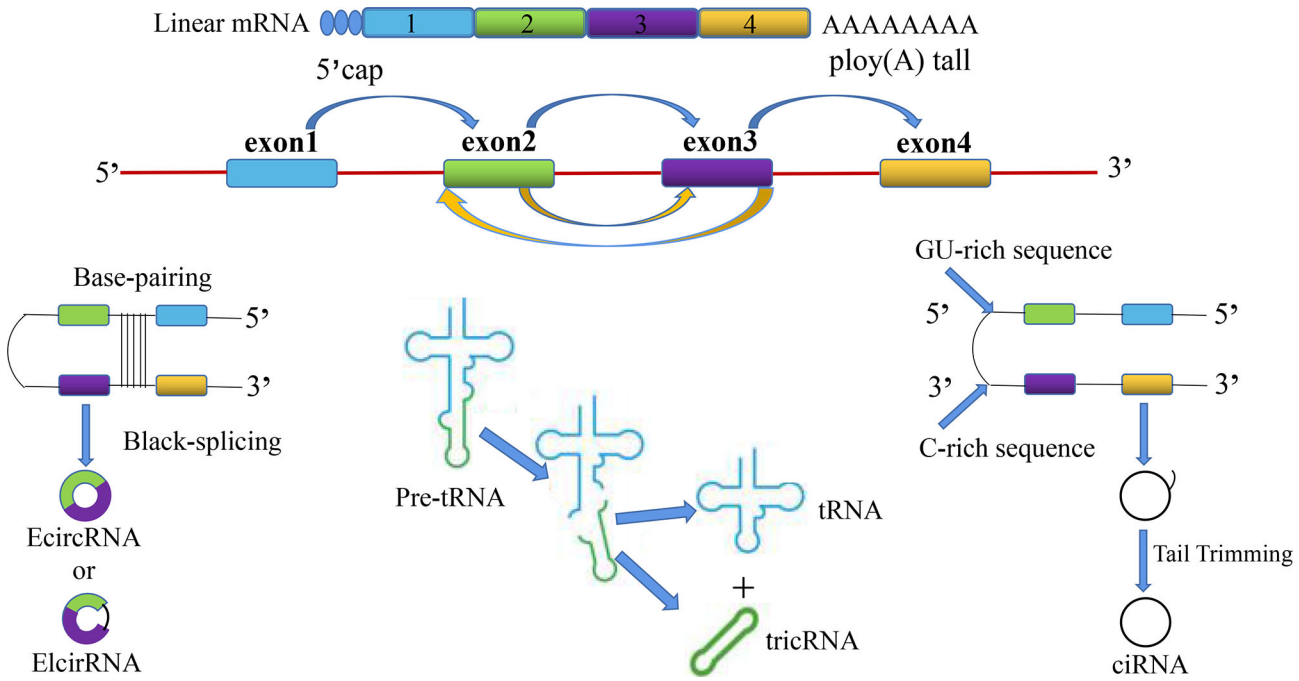

FIGURE 1 | The overview diagram shows four biosynthetic pathways related to the source of circular RNAs: (1) whole-exonic-type circRNAs (EcircRNA); (2) circRNAs with introns and exons (ElcirRNA); (3) lasso-type circRNAs composed of introns (ciRNA); and (4) circRNAs produced by cyclization of viral RNA genomes, ribosomal RNAs, small-nuclear RNAs, and transfer RNAs (tricRNA).

Regulation of circRNA Formation by RNA-Binding Proteins (RBPs)

In *Drosophila melanogaster*, MBL protein (23) can promote the formation of circRNAs by binding to the introns flanking an exon. Some studies (24) have shown that hundreds of circRNAs are regulated during the process of the epithelial-mesenchymal transition (EMT), and the looping formations of more than one-third of circRNAs are dynamically regulated by the splicing factor, Quaking (QKI). The effect of QKI on circRNA abundance depends on the binding motif of the QKI intron. Critically, the addition of a QKI motif is sufficient to re-induce the formation of circRNA from a linear splicing transcript. In contrast to the effect of QKI protein, high expression of ADAR1 protein (22) can inhibit the formation of circRNAs by destroying the RNA pairing flanking the exon.

Some exon-derived circRNAs are localized in the cytoplasm and contain both ribosome-binding sites and evolutionarily conserved termination codons. Exon-derived circRNAs also share the start codon of the host mRNA, which indicates that hundreds of endogenous circRNAs have translation potential (25), and some of their translation products can carry out functions. Previous studies have confirmed that many (31 out of 132) proteins encoded in ribosomal-related circRNAs (ribo-circRNAs) contain at least one identifiable protein domain. Furthermore, circRNA, such as circMbl, can be translated in a cap-independent manner *in vitro* (26). The same phenomenon is found for circ-ZNF609, which is associated with multiple ribosomes. Circ-ZNF609 translates protein in a splicing-dependent and cap-independent manner. This gene controls the proliferation of primary myoblasts and is the key regulatory factor of their growth (27).

Molecular Biological Functions of circRNAs

Efficient microRNA Sponges

One of the most important molecular biological functions of circRNAs is that they indirectly affect the expression of target gene mRNAs by binding to microRNAs (miRNAs). As an important regulator at the post-transcriptional level, the function of miRNAs is mainly to downregulate or hinder the translation of target genes (mRNAs) by binding to the complementary 3′-end non-coding region located on the target mRNA (28, 29). As miRNA sponges, most circRNAs play an important role in transcriptional and translational regulation of mRNAs by competing for intracellular miRNAs (30, 31).

Among these miRNA sponges, Cdr1as circRNA is highly expressed and stable. It contains more than 70 conserved miRNA response elements (MREs) of miR-7 and one MRE of miR-671, which are then widely combined with AGO2, a key element of gene silencing. Bezzi and colleagues (32) provided evidence to support the functional association among Cdr1as, miR-7, and its target gene. A plasmid expressing Cdr1as was injected into zebrafish embryos to knockdown the expression of miR-7. This effect was partially rescued by injecting miR-7 precursor. At the

same time, miR-671 indirectly influenced the expression level of the miR-7 by triggering the degradation of Cdr1as. The interaction between circRNAs and miRNAs plays an important role in tumors, such as breast cancer, liver cancer and cervical cancer (31–35). Han et al. found that circMTO1 was significantly downregulated in human hepatocellular carcinoma (HCC) (36). Silencing of circMTO1, a miRNA-9 sponge, in patients with HCC downregulated the expression of the miRNA9 target gene, p21, thereby promoting the proliferation and invasion of HCC cells. Furthermore, the survival time of HCC cells with low expression of circMTo1 was reduced. This study also found that miRNA9 inhibitors could block the silencing effect of circMTO1. These findings suggest that cycMTO1 may be used as an objective evaluation index of survival prognosis of HCC and may be a potential target for liver cancer treatment. In addition to circMTO1 acting as a sponge for miRNA-9 in HCC, hsa-circRNA-103809 (37) was also found to participate in the pathological mechanism of HCC. It could be used as a sponge for miR-377-3p to increase the expression of its target gene [fibroblast growth factor receptor 1 (FGFR1)] and promoted the proliferation and invasion of HCC cells. Moreover, hsa-circRNA-103809 short-hairpin RNA could act as a tumor inhibitor by downregulating FGFR1 in HCC.

Templates for Translating Proteins

N6-methyladenosine (M6A) is the most abundant base modification of RNA. A previous study found (38) that the m6A motif was enriched in circRNAs, and that a single m6A site was sufficient to drive the initiation of circRNA protein translation in human cells. However, this protein translation of circRNA driven by M6A did not function without the following: the activation factor, eIF4G2; the M6A reader, YTHDF 3; enhancement of the methyltransferase, METTL 3/14; inhibition and upregulation of demethylase during heat shock, FTO. Interestingly, further analysis indicated that m6A-driven circRNA translation is widespread.

Interactions With RBPs

Interactions between circRNAs and RBPs are not only related to their own biological occurrences, but they are also involved in the post-transcriptional regulation of RNAs (39). In general, the cell-cycle protein, cyclin-dependent kinase 2 [also known as cell-division protein kinase 2 (CDK2)], interacts with the cell-cycle proteins A and E to facilitate the cell cycle. However, cyclin-dependent kinase inhibitor 1 (p21) can inhibit these interactions and prevent cell-cycle progression. Du et al. (40) found that circ-Foxo3 was highly expressed in non-cancer cells, forming a ternary complex to inhibit the cell cycle by binding to CDK2 and p21. Additionally, silencing of endogenous circ-Foxo3 promoted cell proliferation. Meanwhile, Zhu et al. (41) showed that circZKSCAN1 exerted its inhibitory effect by competitively binding to the RNA-binding protein (RBP), FMRP. This mechanism blocked the binding of FMRP to the β-catenin-binding protein cell cycle and apoptosis regulator 1 (CCAR1) mRNA, and subsequently inhibited the transcriptional activity of the Wnt signal transduction pathway. In addition, downregulation of the RNA-splicing protein, Quaking5, in HCC tissue caused a decrease of circZKSCAN1. These findings suggest the formation of a Qki5-circZKSCAN1-FMRP-CCAR1-Wnt signaling pathway, which may be useful as a potential therapeutic target for HCC (**Figure 2**).

The above results indicate that circRNAs act not only as miRNA sponges by competing with intracellular miRNA to

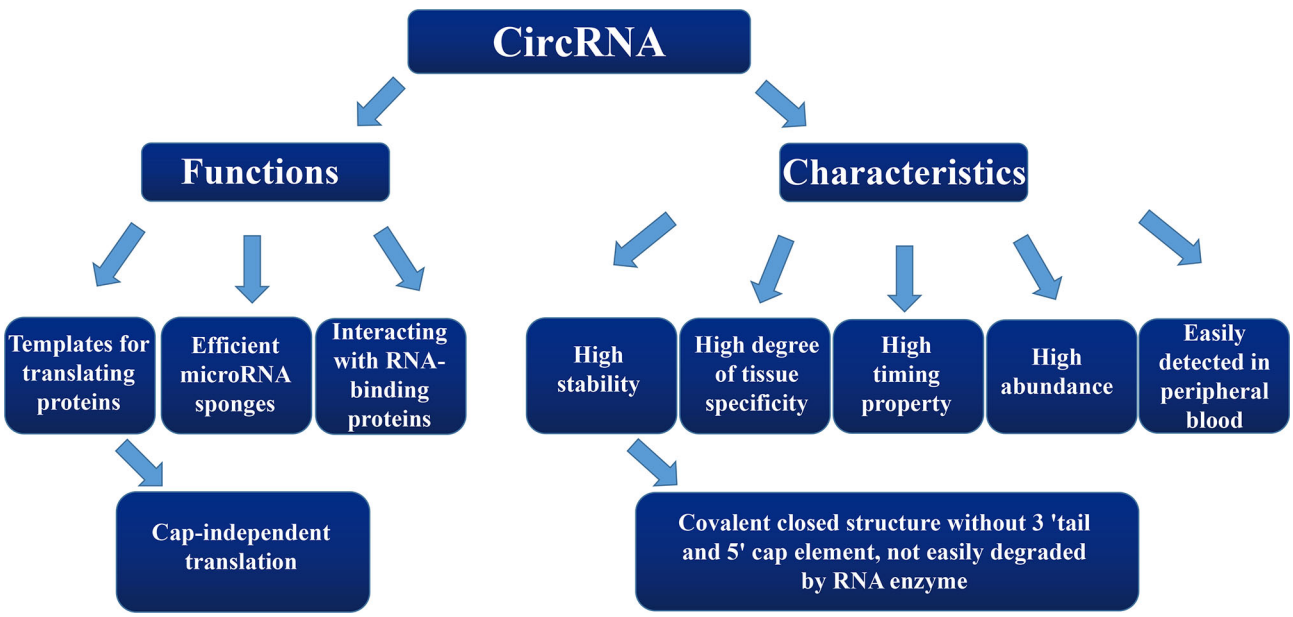

FIGURE 2 | The overview diagram shows multiple biological functions of circular RNAs.

TABLE 1 | Molecular biological function of circRNAs

Author	Main findings
Chen and Sarnow (25)	Hundreds of endogenous circRNAs with ribosome binding sites shared the same start codon of their hosting mRNA, which meant they had translation potential.
Bezzi et al. (32)	Cdr1as knocked down the expression of miR-7, and this effect was partially rescued by injecting miR-7 precursor. At the same time, miR-671 indirectly influenced the expression level of miR-7 by triggering the degradation of Cdr1as.
Yang et al. (38)	N6-methyladenosine (m6A) motif was enriched in circRNA, and a single m6A site was sufficient to drive the initiation of circRNA protein translation in human cells.
Han et al. (36)	Silencing of circMTO1, a miRNA-9 sponge, in patients with HCC could downregulate the expression of miRNA9 target gene p21, thereby promoting the proliferation and invasion of HCC cells.
Legnini et al. (27)	Circ-ZNF609 translated protein in a splicing-dependent and cap-independent manner, which controlled the proliferation of primary myoblasts and was the key regulatory factor of their growth.
Zhu et al. (41)	CircZKSCAN1 exerted its inhibitory effect by competitively binding to the RNA binding protein (FMRP). Therefore, the Qki5-circZKSCAN1-FMRP-CCAR1-Wnt signal axis could be used as a potential therapeutic target for HCC treatment.
Zhan et al. (37)	Hsa-circRNA-103809 could be used as a sponge for miR-377-3p to increase the expression of its target gene [fibroblast growth factor receptor 1 (FGFR1)] and promoted the proliferation and invasion of HCC cells.

CircRNAs can act not only as miRNA sponges by competing with intracellular miRNA to influence the expression of its target genes but also as a translation templates for proteins. They can also combine with RBPs directly and realize the regulation of post-transcriptional RNA, which plays an important role in the occurrence and development of diseases.

influence the expression of target genes, but they also act as translational templates for proteins. More importantly, circRNAs lead to altered gene expression outcomes by splicing their corresponding precursor transcripts, which may play an important role in the occurrence and development of various diseases (**Table 1**).

CIRCRNAS AND SZ

SZ is one of the most common mental disorders, with a global prevalence of 1%, which brings a heavy burden to affected families and society (42). According to the latest research conducted by Huang et al. (43), the prevalence of SZ is 0.559% in China. Symptoms of SZ are divided into positive, negative, and cognitive categories (44, 45). The diagnosis of SZ is mainly based on symptomatic changes and the clinical experience of psychiatrists. However, these rather subjective measures can cause misdiagnoses and missed diagnoses. Therefore, it is necessary to further explore objective indexes to improve the early diagnosis and prognosis of SZ. Since circRNAs are easily detected in peripheral blood and have a high degree of spatiotemporal tissue specificity and stability (46, 47), these properties provide us with a novel approach to further explore the mechanisms underlying the occurrence and development of SZ.

Molecular Mechanisms of circRNAs in SZ

As representative antisense nucleic-acid sponge sequences, circRNAs can competitively bind to miRNAs *via* MREs, which negatively influence the regulatory effects of miRNAs at the post-transcriptional level. MiRNA-320a-3p, miRNA-320b, miRNA-181b-5p, 21-5p, 195-5p, 137, 346, 34a-5p, and hsa-miRNA-206 (48–50) have all been confirmed to play important roles in the occurrence and development of SZ. Wei et al. (51) found that eight miRNAs were upregulated in patients with SZ, as compared to corresponding levels in control participants. Through quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays and a follow-up study on 400 patients with SZ who received regular atypical antipsychotic treatment for 12 months, this study ultimately showed that miR-130b and miR-193a-3p were upregulated in SZ, and the possibility of time-dependent changes were ruled out. Hence, miR-130b and miR-193a-3p may be used as state-independent biomarkers for SZ. A recent study (52) has confirmed that the expression of the network axis composed of miR-30a-5p, its transcription factor, EGR1 and target gene, NEUROD1, changed with the disease state in the patients with SZ before and after treatment. Further receiver operating characteristic (ROC) curve analysis revealed that compared with the single miR-30a-5p, the EGR1-miR-30a-5p-NEUROD1 molecular regulatory axis had higher diagnostic value for predicting SZ. Hence, these findings suggest a possibility for the miRNA-TF-gene regulatory network/axis as a novel diagnostic marker for SZ.

Due to their circular structure, circRNAs are highly conserved across species. Some studies have confirmed that circRNAs are highly expressed in the brain and are differentially expressed in distinct tissues and disease states (53, 54). Moreover, circRNAs exhibit dynamic expression levels during neural development, including neural differentiation and maturation. For instance, circRNAs are upregulated during neuronal differentiation, and compared with their mRNA homologues, they generally exhibit differential expression (55–57). SZ often occurs in late adolescence and early adulthood. Previous studies have suggested that excessive pruning of synapses in the brain during growth and development may contribute to the pathogenesis of SZ (58).

Some studies have suggested synaptic localization of circRNAs. Wolf et al. (56) found that circRNAs in the nervous system were generally upregulated compared to those in the thyroid, liver, and muscle, and that expression varied among different brain regions. Especially in the cerebellum, the expression ratio of circular to linear RNAs was significantly increased, which correlated with a larger number of neurons in the cerebellum compared to that in other brain regions. Furthermore, by measuring high-purity synaptosomal components, this study found that brain-expressed circRNAs had a strong enriching effect at synapses. Another study found that circStau2a was mainly located at synapses, whereas its linear transcript, mRNAStau2, was almost completely localized to the cytoplasm (56). Similarly, circRNA derived from the known neuronal differentiation regulator, RMST, exhibits a high synaptic enrichment rate (59). It has also been found that

during the development of the hippocampus, upregulated circRNAs are produced by the gene locus that simultaneously encodes proteins rich in synapse-related functions. Conversely, no enrichment of encoding of any other functional class of proteins was found in the gene locus that produced a downregulated dynamic expression pattern of circRNAs (29, 60, 61). In summary, the expression levels of circRNAs are regulated by neuronal development. Many circRNAs also change the structures of neurons, which are largely independent of the function of their linear transcripts.

Expression of circRNAs in Postmortem SZ Brains

Our literature review showed that the most extensive studies of circRNAs and SZ have involved the analysis of the expression profiles of circRNAs based on control studies of patients with SZ and the healthy controls matched to them. Mahmoudi et al. used circRNA enrichment sequencing to analyze the expression profiles of circRNAs in the cerebral cortex (BA46) of 35 postmortem patients with SZ as well as those of healthy controls (62). They found that more than 95,000 types of circRNAs in the human dorsolateral prefrontal cortex (DLPFC) showed significant diversity, and that half of them had not previously been reported, such as circHomer1, circKlhl2, circMpped1, and circNell2 (63, 64). A differential analysis of these circRNAs revealed that 390 circRNAs were downregulated and 240 were completely deleted in patients with SZ. In addition to the reduced overall level of gene expression in circRNAs, the total numbers of unique circRNAs found in SZ were also reduced compared to those in heathy controls, and there was a large overlap between them. For instance, circNEll2, which is upregulated after synaptic plasticity induction, was downregulated in patients with SZ. The researchers also explored the correlation between differentially expressed circRNAs and their linear isoforms. Surprisingly, the expression levels of more than half of the circRNAs showed an inverse relationship with those of their linear RNAs, suggesting that both forms of transcripts may sometimes antagonize each other's biosynthesis. Zimmerman et al. (65) also discovered circHomer1a, which is highly expressed and neuron-enriched in the frontal cortex. Interestingly, in the prefrontal cortex (PFC) and induced pluripotent stem cell-derived neuronal cultures in patients with SZ and BD, circHomer1a was found to be significantly downregulated. Additionally, changes in circHomer1a expression in the DLPFC and orbitofrontal cortex (OFC) were positively correlated with the age of onset of SZ. In addition, circHomer1a was found to interact with RNA-binding protein, HuD, through animal-level verification, which was involved in regulating synaptic gene expression and cognitive flexibility. These findings are of great significance for exploring the molecular mechanisms underlying the pathogenesis of mental disorders.

The above results suggest that the overall expression levels of circRNAs are downregulated in patients with SZ, while there are still some unique and significantly differentially expressed circRNAs that differ from this overall trend. These circRNAs may play an important role in the clinical phenotypes of SZ by regulating corresponding cellular metabolic pathways underlying this mental disorder. Furthermore, there is a strong possibility of abnormal regulation of circRNAs in other mental illnesses and behavioral disorders.

Expression of circRNAs in Peripheral Blood of Patients With SZ

CircRNA plays a particularly important role in the regulation of gene expression in SZ (62, 66, 67). Previous studies (68) have identified that differentially expressed circRNAs from peripheral blood perhaps contribute on the development of the diagnosis and treatment of SZ.

A case-control study carried out by Yao and colleagues (69) screened nine kinds of significantly expressed circRNAs. Further verification by qRT-PCR revealed that three circRNAs were downregulated and two circRNAs were upregulated in the SZ group. The expression levels of circRNAs in patients with SZ after 4 and 8 weeks of conventional antipsychotic treatment were then re-quantified to see if there was any change. ROC curve analysis showed that three circRNAs (hsa circRNA 103704, hsa circRNA 101836, and hsa circRNA 104597) were of diagnostic significance, with a sensitivity of 87.25% and specificity of 85.44%. In particular, the sensitivity and specificity of hsa circRNA 104597 were 84.31% and 86.41%, respectively, indicating that it may be useful in the diagnostic and therapeutic evaluation of SZ.

The above results suggest that circRNAs may provide novel ideas for exploring objective biomarkers for SZ diagnosis. However, there has only been one study on this topic, which had some limitations. For instance, the sample size of screening differentially expressed circRNAs was relatively small. Since patients with SZ had been treated with standardized antipsychotic drugs for a relatively short period of time, changes in the expression level of circRNAs before and after treatment may also be due to their own time-dependent changes. Furthermore, only one type of circRNA was found to may represent a novel objective diagnostic value for SZ. Finally, there are still many significantly expressed circRNAs related to SZ that require further exploration (69–71).

CIRCRNAS AND DEPRESSION

Depression (DEP) is one of the most common mental disorders in humans. The World Health Organization (WHO) has reported that more than 800 million people worldwide suffer from DEP (72, 73). Approximately 40–60% of patients with DEP show suicidal ideation or behavior, and the suicide rate has been reported to be as high as 15% (74, 75). The disease burden of DEP is increasing year by year. In 1990, DEP ranked fifth in terms of its global burden as a disease. However, DEP is expected to become the top disease burden in China by 2030 (76). The process of diagnosing this disease is similar to that for SZ and, as with SZ, it can easily lead to misdiagnoses and missed diagnoses. Therefore, it is necessary to further explore the pathological

mechanisms and specific molecular markers related to DEP to improve the early diagnosis and prognosis of DEP.

Expression of circRNAs in Peripheral Blood in Depression

Cui and colleagues randomly selected peripheral blood mononuclear cells (PBMCs) from five patients with DEP and five healthy controls to analyze the expression profile of circRNAs. First, 15 differentially expressed circRNAs (including four upregulated and 11 downregulated circRNAs) were screened (77). Then, four significantly differentially expressed circRNAs were identified through verification in 100 DEP patients, 103 healthy controls, and 30 randomly selected DEP patients after 4 and 8 weeks of antidepressant therapy. However, only hsa-circRNA-103636 showed significant changes in DEP patients after 8 weeks of antidepressant treatment. Further ROC curve analysis showed that the sensitivity and specificity of this gene were 0.73 and 0.65, respectively. Therefore, hsa-circRNA-103636 may have potential diagnostic value of DEP.

Many studies have shown that type-2 diabetes (T2DM) is closely related to the occurrence and development of DEP (78, 79). Tian et al. collected venous plasma from five patients with T2DM and five cases of T2DM with DEP to determine the expression profiles of ncRNAs (including lncRNAs, circRNAs, and mRNAs) in order to discover specific molecular markers associated with DEP. The results of screening identified 28 lncRNAs, 107 circRNAs (including 75 upregulated and 32 downregulated circRNAs), and 89 mRNAs in the differential expression profiles of patients with DEP (80). Compared with that in the T2DM group, circRNA-TFRC had a significantly higher expression level in the DEP group. This circRNA is related to insulin resistance, which indicates that patients with DEP may have more severe insulin resistance symptoms. Some studies (81) have found that the expression level of TFRC is associated with mental disorders, suggesting that TFRC may be useful as a molecular target for studying the pathogenesis of DEP. Another upregulated circRNA-TNIK [Traf 2- and Nck-interacting kinase (TNIK)] is also associated with DEP. TNIK is a serine/threonine kinase that exhibits a high expression level in the brain, which has a good effect on the development and synaptic transmission of dendritic cells. A previous study (80) found that compared with a T2DM group, the expression level of TNIK was significantly increased in the DEP group. This suggests that the function of TNIK merits further investigation.

Jiang and colleagues (82) used microarrays to analyze plasma samples from seven patients with T2DM and seven patients with T2DM and DEP. Compared with the T2DM group, 183 circRNAs were upregulated and 64 circRNAs were downregulated in T2DM patients with DEP. Among them, the upregulated gene, DCP2 (hsa-circRNA-001520), may be used as a type of de-capping enzyme and plays an important role in neuronal development and mental retardation by degrading mRNAs. Another downregulated gene, CSGALNACT1 (hsa-circRNA-001781), is associated with an antidepressant response (83). In addition, Zhang et al. demonstrated that circDYM was also significantly decreased in MDD patients and two depressive-like mouse models [induced *via* chronic unpredictable pressure (CUS) and lipopolysaccharide (LPS)]. Recovery of circDYM levels significantly ameliorated CUS- or LPS-induced depressive-like behavior, which suggests that circDYM may be a novel therapeutic target for DEP (84).

According to the target genes of circRNAs related to DEP predicted by TargetScan and Miranda, 18 miRNAs and 529 mRNAs were found to interact with 4 circRNAs. Interestingly, hsa-miR-761 and hsa-miR-298 are common targets for hsa-circRNA-003251, hsa-circRNA-005019, and hsa-circRNA-015115. A previous study found that hsa-circRNA-003251 and hsa-circRNA-015115 may play important roles in the circRNA-

TABLE 2 | Expression of circRNAs in schizophrenia and depression

Author	Main findings
Ng et al. (59)	CircRNA derived from the known neuronal differentiation regulator RMST had a very high synaptic enrichment rate.
You et al. (60)	During the development of the hippocampus, the upregulated circRNA is produced by the gene locus that simultaneously encodes protein rich in synapse-related functions.
Hanan et al. (57)	CircRNA in the nervous system is generally upregulated compared to the thyroid, liver, and muscle. Especially in the cerebellum, the expression ratio of circular and linear RNA is significantly increased, and the brain-expressed circRNAs, such as circStau2a, have a strong enrichment effect on synaptic-neurons.
Mahmoudi et al. (62)	A total of 390 circRNAs, such as circNEll2, were down-regulated and 240 were completely deleted in patients with SZ. In addition to the reduced overall level of circRNA expression, the total numbers of unique circRNAs found in SZ were also reduced. More than half of circRNAs might sometimes antagonize linear RNA biosynthesis.
Yao et al. (68)	Three circRNAs (hsa circRNA 103704, hsa circRNA 101836, and hsa circRNA 104597) were of diagnostic significance, with a sensitivity of 87.25% and a specificity of 85.44%. In particular, the sensitivity and specificity of hsa circRNA 104597 were 84.31% and 86.41%, respectively, indicating that it might be a new potential biomarker for the diagnostic and therapeutic evaluation of SZ.
Abasolo et al. (81)	The expression level of TFRC is associated with mental disorders, suggesting that TERC could use as a molecular target for studying the pathogenesis of DEP.
Cui et al. (77)	Fifteen differentially expressed circRNAs (including 4 upregulated and 11 downregulated circRNAs) were screened out by a case-control study. However, only hsa-circRNA-103636 showed significant changes in depressed patients after 8 weeks of antidepressant treatment, indicating that it could be used as a new potential biomarker for the diagnosis and treatment of DEP.
Jiang et al. (82)	Compared with the T2DM group, 183 circRNAs were upregulated and 64 circRNAs were downregulated in T2DM patients with DEP. Hsa-circRNA-003251 and hsa-circRNA-015115 might play important roles in the circRNA-miRNA-mRNA network associated with DEP by acting as hsa-miR-761 sponges. In addition to the above two RNAs, hsa-circRNA-100918 and hsa-circRNA-001520 might be involved in the Wnt signaling pathway of thyroid hormone.
An (80)	Compared with the T2DM group, circRNA-TFRC had a higher expression level and circRNA-TNIK was significantly increased in the DEP group. TFRC was related to insulin resistance, which indicated that patients with DEP had more severe insulin resistance symptoms. TNIK had a good effect on the development and synaptic transmission of dendritic cells.

CircRNAs have specific expression profiles in brain regions and peripheral venous blood. Based on these, researchers used case-control studies to explore significantly expressed circRNAs in schizophrenia and depression in order to find the molecular bases of these two diseases.

miRNA-mRNA network associated with DEP by acting as an hsa-miR-761 sponge. Hsa-miR-761 (85, 86) has been shown to be involved in the regulation of mitochondrial networks and to promote the generation of learning and memory. Furthermore, overexpression of hsa-miR-761 inhibits the p38-MAPK signaling pathway (87). Therefore, hsa-miR-761 may participate in the pathological mechanisms of DEP. In addition, KEGG pathway analysis predicted that upregulation of hsa-circRNA-003251, hsa-circRNA-015115, hsa-circRNA-100918, and hsa-circRNA-001520 might be involved in the Wnt signaling pathway implicated in thyroid hormones. Another study showed that thyroid hormones affected mood and promoted signal pathways in the brain (88). These findings suggest that the occurrence and development of DEP might also be related to changes and metabolic disorders involving thyroid hormones (**Table 2**).

Based on the above findings, circRNA is a kind of biological signal that is measurable in physiological, pathological, and therapeutic process and perhaps contribute on predicting risk assessments, early diagnoses, and monitoring of responses in patients with SZ or DEP. At present, although some studies on the specific expression levels of circRNAs in various diseases have emerged, most of them have been at the level of expression profiles. Therefore, further studies are needed to better elucidate the precise associations between circRNAs and clinical symptoms in various diseases.

LIMITATIONS AND FUTURE RESEARCH

At present, an increasing number of studies on circRNAs related to SZ and DEP are emerging. However, there are still many questions to be answered, such as the following: whether changes in a single circRNA or among a group of circRNAs are specific to SZ and DEP; whether levels of circRNAs change with age; whether the expression of circRNAs are affected by smoking or drug abuse; and whether the circRNAs in cellular compartments have any differences in terms of their molecular bases of SZ and DEP (e.g., extra-vesicular circRNAs). These limitations indicate fruitful directions for future studies that may help elucidate the potential diagnostic and therapeutic value of circRNAs for SZ and DEP.

AUTHOR CONTRIBUTIONS

YX designed and supervised the study. ZL and SL drafted the manuscript. XL, WZ, and JL collected some literature.

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REVIEW
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Biomarkers in Psychiatry: Concept, Definition, Types and Relevance to the Clinical Reality

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During the last years, an extraordinary effort has been made to identify biomarkers as potential tools for improving prevention, diagnosis, drug response and drug development in psychiatric disorders. Contrary to other diseases, mental illnesses are classified by diagnostic categories with a broad variety list of symptoms. Consequently, patients diagnosed from the same psychiatric illness present a great heterogeneity in their clinical presentation. This fact together with the incomplete knowledge of the neurochemical alterations underlying mental disorders, contribute to the limited efficacy of current pharmacological options. In this respect, the identification of biomarkers in psychiatry is becoming essential to facilitate diagnosis through the developing of markers that allow to stratify groups within the syndrome, which in turn may lead to more focused treatment options. In order to shed light on this issue, this review summarizes the concept and types of biomarkers including an operational definition for therapeutic development. Besides, the advances in this field were summarized and sorted into five categories, which include genetics, transcriptomics, proteomics, metabolomics, and epigenetics. While promising results were achieved, there is a lack of biomarker investigations especially related to treatment response to psychiatric conditions. This review includes a final conclusion remarking the future challenges required to reach the goal of developing valid, reliable and broadly-usable biomarkers for psychiatric disorders and their treatment. The identification of factors predicting treatment response will reduce trial-and-error switches of medications facilitating the discovery of new effective treatments, being a crucial step towards the establishment of greater personalized medicine.

Keywords: biomarkers, neuropsychiatry, personalized medicine, lymphocytes, peripheral biomarkers, central biomarkers

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INTRODUCTION

According to World Health Organization mental illness presented devastating rates of prevalence, mortality, morbidity and disability. Suffering a serious mental illness reduces average life expectancy in 13 to 32 years (1, 2). Aside from mortality, in most Western countries, mental disorders are the leading cause of disability, responsible for 30-40% of chronic sick leave and costing approximately a

4% of gross domestic product (3). Besides, for all types of mental illness, pharmacological treatment options are scarce and present limited efficacy. Several studies highlighted that, in terms of recovery and remission, current pharmacological interventions showed significant limitations. A series of effectiveness trials sponsored by the National Institute of Mental Health (NIMH) in USA provided relevant data in this regard. In CATIE (Clinical Antipsychotic Trials of Intervention Effectiveness) study, 74% of patients suffering from chronic schizophrenia (SCZ) experienced problems of treatment adherence within 18 months (4). In addition, in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study only 31% of patients with major depressive disorder (MDD) were in remission after being treated with a selective serotonin reuptake inhibitor for a total of 14 weeks (5). An additional study carried out in patients diagnosed with bipolar disorder (BD) (STEP-BD, Systematic Treatment Enhancement Program for Bipolar Disorder) revealed that only 24% of patients experienced a remission of depression during 8 consecutive weeks, outcome similar to those observed in the vehicle group (6).

Several factors contribute to this clinical reality. On one hand, the heterogeneity/complexity of mental disorders. Patients suffering from a mental illness displayed several symptoms related with behavior, thinking, feelings and/or social interaction. To facilitate the diagnoses, mental disorders are classified by diagnostic categories with a broad variety list of symptoms according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-IV), or International Statistical Classification of Diseases and Related Health Problems, Tenth Edition (ICD-11). Consequently, patients diagnosed with the same psychiatric illness present a great heterogeneity in their clinical presentation. In addition, several mental illnesses present symptoms in common, that can often make the diagnosis difficult.

On the other hand, psychiatric diseases present high comorbidity. Approximately 85%–90% of patients with depression also experience symptoms of anxiety, and vice versa (7, 8). Among schizophrenic patients, psychiatric comorbidities are common. Around 50% of patients suffer from depression and more than 47% have a lifetime diagnosis of comorbid substance use disorders (9–11). The simultaneous presence of two or more psychiatric diseases are associated with greater severity, worse response to the pharmacological treatment and have a greater risk of suicide than either condition alone.

Despite these sobering facts, progress in human brain research and the advent of new technologies, such as 'omics' technologies, offers the opportunity for change mental health treatment and outcomes in a near future. In this respect, the identification of biomarkers has become a new promising tool for guiding diagnosis, predicting clinical outcome and, therefore, improving the understanding of the pathophysiology of mental disorders. This review provides an overview about the current state of biomarkers in neuropsychiatry, with the ultimate aim of remarking some goals achieved up to date and the future challenges needed to develop valid, reliable and broadly-usable biomarkers for psychiatric disorders and their treatment. For this purpose, the review includes a definition of biomarker's concept throughout history, describes the different types of biomarkers and their potential role in clinical practice, and emphasizes the samples and techniques commonly used. The role of 'omics' is described in greater detail due to its huge progress in the recent years. A final conclusion remarks the difficulties and limitations of current biomarkers strategies in neuropsychiatry and the future challenges needed to progress in this field.

WHAT ARE BIOMARKERS? EVOLUTION OF BIOMARKERS THROUGH HISTORY

During the last 50 years, the definition of biomarker has been modified according to scientific and clinical progress. The term "biomarker" was used for the first time in 1973 to indicate the presence or absence of biological material. However, the concept is older, referenced as a "biochemical marker" in 1949 (12) and "biological marker" in 1957 (13).

In 2000, the Biomarker Definition Working Group, supported by the U.S. National Institute of Health (NIH), defined a biomarker as "a characteristic that is objectively measured and evaluated as an indication of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (14). This definition has two major limitations. The first one lies in the fact that sometimes a biomarker is measured by subjective parameters. The second one is the fact that additional processes or responses beyond those covered by the definition are excluded.

In 2016, Fitzgerald and colleagues redefined the concept of biomarker as "a functional variant or quantitative index of a biological process that predicts or reflects the evolution of or predisposition to a disease or a response to a therapy" (15). Nevertheless, this description lacks the consideration of structural variants and qualitative index as potential biomarkers.

In order to harmonize the term of biomarker, the Food and Drug Administration (FDA) in collaboration with the NIH Joint Leadership Council convened the FDA-NIH Biomarker Working Group in 2016. This group simplified the biomarker definition being considered as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention" (16). This definition, clearer and more concise, defines a biomarker specifying its principal applications without any unnecessary complexity or contradictory information. Besides, to ensure its clinical use, a good biomarker should be measured with high reproducibility, present a sizeable signal-to-noise ratio and, more importantly, meet the condition of being modified in a dynamic and reliable way as the clinical condition progress. In addition, a biomarker should be accessible for its detection and measurement, as would be the case of a plasmatic parameter or a genetic marker, or being detected by histological or image/neuroimaging techniques (17).

Types and Role of Biomarkers in the Clinical Practice

According to their applications, biomarkers can provide complementary information about the disease or the intervention under consideration. Biomarkers may be identified at any event occurring since the pathogenesis, the onset of first clinical manifestations, diagnosis, treatment outcome or recovery. The FDA-NIH Biomarker Working Group distinguished several types of biomarkers based on their main clinical application: diagnostic, monitoring, pharmacodynamic/response, predictive r, prognostic, safety, and susceptibility/risk biomarkers (**Figure 1**). A biomarker may meet multiple criteria for different uses or present specific features that enable its particular use (18).

Diagnostic Biomarker

Encompasses a variety of biomarkers used to detect or confirm the presence of a disease or medical condition. This type of biomarker can be used to identify disease subtypes. The advent of the era of the precision medicine emphasizes the fact that diagnostic biomarkers are useful not only to identify patients with a disease, but also to redefine its classification. This is an important feature, because many diseases have subtypes with different prognosis or treatment responses. Thus, diagnostic biomarkers would contribute to improve personalized medicine increasing the effectiveness of the therapeutic response. These biomarkers may play also a critical role as *prognostic biomarkers* or *predictive treatment outcome biomarkers* (16, 17). An example could be the concentrations of Aβ42 and total tau (T-tau) in cerebrospinal fluid of patients with dementia as diagnostic biomarker for Alzheimer's disease (19) (**Figure 2**).

Monitoring Biomarker

This category includes biomarkers that are analyzed at different time points to monitor the status of a disease or medical condition, and as a marker of the response to an intervention, including exposure to a medical product or an environmental agent (**Figure 2**) (16). Changes in biomarkers values are considered as indicators of the progression of the clinical condition and as measurements of the pharmacological response and other types of clinical interventions (17). An example of a monitoring biomarker is the elevation of serum creatinine and/or potassium concentrations after a pharmacological or medical intervention,

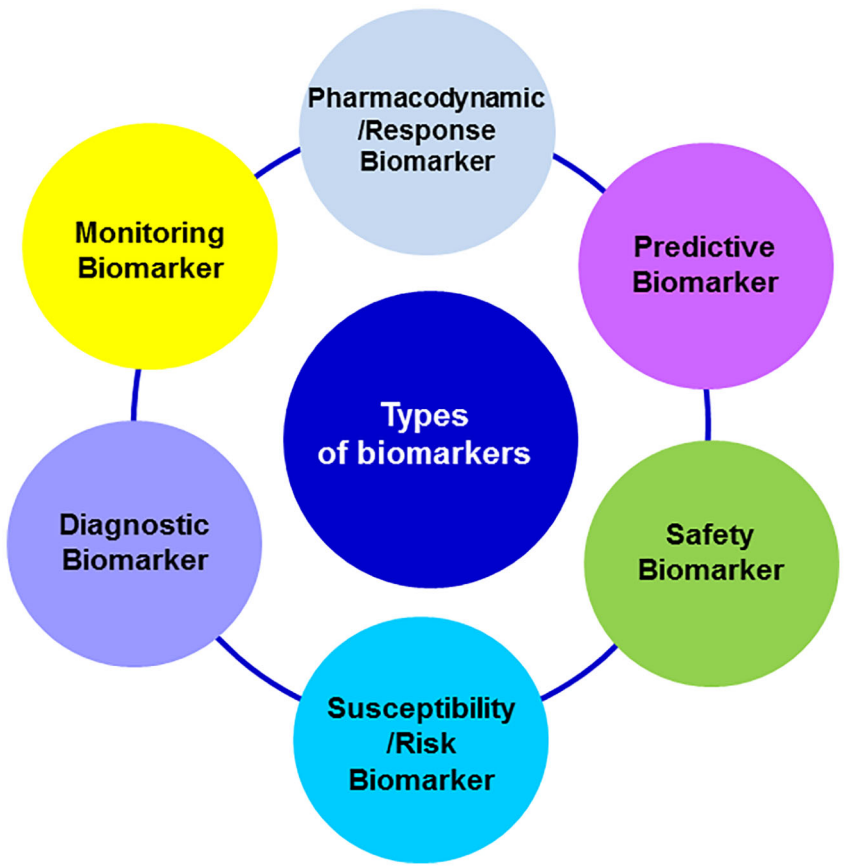

FIGURE 1 | Classification of biomarkers based on its main clinical application.

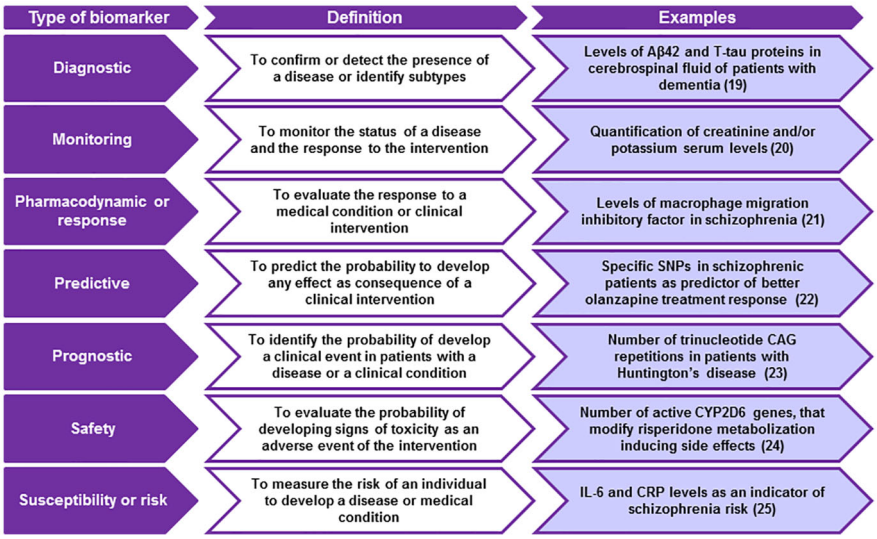

FIGURE 2 | Aims and examples of the main types of biomarkers. SNPs, single nucleotide polymorphisms; CRP, c-reactive protein; IL-6, interleukin 6.

TABLE 1 | Potential role of monitoring biomarkers in neuropsychiatry.

Type of intervention	Utility	References
Clinical care or clinical trial	To evaluate patient's clinical situation during treatment or at the end of the intervention	(21)
Before treatment initiation	To detect signs and/or symptoms of a disease or medical condition as an indicative parameter of the prognosis To determine the need for prompt treatment	(22)
Medical product development	To provide information about the safety and effectiveness of a drug	(23)
Public health	To provide information about the risk of developing any disease or medical condition among the population	(24)
Pharmacodynamics studies	To provide evidences about therapeutic response	(25)

parameters that are commonly used as an indicator of the probability to develop side effects (20). Monitoring biomarkers can be applied in different situations including clinical care or clinical trials, at the beginning of a treatment, for medical product development purposes, as a measure of the risk of developing a disease, or to evaluate the pharmacodynamics of a clinical intervention (**Table 1**).

Pharmacodynamic or Response Biomarker

Proposed to be a potential useful tool in clinical practice providing useful information for patient management. A pharmacodynamic biomarker is modified in response to a medical condition or clinical intervention, including drug treatments (16). Because of the serial nature of their assessment, this type of biomarker is frequently considered as a monitoring biomarker (20). The main utility of this biomarker is to guide the clinical management, providing crucial information for deciding whether or not to continue the treatment. Thus, pharmacodynamic biomarkers determine the progression of the treatment (26) (**Figure 2**).

Another area in which these biomarkers are of special interest is in early therapeutic drug development, being useful to establish the proof that a drug induces pharmacodynamic changes in humans related to its clinical benefit and to guide dose-response studies (18).

Predictive Biomarker

A marker is considered as a predictive biomarker when its presence or modification allows predicting which patient or group of patients are more likely to experience an effect as consequence of being exposed to a medical product or environmental agent (16). This effect could be a symptomatic benefit, an increase in survival rates or an adverse event. These biomarkers are frequently used in randomized controlled clinical trials of new therapies. In this context, the biomarker is used to select patients for participation or to stratify them into intervention groups. If the biomarker predicts a favorable outcome, its presence may indicate a greater effect of the new therapy compared to the control therapy (20). Thus, the use of predictive biomarkers facilitates the selection of specific patients more likely to respond or not to therapy (**Figure 2**). An example of a predictive biomarker is the presence of 12 single nucleotide polymorphisms (SNPs) in Had Chinese schizophrenic

population, that were correlated with greater olanzapine effectiveness (27).

Prognostic Biomarker

Commonly used to identify the probability of developing a clinical event in patients diagnosed with a disease or medical condition (16). These events include death, disease progression or recurrence, or the development of a new medical condition. In clinical trials, prognostic biomarkers are used to identify patients more likely to develop a clinical event or disease progression, allowing to identify populations at higher risk. In this context, prognostic biomarkers are used as inclusion or exclusion criteria (17). An example of a prognostic biomarker is the number of trinucleotide CAG repetitions in patients with Huntington's disease. A high number of CAG nucleotides repetitions are correlated with a greater threshold of disease's severity (**Figure 2**) (28).

An additional utility of prognostic biomarkers is in treatment selection. They can provide information about treatment safety, guiding patient hospitalization or their entrance in intensive care units.

Several factors influence the clinical outcome, including the clinical condition severity, the effects induced by all treatments and the intrinsic characteristics of patients. Some of these characteristics may be used as a prognostic biomarker, allowing to identify patients more likely to experience a clinical event, disease recurrence or progression, and any effect (favorable or unfavorable) induced by a medical product or environmental agent (16, 20).

Safety Biomarker

Is any measure that can be assessed before and after the exposure to a medical intervention, or an environmental agent, allowing to identify the probability of developing signs of toxicity as an adverse event, to detect the presence of toxicity, and for monitoring its extension (**Figure 2**) (16).

For many therapies, monitoring hepatic, renal and cardiovascular functions are critical to detect toxicity ensuring the safety of the therapy under study. All safety biomarkers have in common its ability to detect or predict toxicity prior to theNBSP;onset of clinical signs and before irreversible damage. The toxicity can be determined by the detection or changes in the biomarker level.

Another usefulness of safety biomarkers is the identification of patients in which particular therapies should not be initiated because of significant safety risks. For example, genetic variations in CYP2D6 enzymes modify the response to certain drugs commonly used in psychiatry such as almost 50% of antipsychotics drugs. Alterations in the metabolism of drugs can modify its effectiveness, decreasing the response to the treatment or enhancing toxicity risk in patients (15). In case of the antipsychotic risperidone, there is a correlation between the number of active CYP2D6 genes and its cardiac toxicity. QTc interval is longer in subjects with one active CYP2D6 gene compared to patients with two. The study revealed that the number of CYP2D6 active genes was related with the corrected plasma concentration of risperidone (29). Safety biomarkers are used with this purpose in public health or in epidemiological interventions aimed to control or mitigate risk exposure.

Susceptibility or Risk Biomarker

Is used as a risk measure to develop a disease or medical condition (8). An example is a genetic biomarker that can be detected many years or decades before the onset of clinical signs and/or symptoms of the disease (**Figure 2**) (10). Susceptibility/risk biomarkers are essential for the development of epidemiological studies aimed to evaluate the risk of developing a disease, contributing to establish preventive strategies in clinical practice. In this line, some studies suggested a potential correlation between interleukin-6 (IL-6) and C-reactive protein (CRP) levels and the risk of developing SCZ. Lower CRP levels together with the blockade of IL-6 signaling significantly increase SCZ risk, being proposed as a potential susceptibility/risk biomarkers for this neuropsychiatric disorder (30).

SAMPLES AND TECHNIQUES USED FOR THE SEARCHING OF BIOMARKERS

Biomarkers should be easily measurable, in easily accessible samples and using affordable techniques to ensure its inclusion in the routine clinical practice. Historically, plasma together with tissues obtained from biopsies were one of the most common samples used in the searching for biomarkers. Besides, based on the disease of interest, additional body fluids readily available in large amounts as urine, saliva, tear fluid, sweat, amniotic, cerebrospinal and pleural fluids, cervicovaginal secretion and wound efflux can be used for this purpose (31).

In the case of diseases of the central nervous system (CNS), such as psychiatric and neurological disorders, access to brain samples is of particular interest. In this respect, brain human post-mortem samples, usually provided by brain banks, play a crucial role. However, systematic biochemical investigations using these samples are scarce, limited and unrealistic mainly to the fact that the course of the disease cannot be monitored. In this respect, the progress of functional neuroimaging has allowed to study some neuronal functions including alterations of local cerebral flow, energy metabolism and neurotransmitter receptor density and occupation over the course of disease. Nevertheless, functional neuroimaging fails to provide information at cellular biochemistry level and the access to this technique is limited due to its high economic costs.

In this context, blood lymphocytes have gained special attention in the searching of peripheral biomarkers (32). Lymphocytes can be isolated easily from blood samples and studied on a daily basis allowing to monitor the course of the disease. This is possible due to the fact that receptor properties and transduction processes of lymphocytes are similar to those observed in the CNS. Several studies pointed out a close bidirectional interaction between the CNS and the immune system, in particular with lymphocytes (33). For instance, peripheral cytokines released by lymphocytes modify CNS functions including its autonomic control as well as

neuroendocrine and behavioral responses. Besides, several evidences suggested that alterations in neurotransmitters and hypothalamic-pituitary-adrenal (HPA) axis in the CNS are concomitant with alterations in the function and metabolism of lymphocytes.

To date, some genes such as c-fos, interleukins (IL-2, IL-4, IL-6, IL-10), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), cannabinoid receptors, acetylcholine, $GABA_A$ receptors, B_2-adrenergic receptors, glucocorticoid receptors, mineralocorticoid receptors, D_3 dopaminergic receptor, and serotonin receptors have been analyzed in lymphocytes from psychiatric patients, such as schizophrenic and depressive patients, with promising results as peripheral biomarkers (34–41). Thus, gene expression studies in lymphocytes of psychiatric patients at different stages of the disease, that may reflect alterations in the CNS, would allow to further characterize the mechanisms underlying the pathogenesis of the disease and may contribute to predict the pharmacological treatment response (biomarkers of treatment outcome) (**Figure 3**).

Another crucial factor for the searching of biomarkers is the techniques used. These techniques should have a high-throughput for the application of analytical data through robust dimensional data obtained in high performance tests (42). In this respect, 'omics' technologies, including genomics, proteomics, transcriptomics, metabolomics, and epigenetics, have contributed to the rapid discovery of many potential biomarkers.

'OMICS' BIOMARKERS AND NEUROPSYCHIATRY

This section summarizes the main advantages of each 'omics' technology in the search of biomarkers for assessing risk, diagnosis, monitoring progression and prediction of treatment response in neuropsychiatry disorders.

Genomic Biomarkers

Genomic biomarkers are expanding knowledge for the understanding of disease pathogenesis providing new targets for disease characterization, early diagnosis, and better-targeted treatment (drug discovery, drug development and adverse drug responses) to direct patients towards a more likely benefit based on their unique profile (43). According to the European Medicines Agency (EMEA), a genomic biomarker is defined as "a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions" (44). These measurable features include the expression, function and regulation of a particular gene. In the DNA, these features can be characterized by single nucleotide polymorphisms (SNPs), variability of short sequence repeats, haplotypes, deletions or insertions of (a) single nucleotide (s), copy number variations and cytogenetic rearrangements (translocations, duplications, deletions or inversions) (45). The use of genetic techniques allowed the analysis of candidate genes, genome-wide studies and polygenetic risk score analysis to understand multiple psychiatric disorders such as SCZ (46, 47). These techniques include Comparative Genomic Hybridization (CGH), microarray, exome sequencing, and whole genome sequence. Specifically, pharmacogenomics is crucial to identify genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets, being essential to drug discovery and drug therapy optimization [for review (48, 49)] (**Figure 4**).

Genome-wide association studies have allowed the identification of potential genomic biomarkers in different

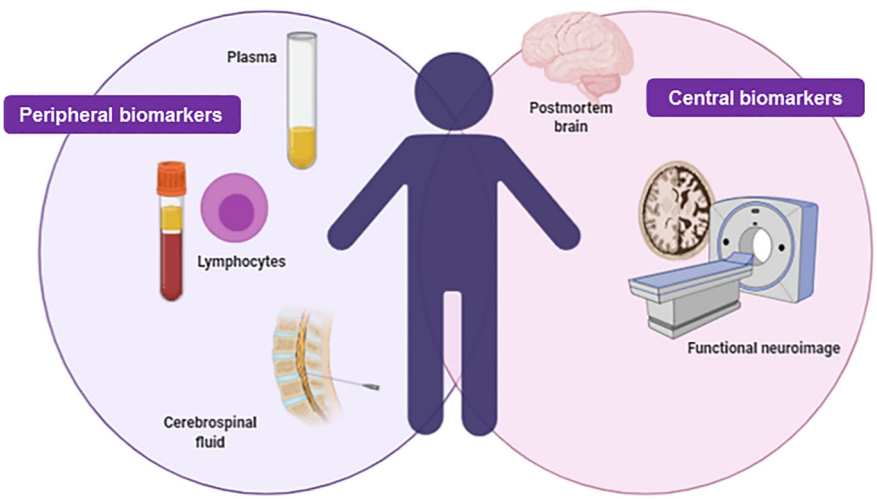

FIGURE 3 | Integrative figure regarding the main samples used in the searching of biomarkers in neuropsychiatry: peripheral and central biomarkers. Created with BioRender.com.

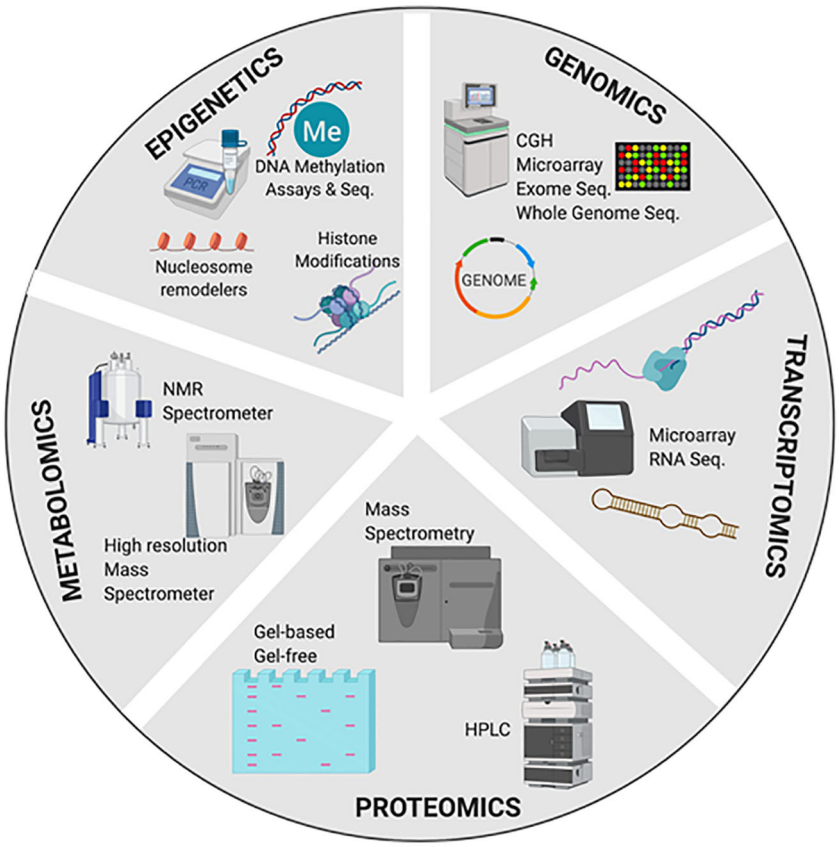

FIGURE 4 | Multi-omics approach for the discovery and validation of biomarkers to probe multidimensional phases of the disease. CGH, comparative genomic hybridization; Seq, sequencing; qRT-PCR, quantitative real time PCR; qPCR, semiquantitative real time PCR; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy. Created with BioRender.com.

neuropsychiatric (50–52) and drug-use disorders (53). For example, the Collaborative Study of the Genetics of Alcoholism (COGA) have correlated the SNPs rs4780836 [A > C; chromosome 16:19974071 (GRCh38.p12)], rs2605140 [A > G; chromosome 17: 18253061 (GRCh38.p12)], rs11690265 [chromosome 2: C > T; chr2:27418655 (GRCh38.p12)], rs692854 [non-functional Se (FUT2) gene; alleles C > A; chromosome 19: 48706207 (GRCh38.p12)], and rs13380649 (alleles A > G; chromosome 16: 19999778 (GRCh38.p12)] with greater vulnerability and predisposition to develop alcohol use disorders (AUD) in European American and African American. Besides, the study has demonstrated that there is a correlation between these SNPs and alterations in electroencephalograms, such as lower posterior gamma, higher slow wave connectivity (delta, theta, alpha), higher frontal gamma ratio and higher beta correlation in the parietal area of patients with AUD (54).

In bipolar disorder (BD), the SNP rs17026688 in the gene encoding glutamate decarboxylase-like protein 1 (GADL1) has been associated with the response to lithium in Chinese patients (55). This SNP has been related to immune dysfunctions in BD patients, such as higher percentages of total T cells, $CD4^{+}$T cells, activated B cells and monocytes. Besides, treatment of BD patients-derived peripheral blood mononuclear cells (PBMCs) with lithium *in vitro* increases the immune response ($CD4^{+}$ cells). These findings suggest that the immune imbalance might not only be a biomarker for diagnosis but also a biomarker of the disease progression and therapeutic response in BD

In addition, a large study carried out through Europe, North America and Australia identified 30 genome-wide significant loci for BD in patients of European descent. These loci contain genes that encode for neurotransmitters transporters, synaptic components, and ion channels, including calcium voltage-gated channel subunit alpha1 C (CACNA1C) and other voltage-gated calcium channel genes. Among the 30 loci identified in BD patients, eight have also been described in SCZ patients (56–58); however, conditional analyses performed in this study suggested that BD and SCZ associations are independent for three of the eight shared loci, providing information that may be useful for understanding the genetics mechanisms underlying these psychiatric disorders that in some cases present symptoms in common that make its diagnosis difficult. Furthermore, the BD subtype polygenic risk score analyses performed in the study supported the nosological distinction between bipolar I (BD1) and bipolar II disorder (BD2) and the importance of psychosis

beyond DSM subtypes. One limitation of the study is the genetic heterogeneity of the samples that may contribute to inconsistent replication in some of the results (59).

Besides, DNA genomic biomarkers as a useful indicator of the state of the disease (severity) also presented relevant consequences for the clinical management of neuropsychiatric diseases. In this respect, a recent study revealed a close relationship between the SNPs rs1360780 in the FK506-binding protein 5 (FKBP5) gene and rs17689918 in the corticotrophin-releasing hormone receptor 1 (CRHR1) gene and greater severity of the disease in posttraumatic stress disorder (PTSD) patients (60).

Transcriptomic Biomarkers in Neuropsychiatry

The transcriptome is defined as the complete set of all RNA molecules in one cell or a population of cells at a specific developmental stage or physiological condition (61). Thus, transcriptome is dynamic and reflects the cellular state. Measuring the expression of an organism's genes as a snapshot in different tissues, conditions, or time points provides information on how genes are regulated and would contribute to a better understanding of human diseases and their pharmacological treatment, allowing to identify potential therapeutic biomarkers when variations in treatment outcomes occur (62, 63). Although first studies for transcriptome began in the early 1990s, technological advances have spread throughout this time. There are two key contemporary techniques in the field: microarrays, which quantify a set of predetermined sequences, and RNA sequencing (RNA-Seq), which uses high-throughput sequencing to capture all RNA sequences (63).

For instance, transcriptomics studies identified that the efficacy of antidepressants is related to gene expression changes at transcriptome-wide scale. In a microarray study, alterations of MMO28 and KXD1 genes encoding for matrix metallopeptidase 28 and KxDL motif-containing protein 1, respectively, were associated with better response to nortriptyline in depressive patients (64). This data could contribute to improve the characterization of the molecular pathways underlying the efficacy of antidepressants.

In addition, a clinical study associated new miRNAs (miR-146a-5p, miR-146b-5p, miR-24-3p, and miR-425-3p) with the effectiveness of antidepressant drugs such as duloxetine, escitalopram, and nortriptyline, in patients with MDD. These miRNAs are ubiquitously expressed and highly correlated in blood and in brain tissue, playing an important role in the regulation of the mitogen-activated protein kinase (*MAPK*) and *Wnt* signaling pathways, closely related with stress response and MDD (65). Interestingly, an additional study revealed that MDD patients responders to antidepressant treatment present a significant reduction of miR-1202 baseline levels compared to non-responders. Moreover, miR-1202 increases as the efficacy of the antidepressant treatment is observed (66).

Besides, a total of 25 miRNAs have been modified in the amygdala of rats exposed to the learned helpless animal model of depression being the miR-128-3p the most affected. Also, a reduction of *Wnt* signaling genes has been also detected. Accordingly, an increase of miR-128-3p expression along with a significant downregulation of key target genes from *Wnt* pathway signaling (WNT5B, DVL, and LEF1) has been identified in the AMY of MDD patients (67).

Especially, RNA studies provide promising results in the searching for biomarkers of suicide. Alterations of RNA editing on the cyclic nucleotide phosphodiesterase (PDE, particularly PDE8A involved in the hydroxylation of cAMP and cGMP) were found in the dorsolateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (ACC) of suicide completers. These alterations have been proposed as a biomarker of risk for attempting suicide in patients with depressive symptoms (68).

Other example of the potential role of these biomarkers in neuropsychiatry is a genome-wide expression study in patients who met the DSM-IV criteria for methamphetamine dependence. The results revealed that treatment with topiramate significantly modified the gene expression of specified genes GRINA, PRKACA, PRKCI, SNAP23 and TRAK2 involved in severe pathways underlying drug addiction and other relevant physiological functions, including neuronal function/synaptic plasticity, signal transduction, cardiovascular function, and inflammation/immune response (69).

Likewise, the microRNA-124 (miR-124) and microRNA-181 (miR-181) were pointed out as potential biomarkers for cocaine use disorder (CUD) (70). The study revealed that these two microRNAs were upregulated in the blood samples of females CUD compared with healthy female controls.

Proteomic Biomarkers in Neuropsychiatry

Proteomics approaches using blood, plasma or serum constitutes a highly desired method for biomarker profiling of psychiatric disorders, due to the fact that these biological samples are used for routine diagnostic analyses in clinical practice, making easier to obtain samples. Besides, in neuropsychiatry, the cerebrospinal fluid (CSF) is a sample of particular interest for the identification of potential proteomic biomarkers due to its proximity to the brain. Although its collecting is very complex, due to the invasive procedure involved, it contains much less proteins than plasma. Thus, the "buffering" of protein composition is much weaker and tend to lead in a reduction of chances to identify potential proteomic biomarkers.

In proteomics, the separation of proteins using gel-based or gel free techniques, commonly followed by mass spectrometry are the mainly techniques used. The strategies for obtaining biological samples are diverse, but it is recommended to reduce the complexity of the sample, and sometimes to employ enrichment techniques improving the levels of certain subcellular fractions of interest or for specific types of proteins (glycoproteins, membrane, secreted, nuclear matrix and phosphorylated proteins) (71).

Diagnostic complications and timely treatment in neuropsychiatric disorders are frequent. Such is the case of SCZ, diagnosed by certain signs and symptoms but not by measurable and identifiable biological characteristics. In this

respect, proteome studies carried out in blood plasma, serum and postmortem brain tissue from SCZ patients identified alterations in proteins that play a significant role in neuronal transmission and synaptic function, calcium homeostasis and signalling, energy metabolism, oxidative stress, cytoskeleton and in immune system and inflammation. These proteins have been proposed as biomarker candidates for prognosis, diagnosis, and medication monitoring in SCZ (72, 73). One of these proteins is zinc finger protein 729 that was found significantly down-regulated in patients with SCZ compared to healthy individuals and patients diagnosed with depression or BD (74). Another example is the study that showed reduced plasma levels of glia maturation factor beta (GMF-β), the brain-derived neurotrophic factor (BDNF), and the 115-kDa isoform of the Rab3 GTPase-activating protein catalytic subunit (RAB3GAP1) in SCZ patients. These biological markers have been proposed as potential biomarkers in this pathology (75).

Besides, the acetyl-l-carnitine (LAC) has been proposed as a proteomic biomarker in MDD. LAC plays an important role in several behavioral features. The reduction of LAC concentrations was associated with abnormal hippocampal glutamatergic function and plasticity. Such alterations suggested that the degree of LAC deficiency was directly proportional with the severity, the age of MDD onset, and the clinical history of treatment-resistant depression (TRD). These findings suggest that LAC may be useful as a diagnostic and prognosis biomarker for MDD (76).

Recently, neurofilaments light chains (NF-L) have been proposed as potential biomarkers for neuronal damage in certain psychiatric diseases. In the plasma of female patients affected by anorexia nervosa, levels of NF-L were significantly elevated, being associated with the neuronal damage observed in AN patients, that partially normalizes with weight recovery (77). An additional study pointed out the potential role of NF-L as a discriminative biomarker between primary psychiatric disorders and neurodegenerative clinical conditions with wide-ranging of behavioral, psychiatric, and cognitive symptoms (78). Interestingly, a reduction of NF-L has been identified in the hippocampus of rodents exposed to an animal model of depression (inescapable stress). In this study, treatment with valproic acid reduces depressive-like behavior and reverses NF-L reduction (79). Besides, elevated concentrations of NF-L have been observed in the CSF of BD patients. Authors demonstrated that there is a positive correlation between CSF NF-L levels and the response to antipsychotics and lithium (80). However, another prospective study failed to observe any association between the high baseline NF-L levels in CSF and clinical outcomes in BD (81). Further studies are needed to identify the role of neurofilaments as biomarkers for psychiatric disorders.

In conclusion, to consider a potential proteomic biomarker, it is necessary to evaluate its sensitivity, specificity and positive or negative predictive values upon the disease of interest (31). In this respect, modern proteomics workflows that enable high throughput studies with large cohorts of well-defined samples represent the opportunity to solve the limited reproducibility of past proteomic workflows (73).

Metabolomics Biomarkers in Neuropsychiatry

Metabolomics biomarkers for drug development are growing. This technology focuses on the presence of small molecules metabolites in various complex matrices like CSF, blood, urine, saliva, and other human fluids. The metabolome is inherently more dynamic and time sensitive than proteome and genome, providing a direct functional measure of cellular activity and physiological status (82, 83). Changes in metabolome are the consequence of the interaction between lifestyle, environmental, genetic, developmental, and pathological factors. Consequently, metabolomics are of particular interest because, in contrast to genomics, captures the dynamic nature of the disease, and in contrast to proteomics, metabolomics measure the final products produced by complex interactions between proteins, signalling cascades and cellular environments.

Metabolomics biomarkers are not characterized by one single metabolite. Rather, they are a set of correlated metabolites defining a specific state of disease or the response to a clinical or pharmacological intervention (84). Currently, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) are the main types of analytical platforms used in the searching for metabolomics biomarkers.

Several studies have focused on the identification of potential metabolomics biomarkers in different psychiatric diseases [for review see (85–88)]. For example, urinary metabolomics have been proposed to be potential useful tools for the identification of pathways that may be involved in the mechanism of action of specific treatments. Such is the case of the study in which 77 urinary metabolites were identified in children with autism spectrum disorder treated with sulforaphane, a supplement that significantly improved the social responsiveness. Some of these metabolites play a role in the regulation of neurotransmitters, hormones, oxidative stress, amino acid/gut microbiome, and sphingomyelin metabolism (89).

Recently, a study conducted in patients with symptoms of depression, tried to found a predictor or a biological correlation of depression recovery after the administration of certain antidepressants including escitalopram, bupropion-escitalopram or venlafaxine-mirtazapine combinations. An increase of phosphatidylcholine C38:1 baseline plasma concentrations was associated with poorer outcome in patients. In contrast, an increased ratio in hydroxylated sphingomyelins after 8 weeks of treatment was linked to symptoms recovery (90).

However, few metabolomics biomarkers, especially in neuropsychiatry, have passed the regulatory standards for their use in clinical practice, mainly due to the lack of robust assays for the routine quantification of potential biomarkers and the heterogeneity of studies. The reduced sample size, particularly of some clinical subgroups, and the limited quantitative power of current mass spectroscopy technology, hampered the identification of robust metabolomics biomarkers, making necessary their validation trough additional assays. Besides, the complexity of samples, such as urine or blood, also contributes to that reality. In this respect, the use of chromatographic

techniques for separation is needed to reduce the potential interferences associated with the complexity of human samples (91).

Epigenetic Biomarkers

Dynamic variations in the structure of chromatin, which do not change the sequence of DNA itself but modified the expression of genes, have been paid attention due to its potential implication in the development of human diseases, including psychiatric disorders (92, 93). Accordingly, epigenetics may provide a functional interface between genotype, environmental exposure and phenotype (94).

To date, different forms of epigenetic regulation have been identified, such as direct methylation of DNA, histone modifications (as methylation and acetylation ubiquitination), exchanges of histone molecules with related isoforms, modification on chromatin by nucleosome remodelers that modify the access to DNA, and additional mechanisms like non-coding RNAs, non-genic DNAs, and differential exosome expression (95). In this way, identifying the aberrant changes in the epigenetic scenery associated to neuropsychiatry diseases and the factors that promote such alterations may allow the identification of potential new biomarkers (96).

An epigenetic biomarker is defined as "any epigenetic mark or altered epigenetic mechanism that can be measured in the body fluids or tissues defining a disease (detection); predicts the outcome of disease (prognostic), responds to therapy (predictive); monitors responses to therapy or medication (therapy monitoring) and predicts risk of future disease development (risk)" (97). So far, several techniques have been designed to analyze not only epigenetic processes at the level of specific genes but also epigenetic changes that occur in defined regions of the genome by epigenome-wide association studies. DNA methylation assays and DNA methylation sequencing are the most employed techniques, but not exclusively. Novel epigenetic techniques, such as those provided by CRISPR/Cas9 system, represent new opportunities in the searching for epigenetic biomarkers (98).

Many of the findings achieved thus far are encouraging, revealing significant associations with epigenetic modulations of genes regulating neurotransmission, neurodevelopment, and immune function in psychiatric diseases (99). One example is the hypermethylation of BDNF gene identified in brain and peripheral blood samples of MDD, SCZ and BD patients (100, 101). Another similar example is the hypermethylation of FKBP5 gene, an important modulator of stress response, detected in peripheral blood samples of PTSD patients (102). In panic disorder, hypomethylation of monoamine oxidase A (MAOA) and glutamate decarboxylases 1 (GAD1) genes have been evident in recent studies (103).

In suicide, advances in epigenetic techniques have allow to characterize epigenetic alterations in key elements of the hypothalamus-pituitary-adrenal axis (HPA-axis), neurotrophic factors, serotoninergic and GABAergic systems, that have been proposed as epigenetic biomarkers for suicide, suicide ideation and suicide attempt (104).

Interestingly, epigenetic biomarkers have been pointed out as potential biomarkers for guiding treatment. Thus, antipsychotic drugs, such as olanzapine, induced DNA methylations alterations through the brain in SCZ patients, changes related with its efficacy (105, 106). For instance, reduced response to antidepressants has been associated with the absence of methylation at a specific CpG site in exon 4 of BDNF in MDD patients (107). Consequently, BDNF exon 4 methylation, and circulating BDNF protein levels may be used together as a predictive tool to personalize treatment of MDD (108).

More interestingly, histone deacetylases (HDAC), that have been demonstrated to control epigenetic programming associated with the modulation of behaviour and cognition, appears to be crucial for reversing dysfunctional epigenetic regulation induced by early life events exposure in preclinical models (109, 110). Additional studies have supported the potential role of HDAC as promising new therapeutic targets for the treatment of MDD (111). In this context, HDAC inhibitors, alone or in combination with current antidepressant drugs, are currently being explored (112–114).

Altogether, epigenetic studies highlight the importance of epigenetic mechanisms on controlling genes or gene complexes. In neuropsychiatry, despite huge advances were achieved, there are still far for providing a clear molecular mechanism underlying these disorders and effective treatment options. The heterogeneity of the techniques and methods used, with a range in sensitivity for detecting effects (115–117); the lack of adjusting the genome-wide results to account for cell specificity (118, 119); the confounding factors such as patient's treatment, population origin and phenotypes included (105, 120); and the lack of further studies to demonstrate the concordance between brain-blood data have hampered the clinical use of epigenetic biomarkers (121).

SUMMARY AND CONCLUSIONS

As set out in this review, there are several proteins, metabolites and genes that have been linked with certain neuropsychiatric diseases mainly due to the advance in 'omics' technologies. However, none of them have demonstrated to be a real and useful biomarker in clinical practice.

Despite each 'omic' presents its limitations and challenges (122–124), three essential key targets are in common to advance in the searching of biomarkers in neuropsychiatry: 1) accurate selection of the clinical population, 2) shortened sampling time and 3) standardization of procedures for sample processing. These items can be applied for any diseases, but are of special interest for psychiatric disorders. The broad spectrum of phenotypes in patients diagnosed from the same psychiatric disorder and the overlapping of some traits or clusters in different neuropsychiatric disorders, which can often make diagnosis difficult, increases the heterogeneity of the clinical population analyzed. To overcome this issue, emerged 'omics' studies have focused on the identification of potential biomarkers for specific traits. However, the reduced number of samples

analyzed per trait/phenotype has made difficult to achieve robust conclusions about the potential clinical use of the proposed biomarkers. In this respect, modern 'omics' workflows that enable high throughput studies with large cohorts of well-defined samples can solve this problem.

Besides, the heterogeneity of procedures for sample processing along with the differences in power and sensitivity of each 'omics' technologies have contribute to that reality. In this respect, new 'omics' with better quantity power and sensitivity would contribute to find robust and realistic biomarkers.

One of the major challenges still lying ahead is the way to integrate the plethora of data obtained from each 'omics' to reach the holistic realization of a 'systems biology' understanding the biological question (125). In this context, bioinformatics tools have been designed to understand the potential of 'omics' technology (126).

Another concern is that current biomarker validation is a lengthy and complex process. In essence, this process includes the validation of the method, determined by the characteristics of the assay employed, and the clinical validation, to provide evidences that the biomarker is linked specifically with the disease or clinical end point under consideration. Is in this aspect in which future longitudinal integrative 'omics' studies can be crucial to provide a rigorously biomarkers validation ensuring its sensitivity, specificity, predictive value, and likelihood ratio, by its assessment in a large cohort (normal clinical population). It is expected that in the following years considerable breakthroughs will occur in these regards.

AUTHOR CONTRIBUTIONS

MG-G and JM conceived the presented idea. MG-G took the lead in writing the manuscript. FN, FS, AG, and AA-O wrote the manuscript in consultation with MG-G. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Association Between *FoxO1*, *A2M*, and *TGF-β1*, Environmental Factors, and Major Depressive Disorder

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Introduction: Investigations of gene-environment (G×E) interactions in major depressive disorder (MDD) have been limited to hypothesis testing of candidate genes while poly-gene-environmental causation has not been adequately address. To this end, the present study analyzed the association between three candidate genes, two environmental factors, and MDD using a hypothesis-free testing approach.

Methods: A logistic regression model was used to analyze interaction effects; a hierarchical regression model was used to evaluate the effects of different genotypes and the dose-response effects of the environment; genetic risk score (GRS) was used to estimate the cumulative contribution of genetic factors to MDD; and protein-protein interaction (PPI) analyses were carried out to evaluate the relationship between candidate genes and top MDD susceptibility genes.

Results: Allelic association analyses revealed significant effects of the interaction between the candidate genes *Forkhead box* (*Fox*)*O1, α2-macroglobulin* (*A2M*), and *transforming growth factor* (*TGF*)*-β1* genes and the environment on MDD. Gene-gene (G×G) and gene-gene-environment (G×G×E) interactions in MDD were also included in the model. Hierarchical regression analysis showed that the effect of environmental factors on MDD was greater in homozygous than in heterozygous mutant genotypes of the *FoxO1* and *TGF-β1* genes; a dose-response effect between environment and MDD on genotypes was also included in this model. Haplotype analyses revealed significant global and individual effects of haplotypes on MDD in the whole sample as well as in subgroups. There was a significant association between GRS and MDD (P = 0.029) and a GRS and environment interaction effect on MDD (P = 0.009). Candidate and top susceptibility genes were connected in PPI networks.

Conclusions: *FoxO1*, *A2M*, and *TGF-β1* interact with environmental factors and with each other in MDD. Multi-factorial G×E interactions may be responsible for a higher explained variance and may be associated with causal factors and mechanisms that could inform new diagnosis and therapeutic strategies, which can contribute to the personalized medicine of MDD.

Keywords: *FoxO1*, *A2M*, *TGF-β1*, depression, G×E interaction, polymorphism

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INTRODUCTION

Major depressive disorder (MDD) is the most common psychiatric disorder and is associated with high morbidity, mortality, costs, and risk of suicide (1–3). The heritability of MDD is approximately 30%–40% (4) and may be greater for recurrent, early-onset disease (5). Establishing the contribution of genetics to MDD can lead to more accurate and timely diagnosis and clinical management, which has significant public health implications.

Studies on main genetic effects in MDD have reported conflicting findings (6). One reason for this is that risk of MDD is polygenic, with many susceptibility genes exerting small effects (7). Additionally, genotypic variations among individuals may increase the risk of MDD only upon exposure to adverse environmental factors, a phenomenon known as gene-environment (G×E) interaction (8). The association between candidate susceptibility genes and disease occurrence has mainly been predicated on the observation that genetic variants not only increase MDD risk but can also explain the underlying biological and molecular mechanisms (9). However, such hypothesis-driven approaches to the study of MDD etiology can only detect a fraction of genetic variants (8). On the other hand, a hypothesis-free approach requires enormous datasets that include both exposure [e.g., stressful life events (10, 11) and childhood adversities (9)] and phenotype (MDD) data. To this end, only three genome-wide environmental interaction studies has been carried out (12–14). In a recent report, a novel omics-based approach was used to identify genetic variants for genome-wide G×E interaction studies based on cross-species and -tissue biological prioritization strategies. However, genomics approaches usually cannot identify causal variants or genes (15). Integrating multiple omics approaches can provide a more comprehensive view of the biology of MDD.

Three candidate MDD susceptibility genes [*Forkhead box* (Fox) *O1*, *transforming growth factor* (TGF)-*β1*, and *α2 macroglobulin* (A2M)] that are ideal candidates for G×E interaction analyses were previously identified using an omics-based approach. It has been suggested that environmental and genetic factors contribute equally to the development of mental illness (16). Distal environmental risk factors such as family history (FH) and culture are important because they increase vulnerability to proximal factors; (17) however, the latter are more relevant for G×E investigations since they are more likely to meet the criteria for risk factors and lend themselves to biologically plausible hypotheses regarding their effects on specific neural systems that underlie psychopathological symptoms.

Genome-wide association studies (GWAS) have identified more than 100 genetic variants associated with MDD (18). Each of these has a small effect and the number of associated genetic markers increases with sample size. However, the utility of individual genetic markers for MDD risk prediction is uncertain (19). A multilocus genetic risk score (GRS)-based analysis has been proposed for combining the relatively small effects of single genes to better assess the complex relationship between genetic markers and MDD (20). GRS analyses have shown that including more weakly associated genetic variants improves the prediction of mental illness risk (21).

Proteins interact to mediate many cellular processes; disrupting one subunit of a protein complex can lead to direct and indirect functional consequences in various diseases and physiological conditions (22). For a specific illness, interactions between proteins encoded by susceptibility genes tend to be more frequent than those between random proteins, as revealed by protein-protein interaction (PPI) network analyses (22).

In the present study, we investigated the association between *FoxO1*, *TGF-β1*, and *A2M* genes and MDD in a large population. G×E, G×G, G×G×E, and environment × environment (E×E) interactions were analyzed in the context of MDD. We also assessed haplotypes of these three genes and the relationship between haplotype and environment. We used a GRS approach to calculate the combined effects of these three genes on the prediction of MDD and the GRS–environment interaction (GRS×E) in MDD. Finally, we examined FoxO1, TGF-β1, and A2M PPI networks to clarify their interactions with each other and with other MDD-related proteins.

MATERIALS AND METHODS

Study Population

From November 2014 to December 2018, 800 MDD patients (564 women and 236 men) were recruited for the study (mean age: 45.64 ± 14.10 years) along with 800 age- and sex-matched control subjects from the same geographic area in Northern China. All subjects were of Chinese Han ethnicity and provided written, informed consent before participating in the study. The study protocol was approved by the ethics committee of Harbin Medical University.

Independent Measures

Participants completed three questionnaires: a socio-demographic questionnaire, the Chinese version of the 24-item Hamilton Rating Scale for Depression (HRSD-24), and the Life Events Scale (LES). The self-rating socio-demographic questionnaire was adapted from the version developed by the Epidemiology Department of Harbin Medical University and was used to collect detailed background information on family psychiatric history, childhood trauma history, and socioeconomic background. The HRSD-24 (23) is widely used to measure depression symptoms; (24–26) a number of patients above the threshold (21 points) were included in the study. The LES questionnaire for measuring negative life events contained 48 items in three dimensions: family life (28 items), work-related problems (13 items), and social and other aspects (seven items). The LES was scored based on the occurrence/absence (1 and 1, respectively) and frequency (0 or more) of SLEs (26, 27).

Genotyping

Single nucleotide polymorphisms (SNPs) in the *FoxO1* (rs2297626, rs7319021, rs28553411, rs17592468, and rs17592371), *A2M* (rs10492115, rs226415, rs10842849, rs11048839, rs10842847, and

rs669), and *TGF-β1* (rs2317130, rs1800469, rs12983775, rs12462166, and rs2241715) genes were selected for genotyping, the candidate SNPs comprised most of the allelic variants with $r^2 > 0.8$ in the Asian population. Genomic DNA was extracted from venous blood samples using the AxyPrep Blood Genomic DNA Miniprep kit (Axygen, Union City, CA, USA). Polymerase chain reaction (PCR) was performed in a reaction volume of 2 μl DNA, 7.5 μl 2× PCR mix, 2 μl primer mix, 0.2 μl *Exo*I enzyme (Fermentas, Burlington, ON, Canada), 0.7 μl *Exo*I buffer (Fermentas), and 0.8 μl FastAP enzyme (Fermentas). Amplification conditions were as follows: at 95°C for 3 min; 35 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s; and 72°C for 3 min. PCR products were purified by incubation with *Exo*I and FastAP at 37°C for 15 min and at 80°C for 15 min. Extension conditions were as follows: 96°C for 1 min, and 30 cycles of 96°C for 10 s, 52°C for 5 s, and 60°C for 30 s. Extension products after denaturation at 95°C for 3 min were analyzed by DNA sequencing (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Haploview v.4.0 software was used to assess Hardy-Weinberg Equilibrium (HWE) and pairwise linkage disequilibrium (LD) and calculate minimal allele frequency (MAF) for genotyped polymorphisms (28). The χ^2 test/Fisher's exact test was used to analyze differences in the distributions of independent variables. The Bonferroni method was adopted for multiple-testing correction. Associations between phenotype and independent variables were analyzed by multivariable logistic regression, with polymorphisms scored as 0, 1, or 2 depending on the carrier status of the minor allele, sex, family history (FH), occurrence/absence or number of SLEs and CAs, and interaction effects (E×E/G×E/G×G/G×G×E/GRS×E). Allele-carrier status and dose-response effect of environmental factors were also determined. Hierarchical regression analysis was performed using RStudio v.1.1.423 software. Alpha levels were corrected with the number of polymorphisms of candidate genes (five polymorphisms for *FoxO1* and TGF-*β1*; six polymorphisms for *A2M*), so that the p values correction was 0.05/5 = 0.01 for *FoxO1* and TGF-*β1*, 0.05/6 = 0.0083 for A2M. Study power was calculated with QUANTO 1.2.4 (http://hydra.usc.edu/gxe/).

Haplotype analysis was performed using UNPHASED v.3.0.11 software (29). Maximum likelihood haplotype frequencies in the study population were assessed with the expectation maximization algorithm. Rare haplotypes with a frequency < 1% were excluded from analyses. The global association and effect of individual haplotypes on phenotype were analyzed in the total sample and in four subgroups classified according to positive/negative environmental factors (SLEs/CAs). The significance level of the global association was determined with the likelihood ratio test. Individual effects of each haplotype, i.e., the difference in effect between a haplotype and all others pooled together, were computed with the score test. A permutation analysis was conducted to assess the reliability of the results; 1,000 random permutations were set to generate empirical P values. Permuted $P < 0.05$ was considered significant in the haplotype analyses.

GRS Analysis

To estimate the cumulative contribution of genetic factors to MDD in an individual by taking into account the reported risk alleles, we used the predictABEL package in RStudio software to compute weighted GRS with the following formula:

$$GRS = \Sigma_{i=1}^{k} \beta_i N_i$$

where GRS is the sum of the effect estimates, k is the number of independent genetic variants with strong association as risk predictors, β_i is the weighted coefficient from logistic regression analysis, and N_i is the number of risk alleles for each locus. The association between GRS and MDD was evaluated by logistic regression analysis of FH, sex, GRS, SLEs/CAs, and the interaction between GRS and SLEs/CAs.

Population Stratification Analysis

Population stratification analysis was performed to eliminate the possibility of false-positive associations using STRUCTURE v.2.3.4 software (http://pritch.bsd.uchicago.edu/structure.html). *FoxO1*, *A2M*, and *TGF-β1* genotype data were used for population stratification analysis of the third-stage sample set. Sixteen SNPs in these genotype datasets were obtained from 311 samples derived from the 1000 Genomes Project, including 99 samples from the Yoruba population of Ibadan, Nigeria (YRI), 109 samples from Chinese Han in Beijing (CHB), and 103 samples from northern and western Europe and the United States (CEU) (1000 Genomes Project Phase 3 at www.internationalgenome.org/data-portal/sample). STRUCTURE assumes that there are K populations in the dataset. The admixture and correlated frequency models had a burn-in length of 10,000 and 10,000 Markov chain Monte Carlo repeats and took into consideration immigration and geography-based genetic isolation. The program was run several times at each K value from 2 to 6 to obtain consistent results.

PPI Analysis

We investigated whether *FoxO1*, *A2M*, and *TGF-β1* genes interact with each other and are involved in the PPI network containing protein products of the top MDD susceptibility genes identified by a recent GWAS (30–32). The PPI network comprises nodes and edges representing protein and physical interactions, respectively. Proteins encoded by MDD susceptibility genes were used as seed proteins. STRING v.11.0 software (https://string-db.org/cgi/input.pl) (33) was used to reconstruct the PPI network.

RESULTS

Descriptive Statistics

Demographic information on the study population is shown in **Table 1**. Genetic markers were successfully genotyped at a rate > 95%. No significant deviation from HWE was observed, and MAF was > 5% for each polymorphism (**Supplemental Table S1**). Pairwise LD D' values are shown in **Figure 1**.

TABLE 1 | Summarized frequencies of demographic and independent variables.

Variables	Frequencies
Gender	
Female	1,023 (63.9%)
Male	578 (36.1%)
Mean age	44.28 (s.d. 11.97)
Exposure to CAs	
No	1,412 (88%)
Yes	189 (12%)
Exposure to SLEs	
No	820 (51%)
Yes	781 (49%)
1	554 (70%)
2	74 (9%)
3 or more	153 (21%)
Family history of psychological problems among first-degree relatives	
FH–	1,440 (90%)
FH+	161 (10%)

FH, family history.

Association Between Independent Variables and MDD

Two polymorphisms of *FoxO1* (rs17592371 and rs2297626), two of *A2M* (rs669 and rs226415), and one of *TGF-β1* (rs12462166) were significantly associated with MDD (**Table 2**). Family history of MDD (χ^2 = 54.823, P < 0.0001), occurrence (χ^2 = 406.993, P <0.0001), and number (χ^2 = 781.522, P < 0.0001) of SLEs were related to MDD. The results remained robust after Bonferroni correction (**Table 2**). The power for association study of *FoxO1* (rs17592371 and rs2297626) was 87.43% and 90.45% and the power of *TGF-β1* (rs12462166) was 86.55%.

Interaction Between SLEs and Candidate Gene Genotypes in MDD

The two *FoxO1* (rs17592371 and rs28553411), three *A2M* (rs10842847, rs10842849, and rs226415), and two *TGF-β1* (rs12462166 and rs12983775) gene polymorphisms showed significant interactions with environmental factors (**Table 3**). In the hierarchical regression analysis, the effect of SLEs on MDD was stronger for the TT genotype (β = 0.612, SE = 0.25, z = 2.450, P = 0.014) than for the TC genotype (β = 0.508, SE = 0.176, z = 2.878, P = 0.003) of *FoxO1* rs17592371; and for the GG genotype (β = 0.692, SE = 0.291, z = 2.379, P = 0.017) as compared to the GT genotype (β = 0.542, SE = 0.166, z = 3.258, P = 0.001) of *TGF-β1* rs12462166 (**Figure 2A**). A dose-response effect of the interaction between number of SLEs and genotypes in MDD was only observed for *A2M* rs10842848, which did not interact with an SLE of 0 (β = 0.099, SE = 0.170, z = 0.586, P = 0.558) but

TABLE 2 | Association of Study Variables with MDD.

Variable	Subgroup	Number		χ^2 Value	P Value	P-Bonferroni
FoxO1		Case	Control			
rs17592371	CC	293	359	9.790	0.007	0.035
	CT	387	318			
	TT	120	123			
rs2297626	AA	293	356	11.424	0.003	0.015
	AG	382	321			
	GG	125	123			
A2M						
rs669	AA	694	644	Fisher's	0.001	0.006
	AG	101	152			
	GG	5	4			
rs226415	AA	6	15	10.395	0.005	0.03
	AG	168	158			
	GG	626	627			
TGF-β1						
rs12462166	CC	132	187	13.679	0.001	0.005
	CT	454	438			
	TT	214	175			
FH						
	Yes	125	36	54.823	<0.001	<0.001
	No	675	765			
SLEs presence						
	Yes	592	189	406.993	<0.001	<0.001
	No	208	612			
SLEs amount						
	0	15	613	781.522	<0.001	<0.001
	1	438	116			
	2	38	36			
	3 or more	110	36			

FH, family history; SLEs, stressful life events.

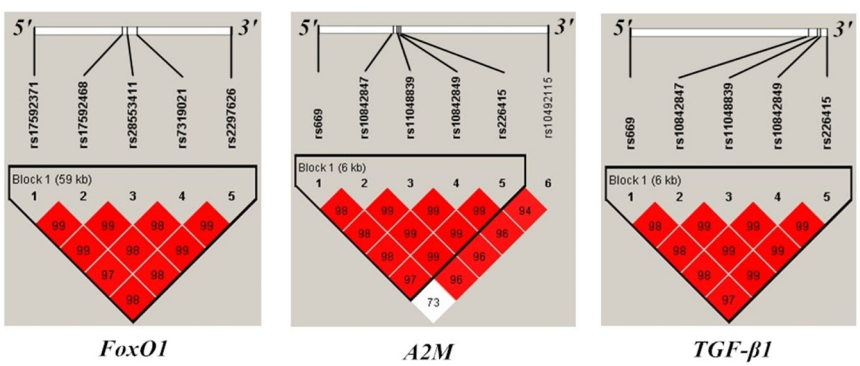

FIGURE 1 | Position and linkage disequilibrium (LD) map of genotyped polymorphisms in FoxO1, A2M, and TGF-β1gene. Pairwise LD statistics in examined genes were calculated with Haploview. Squares are bright red if the |D'| value is high, that is, LD is strong.

TABLE 3 | Effect of Candidate Genes, Environment, and Their Interaction on MDD.

Dependent Variables	β	SE	z	p Value
FoxO1				
rs17592371	–0.051	0.079	–0.651	0.515
CAs	0.521	0.235	2.215	0.026
rs17592371×CAs	0.589	0.250	2.352	0.018
rs17592371	–0.156	0.121	–1.285	0.198
SLEs	1.686	0.170	9.905	<0.001
rs17592371×SLEs	0.468	0.171	2.739	0.006
rs28553411	–1.210	0.148	–8.136	<0.001
CAs	1.622	0.223	7.247	<0.001
rs28553411×CAs	0.616	0.202	3.047	0.002
rs17592371	1.881	0.393	4.781	<0.001
rs28553411	–1.746	0.421	–4.141	<0.001
rs17592371×rs28553411	0.275	0.108	2.547	0.010
rs17592371	1.133	0.679	1.668	0.095
rs28553411	–1.219	0.679	–1.796	0.072
CAs	–0.083	0.202	–0.413	0.679
rs17592371×rs28553411×CAs	0.321	0.109	2.922	0.003
rs17592371	1.104	0.721	1.531	0.125
rs28553411	–1.226	0.721	–1.700	0.089
SLEs	1.704	0.143	11.842	<0.001
rs17592371×rs28553411×SLEs	0.211	0.087	2.409	0.016
A2M				
rs10842847	–0.142	0.120	–1.181	0.238
CAs	–0.133	0.183	–0.725	0.468
rs10842847×CAs	0.894	0.371	2.409	0.016
rs10842847	–0.206	0.176	–1.169	0.242
SLEs	1.152	0.124	9.273	<0.001
rs10842847×SLEs	0.711	0.253	2.811	0.004
rs10842849	–0.166	0.121	–1.372	0.170
CAs	–0.129	0.184	–0.704	0.481
rs10842849×CAs	0.988	0.374	2.639	0.008
rs10842849	–0.172	0.176	–0.978	0.327
SLEs	1.283	0.124	10.309	<0.001
rs10842849×SLEs	0.608	0.254	2.397	0.016
rs226415	2.310	0.194	1.201	0.023
SLEs	2.310	0.134	17.136	<0.001
rs226415×SLEs	–0.697	0.251	–2.775	0.005
rs10842849	0.052	0.615	0.085	0.932
rs226415	0.700	0.673	1.039	0.298
rs10842849×rs226415	–0.676	0.289	–2.341	0.019
rs10842849	–0.123	0.625	–0.197	0.843
rs226415	–0.060	0.631	–0.095	0.924
CAs	–0.141	0.183	–0.768	0.442
rs10842849×rs226415×CAs	1.132	0.378	2.989	0.002
TGF-β1				
rs12461266	–0.206	0.082	–2.493	0.012
CAs	–0.159	0.277	–0.574	0.565
rs12461266×CAs	0.629	0.202	3.106	0.001
rs12461266	–0.276	0.124	–2.218	0.026
SLEs	1.488	0.193	7.667	<0.001
rs12461266×SLEs	0.527	0.167	3.146	0.001
rs12983775	0.066	0.126	0.520	0.603
SLEs	2.363	0.204	11.563	<0.001
rs12983775×SLEs	–0.479	0.167	–2.857	0.004

CAs, childhood adversities; SLEs, stressful life events.

showed interactions with an SLE of 1 (β = 0.420, SE = 0.179, z = 2.339, P = 0.019), 2 (β = 0.647, SE = 0.278, z = 2.330, P = 0.019), and 3 or more (β = 0.749, SE = 0.295, z = 2.535, P = 0.011). That is, high exposure to SLEs was related to an increase in MDD risk (**Figure 2B**).

We evaluated the G×G interaction between *FoxO1* rs17592371 and rs28553411, between the *TGF-β1* rs12462166 and rs12983775, and among *A2M* rs10842847, rs10842849, and rs226415 as well as the three-way interaction between the two genetic markers that were significant in the G×G interaction analysis and LES (**Table 3**). The interaction between the two polymorphisms of the *FoxO1* gene was significant (β = 0.275, SE = 0.108, z = 2.547, P = 0.010), as was the interaction between those of the *A2M* gene (β = −0.676, SE = −0.289, z = −2.341, P = 0.019). The effects of three-way interaction (G×G×E) on MDD were also significant for *FoxO1* (G×G×SLE: β = 0.211, SE = 0.087, z = 2.409, P = 0.016; G×G×CA: β = 0.321, SE = 0.109, z = 2.922, P = 0.003) and *A2M* (G×G×CA: β = 1.132, SE = 0.378, z = 2.989, P = 0.002) (**Table 3**). There results remained significant after controlling for family history and sex in the regression model. The effect of E×E interaction on MDD was significant (β = 0.746, SE = 0.312, z = 2.392, P = 0.016).

Haplotype Analysis

Haplotype analysis was performed for the total sample and subgroups. In the former, 12 haplotypes of five *FoxO1* gene polymorphisms, 15 haplotypes of five *TGF-β1* gene polymorphisms, and 14 haplotypes of *A2M* gene polymorphisms had a frequency > 1%. The haplotypes of the *FoxO1* gene showed a significant global association (χ^2 = 19.732, df = 13, P_{global} < 0.001) and the less common haplotype (G-A-G-C-T-A) of the *A2M* gene was significantly associated with MDD (χ^2 = 4.931, P_{effect} = 0.026). In subgroup samples, haplotypes of the *A2M* gene within the SLE occurrence group (χ^2 = 21.267, df = 11, P_{global} = 0.030) and haplotypes of *FoxO1* (χ^2 = 41.792, df = 11, P_{global} < 0.001) and *TGF-β1* (χ^2 = 34.531, df = 14, P_{global} = 0.001) genes within the CA occurrence group were significant in the global association. Two less common haplotypes (G-A-A-C-G-G and G-A-G-C-T-A) of the *A2M* gene within the SLE occurrence group (χ^2 = 5.815, P_{effect} = 0.015; χ^2 = 5.814, P_{effect} = 0.015) and a less common *A2M* haplotype (G-A-G-C-T-A) in the CA occurrence group (χ^2 = 3.947, P_{effect} = 0.046) were associated with MDD.

GRS Analysis

After controlling for FH and sex, logistic regression revealed that the 16 SNP GRSs comprising risk variants were associated with MDD (β = 0.022, SE = 0.010, z = 2.180, P = 0.029). Interaction analyses indicated that GRSs interacted with SLEs in MDD (β = 0.054, SE = 0.021, z = 2.577, P = 0.009).

Population Stratification Analysis

The combined population of YRI, CHB, and CEU exhibited obvious stratification (**Supplemental Figure S1A**). In the triangle chart with K = 3, each angle represented a potentially independent ancestry and the different colored dots represented individuals in assumed population components that did not cluster with the same color in the triangle, indicating that there was no significant stratification in our sample (**Supplemental Figure S1B**). Consistent results were obtained with K values ranging from two to six. Thus, population stratification was unlikely to be a confounding factor in this study.

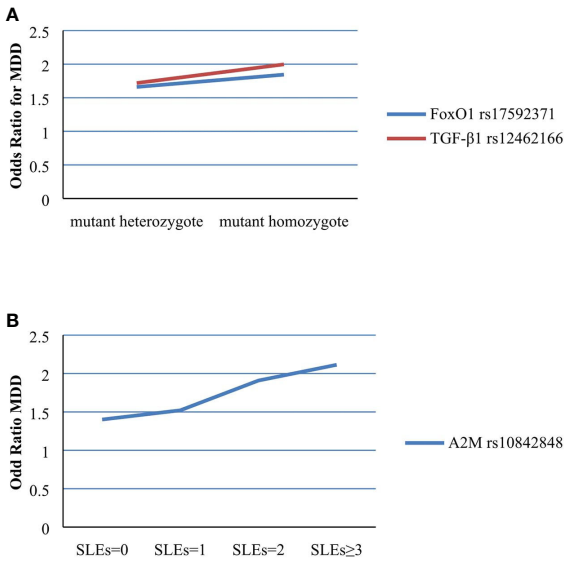

FIGURE 2 | (A) Effect of SLEs on MDD in different genotypes of rs17592371 in FoxO1 and rs12462166 in TGF-β1. **(B)** Effect of rs10842848 in A2M on MDD in different number of SLEs.

PPI Analysis

We detected interactions among FoxO1, A2M, and TGF-β1 proteins and the protein products of multiple MDD risk genes. The top susceptibility genes except for the *calcium voltage-gated channel subunit α1 C* gene, identified in GWASs of MDD (30–32) formed a densely interconnected PPI network (**Figure 3**) that included FoxO1, A2M, and TGF-β1. Interestingly, A2M was found to directly interact with FoxO1 and TGF-β1. TGF-β1 directly interacted with FOS, JUN, cyclic AMP response element-binding protein (CREB) 1, brain-derived neurotrophic factor, AKT1, and Mothers against decapentaplegic homolog (SMAD) 2; and FoxO1 directly interacted with FOS, JUN, CREB binding protein, SMAD2, AKT1, AKT2, and sirtuin 1. These factors have been implicated in MDD genetic risk studies (34–38).

DISCUSSION

The interaction between genetic markers and environment determines the risk of MDD (8). However, most hypothesis-driven studies of MDD to date have not produced reproducible findings. In this study, we investigated candidate MDD risk genes using a hypothesis-free, omics-based, cross-tissue and -species approach. We found that *FoxO1* rs17592371 and rs2297626, *A2M* rs669 and rs226415, and *TGF-β1* rs12462166 alleles were associated with MDD. We also found that *FoxO1* rs17592371 and rs28553411, *A2M* rs10842847, rs10842849, and rs226415, and *TGF-β1* rs12462166 and rs12983775 interacted with the environment in MDD, suggesting a potential relationship between these genes and the etiology of MDD, which could be a bona fide association or the result of linkage. A known allelic association between a gene and a disorder may reflect a G×E interaction, but the absence of such an association does not disqualify a gene as a candidate for disease risk. Some evidences showed that *FoxO1* could express in hippocampus and corpus striatum, *FoxO1* may contribute to the pathological

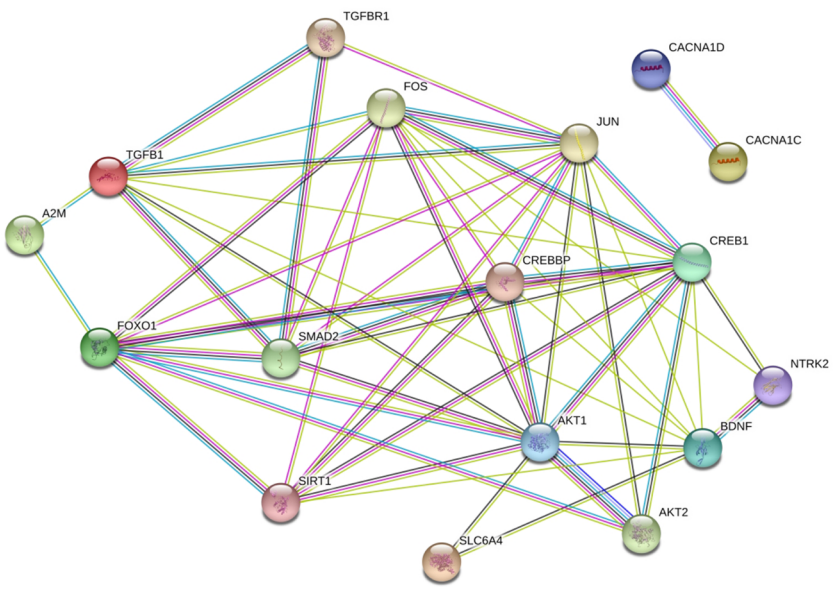

FIGURE 3 | Proteins encoded by FoxO1, A2M, and TGF-β1 in a densely interconnected protein-protein interaction (PPI) network formed by susceptibility genes of MDD. (33) The amaranthine lines indicate known PPIs that have been experimentally determined, and the light blue lines indicate known PPIs from curated databases. The green lines indicate predicted interactions from gene neighborhood, the dark blue lines indicate predicted interactions through gene co-occurrence and the black lines indicate known PPIs from co-expression. The light green lines indicate predicted PPIs through text mining.

process of MDD or other psychiatric disorders (18, 39). It was suggested that immune system associated with MDD and *A2M* played an important role in the immune system, which indicated that *A2M* may associate with MDD. Some studies found that the expression of *A2M* was higher in MDD patients comparing healthy people (21, 40). *TGF-β1* can restrain autoimmune response and maintain immune system stability, some studies found that *TGF-β1* could moderate the imbalance of proinflammatory cytokines and anti-inflammatory cytokines in MDD patients (15, 16). All of these evidences above were consistent with our findings.

The carrier status of the minor allele and number of SLEs were separately used to test different genotypic effects on MDD and the dose-response relationship between SLEs and MDD. The effect of SLEs on MDD was more highly significant for the TT than for the TC genotype of *FoxO1* rs17592371 and for the GG as compared to the GT genotype of *TGF-β1* rs12462166. These results indicated that mutant homozygous genotypes may be more significant than the heterozygous genotype for the effect of SLEs on MDD, as previously suggested (41, 42). We also found that exposure to SLEs was related to an increased risk for MDD, which is consistent with previous reports (42, 43), indicating that although the effect of a single environmental factor may be quite small, the cumulative effect of multiple factors may be large and that strong effects typically result from a chain of related events rather than a single factor.

Because gene variants can interact, we examined the SNPs that showed significant G×E interactions to investigate G×G interactions. We found that two polymorphisms in the *FoxO1* gene and the *A2M* gene showed significant interactions and that the G×G×E interaction was also significant. This was similar to a previous finding that *serotonin transporter-linked polymorphic region* (*5-HTTLPR*) gene and SNP rs140700 both interacted with the environment and with each other in MDD, and that the 5-HTTLPR×rs140700×E interaction was significant (41). Much of the genetic variation associated with a complex trait can be explained by the joint contribution of multiple genetic markers as well as environmental influence (44). G×G and G×G×E interaction analyses are considered as biologically relevant and explain the role of heritability (45–48). We also found that the E×E (CA × SLE) interaction in MDD was significant. CA increases the likelihood of SLE occurrence (49) that is, early negative experiences increase vulnerability to such events later in life, resulting in a sequential E×E interaction. For instance, mistreatment in childhood caused sensitization to the effects of specific types of SLE in adulthood (49). The present study is the first report of an E×E interaction in MDD.

Although direct associations between haplotype and psychiatric disorders have been reported (50, 51), there have been no studies demonstrating an interaction between haplotype and environmental factors. In our study, the global effect of haplotype on MDD was significant for the *FoxO1* and *TGF-β1* genes in the total sample and in subjects that had experienced CA. A significant individual effect and global effect of haplotype on MDD was found for the *A2M* gene in both the total and subgroup samples. The global effect of haplotype on MDD was always significant in combination with environmental factors. Interactions existed not only between the environment and genes, but also between the environment and haplotype in MDD.

GRS analyses have shown that the prediction of mental illness is improved by including more weakly associated genetic variants, suggesting that these influence the risk of mental disorder (21, 40). Our results showed that the association between 16 SNP GRS and MDD was significant, with a significant interaction between GRS and SLEs. Some of the 16 SNPs did not show a significant conditional or interaction effect. GRSs may detect the effects of weaker associated genetic variants (52). The results of the PPI analysis showed that FoxO1, A2M, and TGF-β1 were connected, suggesting that a wide variety of cellular processes are involved in MDD. This could explain the reason for the additive effects of *FoxO1*, *A2M*, and *TGF-β1* gene variants indicated by the GRSs. In addition, several top MDD susceptibility genes were included in this PPI network, although the clinical significance of these associations requires further investigation. The disturbances of certain cellular processes or pathways contributing to the risk of MDD has been emerging and gained evidences supporting (22, 53, 54). Constructing highly interconnected PPI networks between *FoxO1*, *A2M*, and *TGF-β1* protein and proteins of multiple defined risk genes for MDD may reveal the underlying biological mechanisms. *FoxO1*, *A2M*, and *TGF-β1* protein could participate in this PPI network, indicating their potential involvement in the common molecular network modulating the pathogenesis of MDD. It is noteworthy that *A2M* protein directly interacted with *TGF-β1* protein, indicating their connection in the biological process relevant to MDD. A recent study found that *A2M* as an inflammatory fluid proteinase scavenger could bind to a plethora of cytokines, including *TGF-β1*. (55)

We investigated the potential influence of population stratification in our samples using STRUCTURE software v.2.3.4 and the third-stage sample set, but did not find any evidence of stratification, which confirmed that the results were not affected by this confounding factor.

Our study had three major limitations. First, we did not use standard questionnaires that have confirmed reliability and validity to measure CA. Second, we did not analyze additional SNPs in our samples and the third-stage sample set, which can improve the reliability of population stratification analysis. Lastly, including a gene expression microarray analysis in our study would have allowed us to compare the transcriptomes of subjects exposed to SLEs/CAs and non-exposed individuals, which could provide more information for predicting MDD risk.

In conclusion, our data indicated that the hypothesis-free approach is useful for identifying novel genes contributing to the G×E interaction in MDD. *FoxO1*, *A2M*, and *TGF-β1* genes not only interact with environmental factors but also associate with multiple other genes in the MDD G×E interaction. *FoxO1*, *A2M*, and *TGF-β1* genes may serve the clinical diagnose and treatment of MDD by providing biomarkers, which can contribute to personalized medicine. Future studies will need to focus on the complexity of poly-gene–environmental causation to obtain more detailed insight into the etiology of MDD.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZ and LC conducted the statistical analyses and wrote the first draft of the manuscript. ZQ and JZ provided expertise in MDD search. TZ, WZ, and SK did the experiment. XZ, EZ, and XS collected the data. XQ and HP revised the manuscript. YY and XY designed this study and provided expertise. All authors were involved in modifying the secondary-analysis design and editing 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.2020.00675/full#supplementary-material

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Searching for the Needles in a Haystack; Is It Needless? The Search for Peripheral Biomarkers in Psychiatry

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INTRODUCTION

Psychiatric diagnoses rely on syndrome description based on experienced symptoms as reported by patients and further transformed into diagnoses based on a professional's (generally a doctor's) education, knowledge and, not least, clinical experience. This is a major challenge and also an Achilles heel in psychiatry. The lack of more precise biological measures creates exposure to criticism against the use of psychiatric diagnoses: on the one hand, that psychiatric diagnoses are non-existent and, on the other hand, the challenge of demarking severe mental illness (SMI) from many minor psychiatric diagnoses that seem less biologically driven. Nevertheless, research methodologies such as proteomics, transcriptomics, genomics, and brain imaging have advanced the pathophysiological understanding of SMI (e.g. schizophrenia, bipolar disorder, and major depressive disorders) in particular (1–3). Although these efforts may seem promising, we face a translational gap in clarifying to what extent such approaches can prove useful in the clinic and help diagnostics to support a targeted treatment choice and optimize treatment overall. In particular, blood, plasma, and serum are untapped sources of possible clinical and research-useful biomarkers (1). Despite an increasing number of studies on biomarkers, so far the area has not contributed to solid clinically improvements in diagnostics or clinical care.

DISCUSSION

This paucity of progress is likely to be due to several factors that, potentially, could be overcome. First, our categorical diagnostic classification systems—the International Classification of Diseases, 10th revision (ICD-10), and the Diagnostic and Statistical Manual of Mental Disorders, 5th revision (DSM-V)—lump very heterogenic syndromes together based on empirical experience and these categories are not directed towards capturing the underlying biology. Consequently, most clinical research anchored in current diagnostic systems will, by nature, create narrow research questions such as: Do we believe that there are significant biological differences on comparing two severely depressed patients, one with unipolar disorder and one with bipolar disorder? One way to circumvent these limitations is to use initiatives such as the Research Domain Criteria (RDoC)

framework (2), which has been created to integrate the observed behavioral information with neurobiological measures that incorporate multiple dimensions (behavior, thought patterns, neurobiological measures, and genetics), with the overall aim of generating a classification system that can be linked to the underlying dysfunctional pathways. Thus, future studies must look beyond the categorical diagnoses and use a transdiagnostic approach that includes a transdiagnostic assessment procedure for describing the individual behavior thoroughly. Along these lines, a systematic review (3) including the most promising peripheral biomarkers (BDNF, TNF-alpha, IL-6, C-reactive protein, and cortisol) concluded that these were most likely to be non-specific as diagnostic biomarkers (3). However, these biomarkers seem useful as an expression of illness activity and severity, thereby potentially being useful for treatment monitoring (3).

Second, the proteomic biomarker area must recognize that the earliest studies in particular are characterized by significant method limitations (4), such as low study quality (lack of consistency concerning the time of day when the biological samples are withdrawn; laboratory technique with a risk of prolonged storage before the biological samples have proceeded), poorly characterized samples, small sample sizes, and substantial unexplained between-study heterogeneity. Furthermore, limitations such as potential bias in individual studies, indications of publication bias, and lack of standard operating procedures for all aspects of the individual assessment can lead to non-replication and failed studies. These factors point to a need to improve the quality of future research. That said, knowledge from all these studies is essential because it can critically inform future warranted high-quality large-scale studies.

Third, it can be a problem to integrate animal models of SMI. However, close cooperation and infrastructures linking findings from animal models to clinical settings are beneficial (e.g. studying one biomarker in a mouse model and also observing the same potential biomarker in humans for both cases and controls). Integrating animal models is necessary to provide direct insight into the cellular metabolites that are produced during psychiatric processes. In addition, the influence on biomarkers due to short- or long-term medication can be observed under controlled conditions. Thus, a combination of representative animal models and human studies are a promising pathway to improve the potential use of biomarkers in psychiatry (5).

Fourth, there are huge commercial interests in this area. Companies are tempted to promise that their specially designed biomarker will capture the early signs of SMI and lead to better treatment results. This could be overly optimistic and will confuse the area, not least the patients. Thus, a combination of industry and academic groups that need to be funded can lead to being overly optimistic without a solid scientific basis for their promises should also be considered. Nevertheless, the impact of private companies' knowledge, ambitious research, and technology is indispensable and well-described transparent cooperation between university-driven research and industry is necessary.

Fifth, another reason for the many non-replicated findings seems to be the inclusion of very different patient populations: it is difficult to compare biomarkers in a sample of newly diagnosed patients with patients having a late-stage disorder characterized by many admissions, polypharmacy, etc. Furthermore, the present illness state is important because biomarkers often change according to the present state (e.g. depressed, manic, psychotic, or in remission). Therefore, it is mandatory to characterize each illness state and illness course (onset of the disorder, number of episodes) as biomarkers change over time and are sensitive to the impact of environmental factors (smoking, substance use, alcohol use), co-existent physical disorder, age and gender; characterization of the menstrual cycle in females should also be taken into account.

Sixth, the impact of psychotropic medication on biomarker measures is complicated and a major confounder in most studies. This problem is difficult to solve but in larger studies it is possible to include at-risk individuals who do not receive medication (e.g. first-degree relatives to patients with SMI). Twin studies can further help to distinguish between potential environmental and genetic origins of the biomarker signatures (6).

Finally, measuring peripheral biomarkers at only one time point and using a case-control design is not an optimal design. Much more useful information is available if the trajectories of the biomarker are considered; for example, it may help to discriminate trait and state and whether a potential change can be replicated over time. Repeated observations over time including trajectory information are of major interest and also when using biomarkers as monitors of treatment response. Unfortunately, most studies on peripheral molecular biomarkers are cross-sectional studies using one time point to compare patients with controls (3). However, studying biomarkers at several time points makes it possible to evaluate their potential as state and treatment monitors and their use in the prediction of treatment response.

PERSPECTIVES

Despite the above-described obstacles, overall there are, as described, promising peripheral biomarkers (3) that potentially will add to our future clinical tools and improve/impact clinical care. In particular, combining individual biomarkers across tissues and molecular systems seems to be a promising avenue for research in biomarker models (7, 8). However, instead of searching for the needles in the haystack, we need to collaborate and concentrate on describing all the elements in the haystack, integrating the inter-individual and intra-individual variability. At present, there is data and laboratory capacity and extended knowledge spread over multiple international research sites and industrial companies. However, creating well-structured multicenter studies is warranted, including mandatory structures for data sharing and integrated health information systems. Creating infrastructures and pipelines will move the field into the next stage of identifying pragmatically useful clinical biomarkers. This can be achieved by using innovative approaches and advanced technology (e.g. the R-

LiNK initiative aimed at optimizing response to lithium treatment through personalized evaluation of individuals with bipolar I disorder) (9).

Evaluating the potential use of biomarkers or clusters of biomarkers as clinical tools in prevention, to assist in developing personalized medicine and in treatment monitoring, with an overall goal to facilitate more and better treatments for patients with SMI, is clearly needed. Researchers are encouraged to initiate a strategic research agenda (e.g. across a diagnostic biobank consortium) that will require the use of precise biomarker protocols (e.g. in line with the CONSORT 2010 checklist of information to include when reporting randomized trials) (10). Before initiation, a checklist stating a range of criteria, including gold standards, in all aspects using standardized operational procedures for all included studies, should be completed. This could also facilitate harmonization and joint databases, as in other areas of medicine (e.g. the Biomarker for Cardiovascular Risk Assessment across Europe consortium) (11). Overall, improving the opportunities for identifying the needles in the haystack.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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A Novel Network Pharmacology Strategy to Decode Metabolic Biomarkers and Targets Interactions for Depression

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Depression is one of the most prevalent and serious mental disorders with a worldwide significant health burden. Metabolic abnormalities and disorders in patients with depression have attracted great research attention. Thirty-six metabolic biomarkers of clinical plasma metabolomics were detected by platform technologies, including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and proton nuclear magnetic resonance (^{1}H-NMR), combined with multivariate data analysis techniques in previous work. The principal objective of this study was to provide valuable information for the pathogenesis of depression by comprehensive analysis of 36 metabolic biomarkers in the plasma of depressed patients. The relationship between biomarkers and enzymes were collected from the HMDB database. Then the metabolic biomarkers-enzymes interactions (MEI) network was performed and analyzed to identify hub metabolic biomarkers and enzymes. In addition, the docking score-weighted multiple pharmacology index (*DSWMP*) was used to assess the important pathways of hub metabolic biomarkers involved. Finally, validated these pathways by published literature. The results show that stearic acid, phytosphingosine, glycine, glutamine and phospholipids were important metabolic biomarkers. Hydrolase, transferase and acyltransferase involve the largest number of metabolic biomarkers. Nine metabolite targets (TP53, IL1B, TNF, PTEN, HLA-DRB1, MTOR, HRAS, INS and PIK3CA) of potential drug proteins for treating depression are widely involved in the nervous system, immune system and endocrine system. Seven important pathways, such as PI3K-Akt signaling pathway and mTOR signaling pathway, are closely related to the pathology mechanisms of depression. The application of important biomarkers and pathways in clinical practice may help to improve the diagnosis of depression and the evaluation of antidepressant effect, which provides important clues for the study of metabolic characteristics of depression.

Keywords: metabolic biomarkers, depression, network pharmacology, drug-target network, docking score-weighted multiple pharmacology index (*DSWMP*)

INTRODUCTION

Depression is one of the most prevalent and serious mental disorders. In recent years, the number of patients with depression has increased dramatically. In the world's population, the lifetime prevalence of depressed patients is about 17% with a significant burden of disease (1). Previous research reports have found that in the United States, more than 19 million adults suffer from depression in the USA and spend more than 30 billion annually, directly or indirectly (2). In addition, the incidence of depression is about 3 to 5% and currently 26 million Chinese people suffer from depression (3). Depression is a major cause of neuropsychiatric disability worldwide and the accurate diagnosis of depression before treatment is a hub change (4). Increased studies have reported the serotonin and norepinephrine dysfunction in the central nervous system of patients with depression (5). In addition, studies have also reported that hypothalamic pituitary adrenal (HPA) axis is one of the largest neuroendocrine findings of depression (6). Abnormal changes of inflammatory cytokines and endogenous metabolites are also involved in the molecular mechanism of pathology of depression (7, 8). The occurrence and development of depression is a complicated process, the etiology and pathogenesis mechanism of depression represent challenging issues in scientific and medical research.

A new psychological immune neuroendocrine (PINE) network model on depression has been proposed to provide an in-depth understanding of the pathogenesis of depression and the treatment of the disease with antidepressant drugs (1). The PINE network model is composed of three parts of the central nervous system, immune, and endocrine molecular networks, and the three networks are interconnected (9). The three molecular networks consist of many nodes and edges. The nodes in the network can be small molecules with different properties, such as genes, proteins or metabolites. If different nodes are related in biological function, it can be connected by edges. How to determine the key underlying molecular mechanism from PINE network that plays leading roles in the depression is a difficult problem due to the high complexity of the network composition and the incompletely understanding the complex multi-targets mechanism of depression.

Network pharmacology is constructed by integrating pharmacological data and network analysis methods to provide a comprehensive approach to explain the disease pathogenesis and drug treatment mechanism (10). The network pharmacology technology is considered to be one of the next frontiers of new drug research (11). Recently, network pharmacology has been widely used in the pathogenesis of complex diseases and the mechanism of drug action. For example, Huang decoded the mechanism of traditional Chinese medicine in treating depression by analyzing drug target networks and disease target networks (12).

Increased studies have reported metabolic disorders or abnormal metabolic pathways in the plasma of depressed patients (13, 14). Thirty-six metabolic biomarkers of clinical plasma metabolomics were detected by platform technologies, including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and proton nuclear magnetic resonance (^{1}H-NMR), combined with multivariate data analysis techniques in previous our work (13, 14). These metabolic biomarkers are distributed in disordered metabolic pathways and maybe potential diagnostic biomarkers or therapeutic markers. Therefore, how to use the network pharmacology method to mine the existing markers is of great significance to the study of the metabolic mechanism of depression.

The main purposes of this study were to comprehensively analyze plasma metabolites by network pharmacology method and provide valuable information for the pathogenesis of depression. During this process, the relationship between biomarkers and enzymes were collected from the HMDB database and the metabolic biomarkers–enzymes interactions (MEI) network was performed and analyzed to identify hub metabolic biomarkers and enzymes. In addition, the interactions between each MB and each target of the nervous system, immune system and endocrine system was calculated by systemsDock, and then the docking score-weighted multiple pharmacology index (*DSWMP*) was used to assess the importance pathways of hub metabolic biomarkers involved. Finally, the important pathways were verified through published literature. The application of important biomarkers and pathways in clinical practice may help to improve the diagnosis of depression and the evaluation of antidepressant effect, which provides important clues for the study of metabolic characteristics of depression.

METHODS

Metabolites Data Collection and Processing

For the research object, only the metabolic biomarkers of clinical plasma metabolomics in our consideration, because the excavation of metabolites is more meaningful in the same clinical sample. Candidate metabolites refer to the statistical significance found in the original study. Unique metabolites were obtained by removing duplicates. We obtained biological function information and structural data of identified metabolic biomarkers from the Human Metabolome Database (HMDB) (15). The structures of these compounds were downloaded from PubChem (16). Enzymes related to the metabolic biomarkers were summarized from HMDB, while the functional categories of the relevant proteins were found from the UniProt database (17). The target proteins related to the nervous system (**Table S1**), immune system (**Table S2**) and endocrine system (**Table S3**) were retrieved from several Published online databases. The FDA-approved drugs for the nervous system, immune system

Abbreviations: ^{1}H-NMR, Proton nuclear magnetic resonance; APT-2, Acyl-protein thioesterase 2; ATP, Adenosine triphosphate; CMS, Chronic mild stress; CNS, Central nervous system; CoA, Acetyl-coenzyme A; CPLA2, Cytosolic phospholipase A2; EPA, Eicosapentaenoic acid; DSWP, Docking score-weighted polypharmacological index; HMDB, Human Metabolome Database; HPA, Hypothalamic pituitary adrenal; HRAS, HRas Proto-Oncogene; LC–MS, Liquid chromatography–mass spectrometry; GC–MS, Gas chromatography–mass spectrometer; MEI, Metabolic Biomarker–enzyme interactions; PTEN, Phosphatase and tensin homolog; MTI, Metabolic Biomarker–target interactions; MTOR, Rapamycin Kinase; PIK3CA, Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit alpha isoform; PINE, Psycho-immune-neuroendocrine; TP53, Tumor Protein P53; TNF, Tumor necrosis factor.

and endocrine system with their targets were collected from DrugBank (18). The depression-related proteins were retrieved from Genecards (19), OMIM (20) and TTD (21). Finally, we carefully checked the target proteins in the literature. Integrate all target proteins and divide them into three categories, including the nervous system, immunity and endocrine.

Molecular Docking

Molecular docking was used to investigate the affinity between metabolic biomarkers and targets. The protein 3D structures were achieved from the RCSB protein data bank (http://www.rcsb.org) (22). We chose structures with more complete peptide chains, higher resolution, and better ligands as the selection criteria for the most appropriate protein structure.

SystemsDock (http://systemsdock.unit.oist.jp/) (23) was used for network pharmacology prediction and analysis, including four specific steps, selecting proteins by different parameters, defining binding sites by interactive molecular visualizer, preparing metabolic biomarkers for the test, and performing docking simulation and evaluating the result. The docking score calculated by systemsDock was used to assess the interactions between the metabolic biomarkers and the target proteins.

Mapping Metabolic Biomarkers–Target/Enzyme Network

Metabolic biomarker–enzyme relationships were collected from HMDB and visualized by using Cytoscape 3.4.0 (24). The network topological properties of nodes (metabolic biomarkers and enzymes) were calculated by the NetworkAnalyzer Cytoscape plugin (25). In an undirected network, the degree centrality of a node denotes the number of edges connected to this node is commonly used to measure the importance of a node in a single-layer network (26, 27). Degree centrality is a key indicator in analyzing the network, thereby reflecting the importance and influence of a metabolome biomarker or an enzyme in the metabolic biomarkers–enzyme interactions (MEI) network.

The threshold of the docking score was set to 5.52 (pK_d), which was equal to the dissociation constant (K_d) of 3 μM. A docking score greater than the threshold was considered to be a very good correlation between the metabolic biomarker and the target protein (23). Based on previously reported works and experimental findings, a high accuracy level (80–83%) was found to evaluate the binding of a metabolomic biomarker and a protein, when the threshold score was in the range of 4.82 to 6.11 (pK_d). The metabolic biomarkers-target interactions (MTI) network was visualized by Cytoscape software.

Docking Score-Weighted Multiple Pharmacology Index

The target-related pathways were collected from Kyoto Encyclopedia of Genes and Genomes (KEGG) (28). One metabolic biomarker can be combined with many targets, and one target can be enriched to function in many different biological pathways. Therefore, a metabolic biomarker involves multiple different biological pathways. At present, there is no method to directly confirm that the metabolic biomarkers are directly enriched in the pathway through the targets, so we have defined a docking score-weighted multiple pharmacology index (*DSWMP*):

$$DSWMP \quad (P_k) = \frac{\Sigma_i^N \Sigma_j^M DS_{BiTj}}{Count \quad (B_i)}, \quad T_j \quad \in \quad TP_k \qquad (1),$$

where DS_{BiTj} is the affinity between metabolic biomarker B_i and target T_j, and TP_k is a group of targets involved in pathway P_k. N and M are the numbers of metabolic biomarkers and targets, respectively.

Experimental Evidence of Important Pathways

To find experimental evidence for the predicted important pathways of depression by *DSWMP* calculation, the published literature will be identified by searching Pubmed, Embase, Cochrane Central Register of Controlled Trials and Web of Science. In the end, we chose the literature to verify the important pathways we found and provide references for the next step of research.

RESULTS AND DISCUSSION

In this report, a novel network pharmacology strategy was designed to detect the important pathway and elucidate the molecular mechanisms of depression (**Figure 1**). Firstly, the relationship between biomarkers and enzymes were collected from the HMDB database and the MEI network was performed and analyzed to identify hub metabolic biomarkers and enzymes. Secondly, the interactions between each MB and each target were calculated by systemsDock, and then the *DSWMP* was used to assess the important pathways of hub metabolic biomarkers involved. Thirdly, the important pathways verified through published literature.

Metabolic Biomarker–Enzyme Interactions (MEI) Network Analysis

The information of 36 metabonomic biomarkers and 350 enzymes are shown in **Table S4**. Most enzymes related to metabolic biomarkers belong to hydrolases, transferases and acyltransferases, which is consistent with our previous results. These enzymes mainly regulate metabolites such as energy metabolism, amino acid metabolism, gut microbe metabolism, glycerophospholipid metabolism and sphingolipid metabolism (13, 14). These enzymes play an important role in the regulation of metabolic pathways, which affect the molecular mechanism of depression. Overall, the results show that functional studies of enzymes related to metabolic biomarkers can reflect the molecular mechanism of depression, which also provides a reliable reference for the next network analysis.

The MEI network was used to characterize the relationship between enzymes and metabolic biomarkers, as shown in **Figure 2**. In MEI network, the node represents a metabolic biomarker or an enzyme, and the edges represent the interactions between them originated from HMDB. It turns out that the concentrations of some biomarkers in depression patients are up-regulated, while

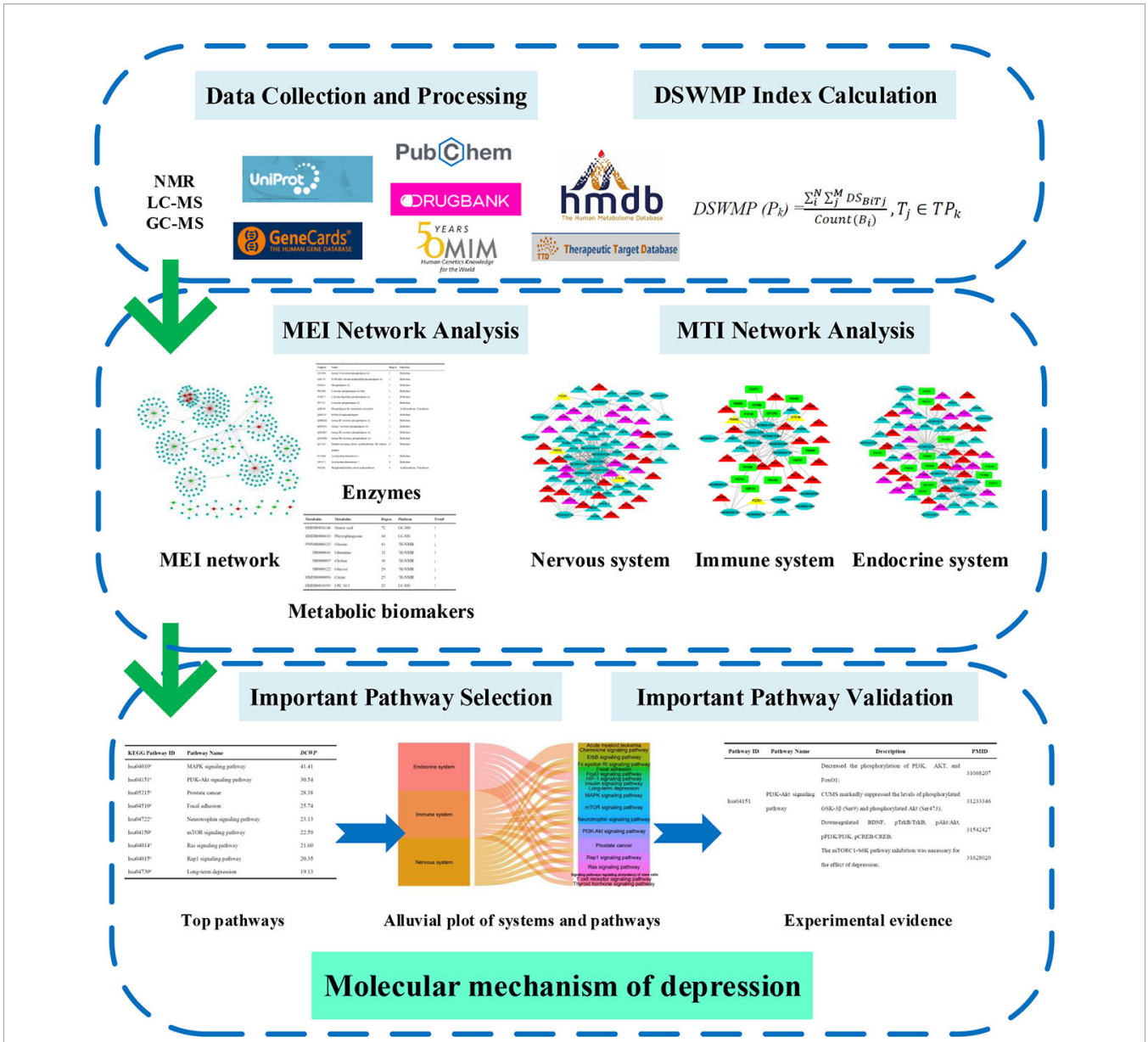

FIGURE 1 | The flowchart of network pharmacology approach.

others are down-regulated. Among these regulated biomarkers, their related enzymes form a particularly large cluster around them, suggesting that multiple enzymes regulate the same metabolite. **Tables 1** and **2** show the important enzymes and biomarkers identified by the network pharmacology approach, respectively. Among these data, previous literatures reported that cytosolic phospholipase A2 (cPLA2) is an important enzyme for PUFA metabolism and PGE 2 synthesis, which is the pivotal to the mode action of mood stabilizers in animal studies (29, 30). In addition, in peripheral blood cells of patients with depression, the mRNA expression of the gene encoding COX-2 increases sharply, which plays an important role in the pathogenesis of depression (31).

Among these metabolite-related enzymes, the phospholipase A2 family is related to many metabolites, so it is also important to study the function of depression. Previous research reports suggest that different phospholipase A2 types are associated with somatic symptoms of depression (32). It is believed that genetic variation of the phospholipase A2 gene increases the risk factor of depression induced by IFN-α (32). In addition, phospholipase A2 inhibitors have potential therapeutic effects in treating inflammation-related diseases, such as depression (33). Moreover, eicosapentaenoic acid (EPA) could up-regulate the expression of cytosolic phospholipase A2 gene and play an antidepressant effect in clinic, which shows the superiority of EPA antidepressant effect (34). Another research group also found that the same G allele of

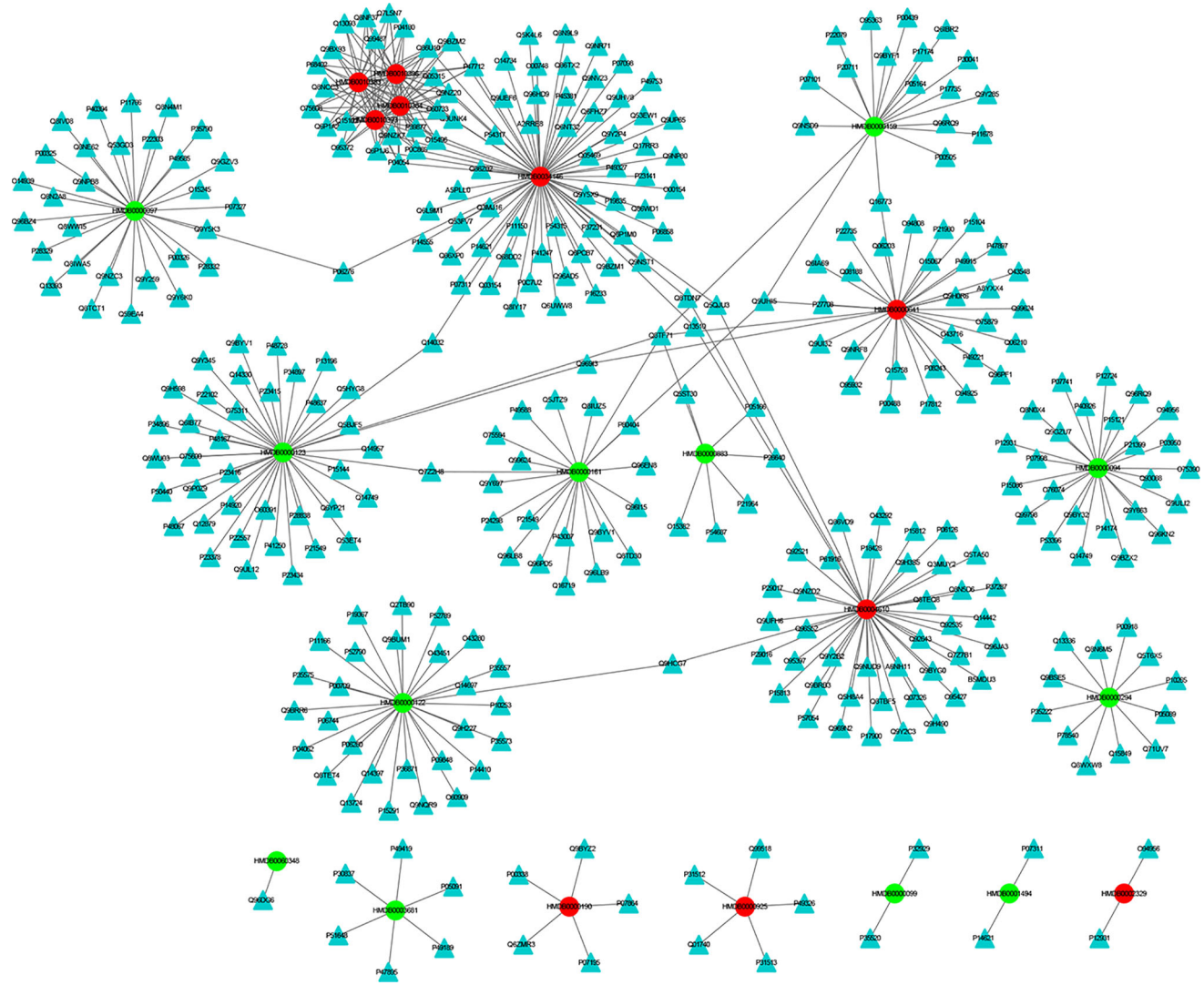

FIGURE 2 | The MEI network. The cycle and triangle represent metabolic biomarker and enzyme, respectively. The color of cycle is green or red if the concentration of the metabonomic biomarker is down-regulated or up-regulated, respectively.

TABLE 1 | Important enzymes identified by network topology analysis of MEI. network.

Uniprot	Name	Degree	Function
O15496	Group 10 secretory phospholipase A2	5	Hydrolase
O60733	85/88 kDa calcium-independent phospholipase A2	5	Hydrolase
P04054	Phospholipase A2	5	Hydrolase
P0C869	Cytosolic phospholipase A2 beta	5	Hydrolase
P39877	Calcium-dependent phospholipase A2	5	Hydrolase
P47712	Cytosolic phospholipase A2	5	Hydrolase
Q6P1J6	Phospholipase B1, membrane-associated	5	Acyltransferase, Transferase
Q86U10	60 kDa lysophospholipase	5	Hydrolase
Q9BZM2	Group IIF secretory phospholipase A2	5	Hydrolase
Q9NZ20	Group 3 secretory phospholipase A2	5	Hydrolase
Q9NZK7	Group IIE secretory phospholipase A2	5	Hydrolase
Q9UNK4	Group IID secretory phospholipase A2	5	Hydrolase
Q15102	Platelet-activating factor acetylhydrolase IB subunit gamma	4	Hydrolase
O75608	Acyl-protein thioesterase 1	4	Hydrolase
O95372	Acyl-protein thioesterase 2	4	Hydrolase
P04180	Phosphatidylcholine-sterol acyltransferase	4	Acyltransferase, Transferase
P68402	Platelet-activating factor acetylhydrolase IB subunit beta	4	Hydrolase
Q13093	Platelet-activating factor acetylhydrolase	4	Hydrolase
Q6P1A2	Lysophospholipid acyltransferase 5	4	Acyltransferase, Transferase
Q7L5N7	Lysophosphatidylcholine acyltransferase 2	4	Acyltransferase, Transferase
Q8NCC3	Group XV phospholipase A2	4	Acyltransferase, Hydrolase, Transferase
Q8NF37	Lysophosphatidylcholine acyltransferase 1	4	Acyltransferase, Transferase
Q99487	Platelet-activating factor acetylhydrolase 2, cytoplasmic	4	Hydrolase
Q14032	Bile acid-CoA:amino acid N-acyltransferase	2	Acyltransferase, Hydrolase, Serine esterase, Transferase
P06276	Cholinesterase	2	Hydrolase, Serine esterase
Q13510	Acid ceramidase	2	Hydrolase
Q16773	Kynurenine–oxoglutarate transaminase 1	2	Aminotransferase, Lyase, Transferase
Q5QJU3	Alkaline ceramidase 2	2	Hydrolase
Q8TDN7	Alkaline ceramidase 1	2	Hydrolase
Q969I3	Glycine N-acyltransferase-like protein 1	2	Acyltransferase, Transferase
Q9HCG7	Non-lysosomal glucosylceramidase	2	Glycosidase, Hydrolase

TABLE 2 | Important metabolic biomarkers identified by network topology analysis of MEI network.

Metabolite	Metabolite	Degree	Platform	Trend[a]
HMDB0034146	Stearic acid	72	GC–MS	↑
HMDB0004610	Phytosphingosine	44	LC–MS	↑
HMDB0000123	Glycine	41	^{1}H-NMR	↓
HMDB0000641	Glutamine	32	^{1}H-NMR	↑
HMDB0000097	Choline	30	^{1}H-NMR	↓
HMDB0000122	Glucose	29	^{1}H-NMR	↓
HMDB0000094	Citrate	25	^{1}H-NMR	↓
HMDB0010393	LPC 10:3	25	LC–MS	↑
HMDB0010383	LPC 16:1	25	LC–MS	↑
HMDB0010396	LPC 21:4	25	LC–MS	↑
HMDB0010384	LPC 18:0	25	LC–MS	↑
HMDB0000161	Alanine	21	^{1}H-NMR	↓
HMDB0000159	Phenylalanine	19	^{1}H-NMR	↓
HMDB0000294	Urea	12	GC–MS	↓
HMDB0000883	Valine	7	^{1}H-NMR/GC–MS	↓
HMDB0003681	4-Acetamidobutanoic acid	6	LC–MS	↓
HMDB0000190	Lactate	5	^{1}H-NMR	↑
HMDB0000925	TMAO	5	^{1}H-NMR	↑
HMDB0002329	Oxalic acid	2	GC–MS	↑
HMDB0000099	L-Cystathionine	2	LC–MS	↓
HMDB0001494	Acetylphosphate	2	LC–MS	↓
HMDB0060348	4-oxohex-2-enedioic acid	1	LC–MS	↓

[a]*:The fold change was calculated as the ratio of the concentration of metabolite of the depression to that of healthy control.*

the PLA2 BanI polymorphism is one of the risk factors for depression in Korean populations (35). The expression levels of acyl protein thioesterase 2 (APT-2) and oleamide are increased in chronic mild stress (CMS). This provides a reference for the treatment of depression by targeting these proteins (36). At present, there is no literature on the relationship between acetylhydrolase and depression, which may be a novel research point.

Fourteen metabolic biomarkers are associated with more than ten enzymes in MEI network (**Table 2**). The concentrations of most MB were down-regulated, while stearic acid, phytosphingosine, glutamine and phospholipids were up-regulated. It is well known that fatty acids are an important source of human body production and storage. Acetyl-coenzyme A (CoA) produced by oxidation of β-fatty acids can produce adenosine triphosphate (ATP) in the TCA cycle and could be converted to ketone bodies for storage in the kidney and liver (37). There are reports showing that the concentration of stearic acid in plasma of patients with depression is significantly increased, which may lead to blockage of fatty acid transport and inhibition of TCA cycle, which is consistent with the previous research results (38, 39). Moreover, the AUCs for oxalic acid and stearic acid were >0.7, indicating a great clinical diagnostic value. Therefore, stearic acid may be a diagnostic indicator of depression. These results indicate that the metabolic biomarkers in MEI network are involved in the molecular mechanism of depression.

In addition, most metabolic biomarkers belong to long-chain fatty acids and phytosphingosine, which indicates that the lipid metabolism disorder plays a crucial role in the mechanism of depression. These metabolic biomarkers are involved in glycerophospholipid metabolism and sphingolipid metabolism. Among them, sphingosine involves various biological processes, including cell–cell interactions, cell proliferation, differentiation and apoptosis. Studies have reported that elevated levels of hemolytic phosphatidylcholine also increase oxidative stress, which is a key factor in the onset of depression (40). The results indicate that the long-chain fatty acids and phytosphingosine in MEI network is involved in the pathological mechanism of depression.

Glutamine and its related enzymes form an independent part of the MEI network, suggesting that glutamine may play an unusual role in the pathogenesis mechanism of depression. Glutamate is associated with the neurobiology of depression and can cause neurotoxicity if over-released (40). In addition, glutamine and glutamate can be converted between neurons and astrocytes, which is necessary for the steady state of the glutamine-glutamic acid cycle (41). Therefore, the increase in glutamine in the plasma of depression patients may be a compensatory adaptation to glutamate-induced neurotoxicity. The results show that the glutamine in the MEI network is involved in the pathological mechanism of depression.

Metabolic Biomarker–Target Interactions (MTI) Network Analysis

Tables S1–S3 show the nervous system, immune system and endocrine system target proteins, and the nervous system,

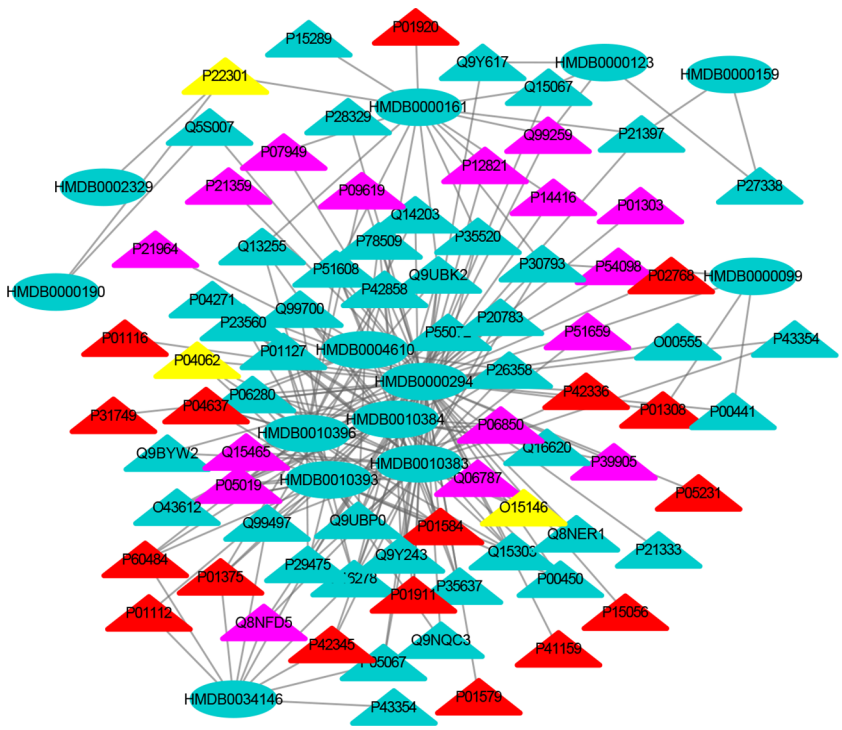

FIGURE 3 | The MTI network of the nervous system. The ellipses refer to metabolic biomarkers, and triangles refer to targets, respectively. The color of the nodes indicates that the three systems connected to depression are different, including nervous system, immune system and endocrine system. The colors of the nodes are blue, yellow, and red, indicating that the target is connected to one system, two systems, and three systems, respectively.

immune system and endocrine system target numbers are 155, 60 and 125, respectively. The interactions between each metabolic biomarker and the target protein were performed by molecular docking. The MTI network of nervous system, immune system and endocrine system were visualized by Cytoscape, respectively (**Figures 3**–**5**). In MTI network, the node represents a metabolic biomarker or a target, and the edges represent the interactions between them from the docking score meeting the set threshold. The metabolic biomarker links the target to a highly interconnected cluster. In order to analyze the correlation between metabolic biomarkers and targets and to mine important molecules, we calculated the topological parameter of the network, namely degree centrality.

The MTI network of the nervous system (**Figure 3**) contains 13 metabolic biomarkers and 78 targets, resulting in 232 edges associations between them. The average number of targets for each metabolic biomarker is 17.85 and the average number of metabolic biomarkers for each target is 2.97. Similarly, the MTI network of the immune system (**Figure 4**) contains 13 metabolic biomarkers and 37 targets, resulting in 107 edges associations between them. The average number of targets for each metabolic biomarker is 8.23 and the average number of metabolic biomarkers for each target is 2.89. Moreover, the MTI network of the endocrine system (**Figure 5**) contains 11 metabolic biomarkers and 67 targets, resulting in 78 edges associations between them. The average number of targets for each metabolic biomarker is 15.91 and the average number of metabolic biomarkers for each target is 2.61. This indicates that the molecular mechanism of depression in the nervous system, immune system and endocrine system has the characteristics of multiple metabolic biomarkers and multiple targets.

In these three MTI networks, the three metabolic biomarkers of urea, LPC 16:1 and LPC 18:0 have a higher degree centrality in the network. The number of targets that the three metabolic biomarkers could bind to in the depression-related nervous system, immune system and endocrine system are 68, 33, 61; 40, 18, 25; 29, 13, 20, respectively. The role of these metabolic biomarkers in the pathogenesis of depression is worthy of our in-depth study, because these metabolic biomarkers play a crucial role in the three networks. Among these metabolic biomarkers, urea, LPC 16:1 and LPC 18:0 can be classified as organic carbonic acids or glycerophospholipids, and further research is needed on their role in the pathogenesis of depression.

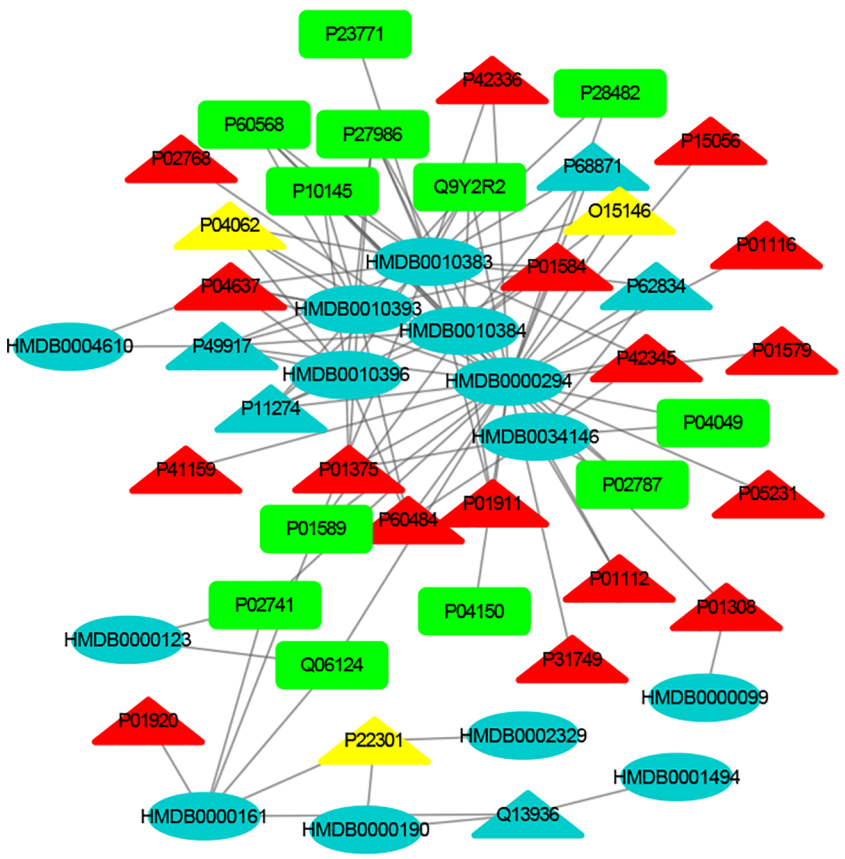

FIGURE 4 | The MTI network of the immune system. The ellipses refer to metabolic biomarkers, and triangles refer to targets, respectively. The color of the nodes indicates that the three systems connected to depression are different, including nervous system, immune system and endocrine system. The colors of the nodes are blue, yellow, and red, indicating that the target is connected to one system, two systems, and three systems, respectively.

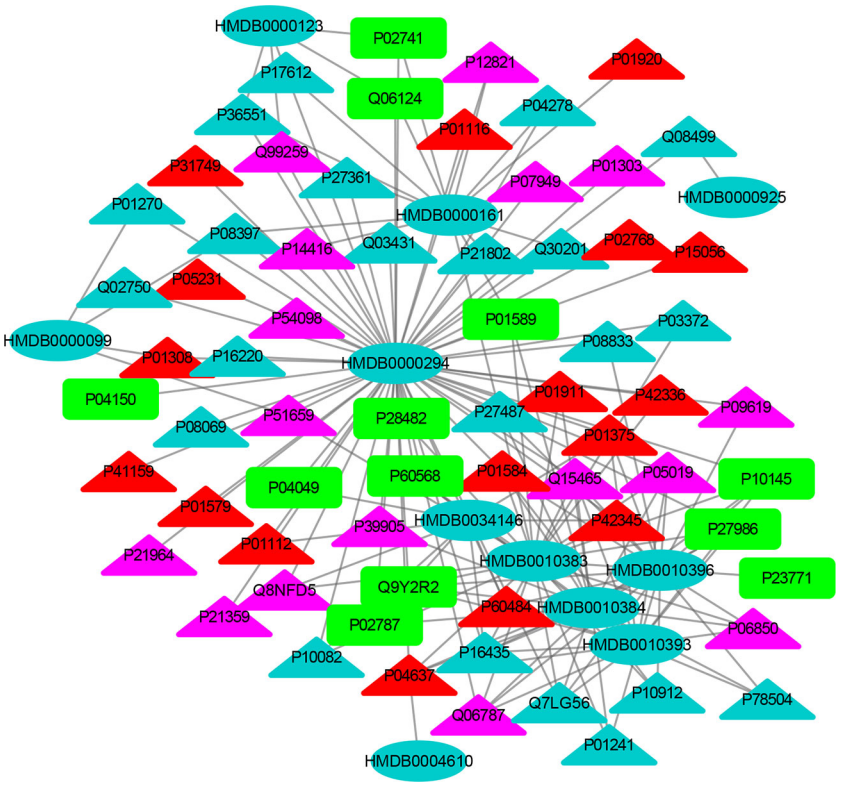

FIGURE 5 | The MTI network of the endocrine system. The ellipses refer to metabolic biomarkers, and triangles refer to targets, respectively. The color of the nodes indicates that the three systems connected to depression are different, including nervous system, immune system and endocrine system. The colors of the nodes are blue, yellow, and red, indicating that the target is connected to one system, two systems, and three systems, respectively.

There are nine identical targets in these three systems, which simultaneously play an important role in the three MTI networks involved in the pathogenesis of depression. Among these targets related to metabolic biomarkers, P04637 (Tumor Protein P53), P01584 (Interleukin 1 Beta), P01375 (Tumor Necrosis Factor), P60484 (Phosphatase And Tensin Homolog), P01911 (Major Histocompatibility Complex, Class II, DR Beta 1), P42345 (Mechanistic Target of Rapamycin Kinase), P01112 (HRas Proto-Oncogene, GTPase), P01308 (Insulin), P42336 (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), were widely involved in the nervous system, immune system and endocrine system.

The minor allele 72C of the tumor protein P53 (TP53) gene plays a protective role in the occurrence of depression. It participates in the pathological mechanism of depression through the cell survival and death regulation (42). Previous studies have reported that proinflammatory cytokines such as IL-1 beta (IL1B) (43) and tumor necrosis factor (TNF) (44) have been implicated in the pathogenesis molecular mechanism of depression. The evidence provided by Liu suggests that the rs701848, rs2735343 and rs112025902 polymorphisms in the phosphatase and tensin homologous gene (PTEN) genes may be related to the risk of depression in Chinese (45). Major Histocompatibility Complex (HLA-DRB1) plays a significant role in the immune system by presenting peptides derived from extracellular proteins (46). HRas Proto-Oncogene (HRAS) (46) can encode some genes in signal transduction pathways. These proteins can be linked to GTP and GDP, and they have their own GTPase activity. In addition, rapamycin kinase (mTOR) is a serine/threonine kinase that regulates cell proliferation (47). Li research found that the activation of mTOR in the prefrontal cortex of rats was one of the important mechanisms by which ketamine exerts antidepressant effects (48). Some related studies have also found that acute ketamine administration will activate mTOR in the peripheral blood of patients with depression (49). The antioxidant alpha lipoic acid has been shown to increase insulin (INS) sensitivity and has been used to treat diabetic patients. INS also plays an important role in the pathogenesis of depression. Therefore, the nutrient alpha lipoic acid should be clinically tested as an adjunct treatment for depression. Therefore, some scholars suggested that the nutrient alpha lipoic acid should be used as an adjunct treatment for depression (50). Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA) inhibitors used in bipolar disease and depression (51). Consequently, these results demonstrated that the crucial roles of nine identical targets in the treatment of depression and further confirmed that drug works in a multi-targets manner to treat depression.

TABLE 3 | Top ten pathways for the nervous system, immune system and endocrine system.

KEGG Pathway ID	Pathway Name	*DCWP*
hsa04010[a]	MAPK signaling pathway	41.41
hsa04151[a]	PI3K-Akt signaling pathway	30.54
hsa05215[a]	Prostate cancer	28.38
hsa04510[a]	Focal adhesion	25.74
hsa04722[a]	Neurotrophin signaling pathway	23.13
hsa04150[a]	mTOR signaling pathway	22.59
hsa04014[a]	Ras signaling pathway	21.60
hsa04015[a]	Rap1 signaling pathway	20.35
hsa04730[a]	Long-term depression	19.13
hsa04012[a]	ErbB signaling pathway	18.50
hsa04151[b]	PI3K-Akt signaling pathway	25.21
hsa04062[b]	Chemokine signaling pathway	23.30
hsa05215[b]	Prostate cancer	22.58
hsa04150[b]	mTOR signaling pathway	22.29
hsa04919[b]	Thyroid hormone signaling pathway	19.97
hsa05221[b]	Acute myeloid leukemia	18.45
hsa04012[b]	ErbB signaling pathway	18.45
hsa04664[b]	Fc epsilon RI signaling pathway	18.41
hsa04722[b]	Neurotrophin signaling pathway	18.10
hsa04910[b]	Insulin signaling pathway	17.49
hsa04151[c]	PI3K-Akt signaling pathway	37.33
hsa04150[c]	mTOR signaling pathway	25.73
hsa04068[c]	FoxO signaling pathway	25.71
hsa05215[c]	Prostate cancer	25.11
hsa04660[c]	T cell receptor signaling pathway	25.07
hsa04066[c]	HIF-1 signaling pathway	23.17
hsa04015[c]	Rap1 signaling pathway	23.16
hsa04014[c]	Ras signaling pathway	23.14
hsa04010[c]	MAPK signaling pathway	21.95
hsa04550[c]	Signaling pathways regulating pluripotency of stem cells	20.65

[a,b], and [c]: the pathway was related to target of nervous system, immune system and endocrine system, respectively.

Important Pathways Selection and Validation

In order to improve the therapeutic effect while reducing side effects, drug treatment of diseases usually go through a variety of target methods (52). These targets involve multiple different pathways of disease pathology. The target-pathway approach is a disease-specific research module, which has been widely used in disease pathology exploration and drug development. To find the important pathological mechanism of depression, the strategy of *DSWMP* index was applied and calculated to select crucial pathway, which provides a methodological reference for the research and development of disease. The *DSWMP* index is an indicator to evaluate the importance of the pathway. The size of the DSWMP index is determined by the number of metabolic biomarkers binding targets and the binding energy between them. In other words, the DSWMP index method can be used to evaluate the importance of metabolic biomarkers in pathways.

There are 105, 113 and 125 metabolic biomarkers-related targets involved in the nervous system pathways immune system and endocrine system of depression, respectively. In each function, the top 10 pathways of *DSWMP* index are shown in **Table 3** and **Figure 6**. Among these pathways, the pathways of hsa04151 (PI3K-Akt signaling pathway) and hsa04150 (mTOR signaling pathway) are top-ranked in the pathological mechanism of depression among the nervous system, immune system and endocrine system, indicating that these two pathways play an important role in the pathogenesis of depression. The hsa04151 has been reported involved in the inhibition of apoptosis, cell proliferation and expression of inflammatory cytokines (53, 54). The hsa04150 pathway connects intracellular and extracellular signaling communication, and plays an important role in the protein

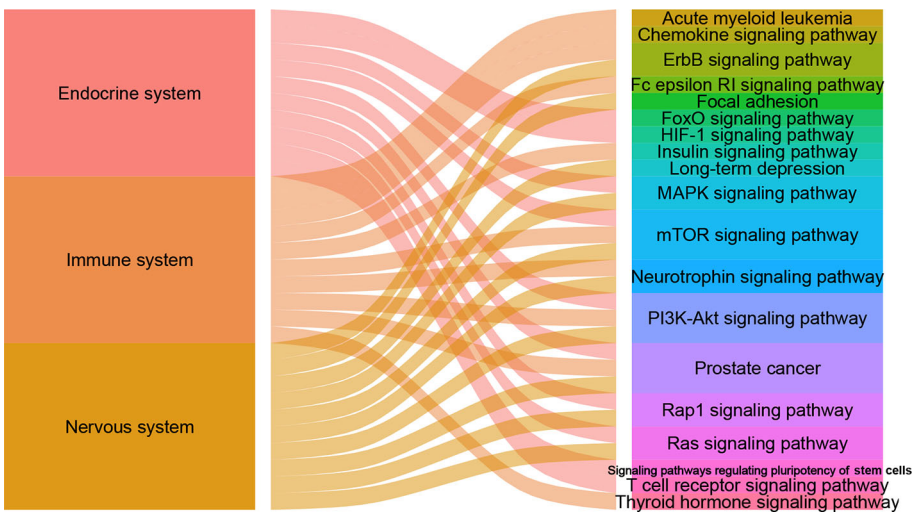

FIGURE 6 | The alluvial plot of systems and pathways. The left column represents nervous system, immune system and endocrine system, the right represents pathways, and the edge represents the relationship between them. A larger edge width indicates the number of pathways-linked systems.

TABLE 4 | Experimental evidence for the seven important signaling pathways with most significant therapeutic relationships of depression.

Pathway ID	Pathway Name	Description	PMID
hsa04151	PI3K-Akt signaling pathway	Decreased the phosphorylation of PI3K, AKT, and FoxO1;	31068207
		CUMS markedly suppressed the levels of phosphorylated GSK-3β (Ser9) and phosphorylated Akt (Ser473);	31233346
		Downregulated BDNF, pTrkB/TrkB, pAkt/Akt, pPI3K/PI3K, pCREB/CREB;	31542427
hsa04150	mTOR signaling pathway	The mTORC1-S6K pathway inhibition was necessary for the effect of depression;	31628020
		Reduction in the levels of mature BDNF and mTOR (Ser2448) phosphorylation in the hippocampus;	31078612
		UCMS-induced reductions of p70S6K and post-synaptic density 95 (PSD-95) mRNA levels, and of phospho-mTOR and phospho-4EBP1 in the prefrontal cortex, hippocampus, hypothalamus, and olfactory bulb;	27374162
hsa04010	MAPK signaling pathway	Downregulated expression of the mitogen activated protein kinase (MAPK) phosphatase 1 (MKP-1) and the downregulated phosphorylation of extracellular signal-regulated kinase (pERK) in the anterior cingulate cortex (ACC) of mice;	32109506
		CTRP3 may be an innovative therapeutic target for treating patients with depression through regulating p38 and JNK signaling;	31629950
		The MAPK/ERK signaling pathway has been shown to be involved in the pathogenesis of MDD and the rapid onset of action of antidepressant therapies;	30859414
hsa04012	ErbB signaling pathway	The stressed rats showed elevated expression of NRG1 and phosphorylated ErbB4 (pErbB4) in the myocardium, whereas ErbB2 and pErbB2 were inhibited;	27133902
		The stressed rats displayed elevated expression of NRG1 and phosphorylated ErbB4 (pErbB4) in the prefrontal cortex, whereas ErbB2 and pErbB2 were inhibited;	26626816
hsa04722	Neurotrophin signaling pathway	Decreased neurotrophic factors expression and neurotrophin receptors signaling have repeatedly been reported in association with stress, depression, and neurodegenerative disorders;	28315978
		p75 neurotrophin receptor/nerve growth factor signaling and innate immune toll-like receptor signaling in MDD;	31889537
		Reduced synaptic markers in hippocampus, demonstrated by reductions in β III-tubulin (neuronal marker), PSD-95, SNAP-25, and neurotrophin-3;	28451885
hsa04015	Rap1 signaling pathway	Rap1-MKK3/6-p38 MAPK pathway in the induction of mGluR-dependent long term depression (LTD) by directly coupling to receptor trafficking machineries to facilitate the loss of synaptic AMPA receptors;	14709549
		The small G-protein Rap and the transcription factor STAT-3 are also involved since reducing the levels of Rap1 (using small interfering RNA) or STAT-3 (using dominant negative STAT3) significantly blocks 5-HT1A-receptor-mediated neurite outgrowth;	15925428
hsa04014	Ras signaling pathway	Blood mononuclear cell proteome suggests integrin and Ras signaling as critical pathways for antidepressant treatment response;	24607422
		Ras-GRF proteins contribute to forms of synaptic plasticity that are required specifically for mature hippocampal function;	16467520
		RAS-GRF1 mediates NMDA-type glutamate receptor (NMDAR)-induction of long term depression in the CA1 region of the hippocampus of mice	23766509

synthesis process of new synaptic connections (55). Recent research supports the hypothesis that major depression may be the result of disruption of mTOR-dependent translational regulation (13, 14). This result indicates that this pathway plays a crucial role in the molecular mechanism of depression. In addition, there are another five pathways, including hsa04010 (MAPK signaling pathway), hsa04012 (ErbB signaling pathway), hsa04722 (Neurotrophin signaling pathway), hsa04015 (Rap1 signaling pathway) and hsa04014 (Ras signaling pathway), were involved in two of the nervous system, immune system and endocrine system related to depression. Finally, the experimental evidence for the seven important signaling pathways with the most significant therapeutic relationships of depression is shown in **Table 4**. These pathways could have closely related to the pathological process of depression and need to research in depth.

It is undeniable that there are several limitations in this study. First, it is not sufficient to study based on the data currently available, because the technique of identifying metabolites for depression is still a continuous improvement process. Second, although we have used published literature to verify some results, validation of molecular mechanisms based on clinical samples are necessary to analysis the complex pathogenesis of depression. Our results provide good ideas and methods for studying the pathogenesis of depression.

CONCLUSION

We identified 36 metabolic biomarkers of clinical plasma metabolomics using NMR and MS. The relationship between biomarkers and enzymes were collected from the HMDB database. The results show that stearic acid, phytosphingosine, glycine, glutamine and phospholipids were important metabolic biomarkers. Hydrolase, transferase and acyltransferase involve the largest number of metabolic biomarkers. The important metabolites and enzymes screened by the topology of the network may play a key role in the underlying molecular mechanism of depression.

The nervous system, immune system and endocrine system are mainly involved in the underlying pathological mechanism of depression. The *DSWMP* index was used to assess the importance pathways of hub metabolic biomarkers involved. Nine proteins (TP53, IL1B, TNF, PTEN, HLA-DRB1, MTOR, HRAS, INS and PIK3CA) are widely involved in the nervous system, immune system and endocrine system. These targets may be important targets for antidepressants in the treatment of depression. Seven important pathways, such as PI3K-Akt signaling pathway and mTOR signaling pathway, are closely related to the pathogenesis molecular mechanisms of depression and require further investigation. A combination of network

pharmacology strategy and metabolomics approach has great potentials in comprehensively and deeply understanding the molecular mechanism of depression. The application of important biomarkers and pathways in clinical practice may help to improve the diagnosis of depression and the evaluation of antidepressant effect, which provides important clues for the study of metabolic characteristics of depression.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

X-MQ and J-ST provided the concept and designed the study. YG, TX, Y-XZ, TL-H and S-BL conducted the analyses and wrote the manuscript. YG, TX, Y-XZ, TL-H and S-BL participated in data analysis. X-MQ and J-ST contributed to revise 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.2020.00667/full#supplementary-material

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REVIEW
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Neuropsychophysiological Measures of Alcohol Dependence: Can We Use EEG in the Clinical Assessment?

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Addiction management is complex, and it requires a bio-psycho-social perspective, that ought to consider the multiple etiological and developmental factors. Because of this, a large amount of resources has been allocated to assess the vulnerability to dependence, i.e., to identify the processes underlying the transition from substance use to dependence, as well as its course, in order to determine the key points in its prevention, treatment, and recovery. Consequently, knowledge \from neuroscience must be taken into account, which is why different initiatives have emerged with this objective, such as the "Research Domain Criteria" (RDoC), and the "Addiction Neuroclinical Assessment" (ANA). Particularly, neuropsychophysiological measures could be used as markers of cognitive and behavioral attributes or traits in alcohol dependence, and even trace clinical change. In this way, the aim of this narrative review is to provide an overview following ANA clinical framework, to the most robust findings in neuropsychophysiological changes in alcohol dependence, that underlie the main cognitive domains implicated in addiction: incentive salience, negative emotionality, and executive functioning. The most consistent results have been found in event-related potential (ERP) analysis, especially in the P3 component, that could show a wide clinical utility, mainly for the executive functions. The review also shows the usefulness of other components, implicated in affective and substance-related processing (P1, N1, or the late positive potential LPP), as well as event-related oscillations, such as theta power, with a possible use as vulnerability or clinical marker in alcohol dependence. Finally, new tools emerging from psychophysiology research, based on functional connectivity or brain graph analysis could help toward a better understanding of altered circuits in alcohol dependence, as well as communication efficiency and effort during mental operations. This review concludes with an examination of these tools as possible markers in the clinical field and discusses methodological differences, the need for more replicability studies and incipient lines of work. It also uses consistent findings in

psychophysiology to draw possible treatment targets and cognitive profiles in alcohol dependence.

Keywords: alcohol dependence, electroencephalogram, endophenotypes, incentive salience, negative emotionality, executive dimension, event-related potential

INTRODUCTION

Addictions continue to be a public health problem, despite the continued efforts by different types of professionals. That is why great efforts are being made from a preventive and therapeutic point of view, although it seems that these efforts are not being effective. Both preventive and therapeutic strategies include information from a psychosocial approach, and more recently from the neurosciences, not only from the field of psychopharmacology but also genetics and neuroimaging (1).

However, the results of these procedures, although highly evidenced and confirmed, are quite heterogeneous, possibly in relation to the clinical and methodological characteristics of the studies available in this field (1). For instance, different types of patients, type of consumption, withdrawal periods, and of course different type of interventions. In fact, treatment does not bring with certainty the abstinence maintenance and a great number of patients go through relapse in the first follow-up year (2–4).

To cope with this complexity, there is a consensus that in order to improve the outcomes of behavioral interventions in alcohol use disorders (AUD) it is necessary to understand the mechanisms underlying the behavioral change in effective treatments. From this standpoint, building a strong foundation for alcohol dependence treatment includes answering the question of why and how, not just whether, a treatment is effective (5).

Research on the mechanisms of effective AUD treatments that underlie behavior change has been focusing on cognitive neuroscience. Dysfunctional processes that maintain AUD, such as craving, withdrawal, lapse, and relapse are understood by studying the functioning of cognitive processes, such as attention or motivation, and the underlying neural systems.

The Research Domain Criteria (RDoC) is a framework that allows the study of psychiatric disorders from this point of view since its objective is the analysis of what may cause the symptoms, rather than the symptoms themselves. It even allows the search and validation of biomarkers (6). It takes into consideration five dimensions for all possible pathologies: negative valence systems, positive valence systems, cognitive systems, social systems, and activation supervising systems (7). Therefore, patients could be evaluated in each one of these dimensions at different levels of analysis, from basic disciplines involving genetics, molecules, cells or neuroanatomical circuits, including physiology to clinical or behavioral measures, such as neuropsychological assessment, self-informed measures or behavioral paradigms (8, 9).

This approach makes it possible to characterize the psychological attributes by being analyzed in cognitive, social, emotional, and behavioral terms, with measures coming from basic science and clinical area (10).

Following this framework, the Addiction Neuroclinical Assessment (ANA) considers the same levels of analysis, selecting three domains as essential; these are the incentive salience, negative emotionality, and executive abilities (8). Therefore, each level of analysis within the dimensions proposed by ANA could be considered as a follow-up measure of the course of substance dependence, perhaps understanding it as an intermediate endophenotype. The disorder is understood as an active process, not an endpoint, and each of the measures obtained at different levels of analysis would be considered a marker of this process (7, 11). In this way, in this study we summarize the relationship between the pathological consumption of alcohol and the use of EEG as a measure of cognitive components assessment, according to ANA dimensions.

Besides the changes produced by moderate alcohol consumption in the brain electrical activity (12–14), in alcohol dependence EEG measures have been employed as markers of structural and functional changes that rise as a consequence of continuous and pathological consumption. Hence, there is considerable bibliography available with respect to these changes (15–17). By using the brain electrical activity, we can obtain different types of information, useful for understanding alcohol dependence processes; the most frequent one in the scientific literature is the one provided by event related potentials, in the sensorial, cognitive or motor modality. They are represented by the average electrical activity appearing after each event, and are composed by different components, which are named according to their polarity (positive or negative) and the moment when they appear (in milliseconds).For instance, the P300 component would reflect a positive deflection approximately 300 ms after stimulus presentation (16). A different type of analysis is the one brought by brain oscillations, their pattern would reflect the sum of postsynaptic potentials generated by a neuronal field close to an electrode. In this way, the number of oscillations (measured in Hz) determines the traditionally known frequency bands (e.g., delta, theta, alpha, beta, and gamma bands). All of them have been explored in alcohol dependent patients, however, the most frequent findings point to beta rhythms (12–28 Hz) alterations, observed even during the abstinence period and their offspring (15). According to these basic concepts different, conditions of EEG recordings arise (e.g., resting-state or event-related activity), as well as different methods of analysis, which are addressed later in this work, such as the synchrony in brain rhythms between different locations.

The measures obtained by means of neuropsychophysiological assessment are an example of possible biomarkers that reflect, in an indirect but objective way, the cognitive processes that give rise to the problematic behavior of alcohol consumption. Several

benefits could be drawn from these type of measures: 1) It allows the reflection of the related brain activity, with a great temporal, "instantaneous and continuous" precision, in words of Campanella (18); 2) When the employed paradigm is well-characterized from the cognitive and methodological points of view, the neural pattern can reflect the psychological features that contribute to the disorder, even when no observation was made from the psychopathological point of view (18, 19), 3) In addition, it is a measure that escapes the patient's voluntary control, for example, it reflects the lack of inhibitory control or craving, even when the patient is not aware of it (20).

The suggestion that neuropsychophysiological measures, namely, event-related potentials (ERP) could be biomarkers, e.g., the P300 component, or other intermediate endophenotypes, such as N170 or N200, is a well-established line of work, from the research standpoint. Nonetheless, these are not consistently used in clinical evaluation. The aim of this revision is to provide an overview, through a narrative revision of the literature, of the most solid results in the different neuropsychophysiological measures that can be obtained by using electroencephalographic (EEG) measures.

DIMENSIONS OF ADDICTION NEUROCLINICAL ASSESSMENT AND NEUROPSYCHOPHYSIOLOGICAL EVALUATION

Incentive Salience

Incentive salience is one of the main objectives of evaluation inside ANA frameworks. As defined by early theories of addiction and I-RISA model (21), incentive salience can be understood as the narrowing of the attentional focus on the substance and the related contextual cues, that gain in motivational value and appetitive properties, in detriment of natural reinforcers, changing the entire reward system. A sensitization to the substance takes place, based on associative and conditioned reinforcement mechanisms, where the mere presence of related cues can drive substance-seeking behavior (22, 23), by changing activation and craving states (24, 25). In alcohol dependence, alterations in the affective information processing and preferential attention toward substance cues have been described (26, 27) as well as attentional bias and interference control effects (28–30). Moreover, cue exposure is related to a higher desire and urgency consumption and increased expectancies regarding alcohol effects, as well as dependence severity (26, 27). Changes in the reward system are also observed, resulting in difficulties regarding the delay of gratification and poor loss and gain evaluation (20, 31–34). Thereby, the incentive salience construct, together with cognitive and motivational processes involved seem essential in the search for clinical markers of change through the dependence process and as vulnerability factors involved in maladaptive behaviors. These can be measured through a great variety of tools, including self-informed measures, neuropsychological tests, and behavioral paradigms, sometimes combined with neuroimaging and psychophysiological techniques. ANA model proposes specific materials for evaluating the incentive salience domain, including behavioral paradigms such as the dot-probe attentional bias task (29, 35, 36), cue-reactivity task (37) or the monetary incentive delay task (38).

Nonetheless, initiatives like ANA or RDoC encourage the use of neuroimaging tools, like psychophysiological measures. Taking into consideration that the motivational and attentional allocation changes toward substance-related cues are produced in parallel with structural and functional changes of reward and salience circuitry of the brain, the neural underpinnings of these processes become relevant in the discovery for new markers of clinical changes and vulnerability related to the dependence process. In this way, neuropsychophysiological measures enable the assessment of early stimuli evaluation (perceptive and preattentional operations), together with distractibility and the attentional biased produced by substance-related stimuli, as well as mental operations during reward processing and delay of gratification.

Early visual components, such as P1, N1, or P2, as well as the late positive potential (LPP) are ERP components usually related to early stimulus evaluation and attentional processes that can be found altered in AUD in cue reactivity tasks or attentional bias paradigms. One of the most frequent ways to evaluate the influence of alcohol cues on neuropsychophysiological activity is by using cue reactivity tasks that usually consist in the presentation of visual substance-related stimuli (images or words), and even olfactory or gustatory cues. These can be passively visualized (e.g., a bottle of whiskey or neutral content stimuli, such as a book or a pen) or can be part of a classification or a discrimination task while the electrical brain activity is being recorded.

There is evidence in the literature for an early preferential processing of substance-related cues in AUDs, shown by greater N1 amplitudes (39), an exogenous and automatic attention index, indicating a preferential attentional processing of alcohol cues, in line with motivated attention (40) and theories of addiction (41). P3 to alcohol cues in an oddball task is also found increased in alcohol dependence, indicating the alteration of higher-order processing such as controlled attentional allocation (42). This preferential processing of alcohol cues also appear in individuals at risk, ERP changes being observed in social drinkers (43) with heavy use (44), that show increased P1 latencies and LPP amplitudes toward alcohol images, and in individuals with a low sensitivity to alcohol effects, that have increased P3 amplitudes (45, 46). In this way, since early stages of alcohol consumption and in individuals at risk (low alcohol sensitivity), substance-related cues seem to be processed faster and with a high motivational salience, and ERP monitoring could help predict substance-related problems.

Alcohol salience can even dampen other demanding processes in course, heavy drinkers (47) and recently detoxified individuals (48) showing higher N2 amplitudes toward substance cues during inhibitory processes (during NoGo conditions). Thus, alcohol salience can be so powerful that other important

executive processes could be affected, such as inhibitory control, essential for adaptive behaviors.

Regarding the attentional bias produced by substance-related cues, a common task is the dot-probe paradigm, that consists in the displaying of pairs of images (substance-related and neutral ones) appearing side by side, usually for 500 ms, prior to the presentation of a probe (a dot or an asterisk) replacing one of the pictures (on the left or right side of the screen). The subject must respond by indicating as quickly as possible, on which side the point has appeared, pressing one key for the right side and another for the left side. The attentional bias is typically calculated using the differences in reaction times when the dot is presented on the same side where alcohol-related pictures were (congruent trials) and when it is on the opposite side (incongruent trials). ERP analysis, in this case, would help to elucidate what happens with brain activity at several stages of the cue processing, that is, sensorial filtering, attention orientation and re-orientation, stimuli salience and arousal, and also more controlled attentional processes, even in absence of behavioral changes in reaction times or self-report measures. In the same way as cue tasks, visual probe paradigms display in individuals at risk (low alcohol sensitivity) a preferential early attention orientation, indicated by larger P1 amplitudes, and difficulties reorienting attention away from alcohol cues, reflected by larger negativity between 220 and 280 ms (49).

With respect to reward system changes, monetary incentive or choice tasks are a usual tool of evaluation of motivational decision making. This type of task usually consists in gambling tasks where participants take low or high risks in order to gain a prize (generally money). The subject must decide between gambling alternatives with higher rewards and losses (higher risk) or with lower rewards and losses (lower risk). These tasks allow us to evaluate not only risk-taking and the capacity of delaying gratification, but also the evaluation of the own decisions and responses through the task. Alcohol-dependent individuals (50) and youth at risk with families densely affected by alcoholism (51) seem to show lower amplitudes of the P3 component and lower activity in frontal areas (e.g., cingulate gyrus), during both gain and loss conditions of gambling tasks. There even seems to be a relation between risk-taking features and impulsivity (50). In this way, ERP analysis of risk and outcome evaluation could help in the discovery of vulnerability markers. Even recent alcohol intoxication seems to affect neuropsychophysiological activity during reward processing, reducing ERP positivity in 250 to 400 time-window (52), indicating an affectation of performance monitoring and feedback that drives the decision-making process during the task. This is also exposed in binge drinkers, that show alterations in automatic error processing (larger error-related negativity [ERN]) in a Go-NoGo task and in motivational processing (delayed error positivity Pe) in a risk task (53). This would indicate an affectation of controlled processes such as monitoring self-behavior since early stages of consumption.

Taking into consideration the importance of the incentive salience and its effects across the dependence course, its evaluation through ERP measures can bring more light upon the specific processes that underlie to appetitive changes, attentional interference produced by alcohol cues and craving, that motivationally drive the decision making. Moreover, changes in psychophysiological activity appear early in the course of continuous consumption, indicated by the early attentional and motivational capture by alcohol stimuli and by changes in the reward system in acute effects of alcohol administration and social drinkers. Some studies even find these changes in population at risk, namely individuals with low sensitivity to alcohol and individuals with families densely affected by alcohol dependence. Hence, early informational processing components, such as P1 or N2 or those involved in controlled attention, N2 and P3 could be assessed as possible vulnerability markers or as markers of cognitive efficacy change through time.

Negative Emotionality

Within the ANAs framework, negative emotionality refers to the propensity to experience and react with negative emotions, such as sadness, anxiety, fear, and anger to environmental cues (54). In fact, there is enough evidence that shows how dependent individuals have clear difficulties within the emotional regulation process, namely a diminished emotional awareness and a reduced acknowledgment of other people's emotions and their own, giving rise to an inadequate emotional adjusting in relation to environmental demands (55, 56). Furthermore, tolerating negative emotional states might become difficult and even bring people to act impulsively, making them to engage in behaviors that they find rewarding in the short-term without fully considering its risks, in order to diminish the experience of this negative affect. These negative reactions to environmental cues seem to be highly related to alcohol consumption. So much that different theoretical models consider that negative emotionality is present during different stages of the alcohol consumption cycle.

Among them, classical theoretical approaches (57) propose that deregulation in the reward system is characterized by the transition of the experience of pleasant sensations every time an individual consumes alcohol, to a progressive shift toward feelings of relief when he takes the substance, that is, taking alcohol with the whole purpose of avoiding the negative emotionality states experience during withdrawal stages (58). This leads to further consumption as a way to avoid this negative state, reinforcing the cycle. Additionally, neuroadaptations seem to persist even after prolonged abstinence, increasing the risk of relapse (59).

There is also broad evidence of the existence of negative emotionality prior to the development of AUD. This seems to play a key role in the engagement of problematic consumption as a self-regulation mechanism. Hagan et al. (60) found that a higher negative emotionality in children predicts future alcohol consumption, stress, and internalizing symptoms during adulthood. Additionally, there is evidence that the increase of alcohol consumption in adolescents is highly related to a reduction of the positive affect and an increased negative affect, giving rise to the usual anhedonia symptoms in this type of

patients (61). Implying then, that negative emotionality can also be considered an intermediate endophenotype of alcoholism.

This negative emotionality is reflected at a biological level, hence psychophysiological changes can be assessed with EEG measurements. The use of EEG techniques in affective process evaluation can be challenging, due to its complexity. However, the correct implementation of EEG measures ensures a direct measurement of the affective processing, providing key temporal information of all stages of emotional processing, allowing then a better understanding of some of the alterations found in AUD patients. However, the number of studies of emotional alterations in AUDs with EEG measures is scarce.

ERP analysis is one of the most used EEG measures to study emotional processing with psychophysiological techniques. Among the ERPs related to emotional processing, we can mention the Early Posterior Negativity (EPN), P1, P2 and the LPP (62). The modulation of these components by affective information is reliable and systematically observed (63–65). LPP is characterized by a positive centro-parietal deflection, starting around 200 ms after the stimulus onset, and it is prolonged several milliseconds in time. It is modulated by the emotional content of stimuli, showing an increase in comparison with its activity under neutral stimuli. However, cognitive reappraisal related to positive emotional regulation seems to reduce LPP amplitude (66–68). In this type of tasks, subjects passively visualize images with high emotional content, and they are asked to classify them according to three dimensions: valence, arousal, and dominance. The emotional content of these images is usually related to positive appetitive stimuli (e.g., sex-related), negative threatening ones (e.g., aggressions) and sometimes motivationally relevant stimuli related to the substance (e.g., a beer).

Studies with paradigms of viewing of neutral and affective images found that alcohol consumption selectively reduces the processing of negative cues, specifically there is a decreased amplitude of LPP during the viewing of negative images, and this has been seen in healthy population after the intake of a small doses of alcohol (69) and in population at risk, such as binge drinkers (70). A reduced LPP amplitude would be indicating an early effect of alcohol consumption on the impact of negative-valence content in later information processing stages. This could be in line with theories regarding negative affect evaluation and processing alterations in alcohol dependence.

Prolonged alcohol consumption is also related to alterations in the recognition of emotional facial expressions. In emotion recognition tasks, the person is asked to identify emotional expressions on faces, usually without any context. When AUDs had to determine an emotional face expression they presented a distinct neuropsychophysiological response, indexed by a decreased amplitude of early (P1, N10, N170) and late (LPP) ERPs (71). This alteration persists after a prolonged abstinence (59, 72). These difficulties for recognizing emotional facial expressions are highly related to more interpersonal difficulties, probably leading to social isolation and to an increase of their negative emotionality (59).

In summary, both early processing (P1, N1, N170) and later (LPP) components can be used as potential markers when evaluating negative emotionality aspects, such as affective processing, emotional recognition, and appraisal. For instance, alcohol seems to affect LPP from the beginning of consumption and together with N1, they are affected even in prolonged abstinence, indicating a possible role for these components as possible biomarkers. Moreover, considering its modularity by re-appraisal, LPP could be measured through time, in relation to cognitive and emotional regulation therapy.

Executive Dimension

Under the ANA framework, executive functions would be included within the executive domain, which comprises those higher-order processes mainly involved in the organization of behaviors, aimed at achieving future objectives (8, 73). Specifically, this domain is focused both on those processes of temporary organization of behavior such as attention, inhibition of response, planning, working memory and behavioral flexibility, as well as evaluation of future events (8). For an adequate measurement of the functions included within this domain, the authors of the ANA propose a series of assessment tests that can be widely used by both researchers and clinicians (8). However, in addition to the behavioral and self-reporting tests proposed by the ANA authors, they also recommend supplementing the use of these tests with other measures from neuroscience (8), but without specifying on any particular measure. This is because the evaluation of the executive dimension with behavioral and self-reporting tests exclusively may not identify aspects underlying these measures, such as inefficient brain functioning (74). This inefficient brain functioning may not manifest itself behaviorally and/or consciously in controlled contexts (e.g., attentional evaluation in clinical consultation), while in everyday contexts it does, putting at risk the maintenance of abstinence. In this case, psychophysiology can be very useful, because it can be sensitive to this type of information. Along these lines, quite interesting results have been found by combining different evaluation tests and different types of analysis of the electrophysiological signal.

One of the most studied tasks with the objective of obtaining a neuropsychophysiological marker of attentional control in alcohol addiction is the oddball paradigm. This task consists in the presentation of a series of infrequent stimuli (targets) during the presentation of stimuli in a frequent way (standards), allowing to evaluate the attentional processing from bottom-up as well as from top-down (75). The analysis of the electrophysiological activity recorded during the performance of this task that has been mostly carried out is that of ERPs (76–81). The task leads to the generation of different electrophysiological components, being P3a and P3b the most studied ones (76–81). These two electrophysiological components appear between 300 and 700 ms after the stimulus, and differ mainly in the cause that generates them and in the topographic distribution that they have. The first of these is the P3a component, whose evocation is produced by the

absence of explicit instruction to attend to the infrequent stimulus, and has a frontal topographical distribution (17). This component would reflect the bottom-up attentional processing, because there is no controlled processing of the stimuli presented. The second component is the P3b, which has a parietal topographic distribution, and whose evocation is produced by the explicit instruction to attend to the infrequent stimulus (17). In this case, this component would reflect the top-down attentional processing, since, unlike the P3a component, here there is a controlled processing of the stimuli presented in the task.

A large number of studies using this oddball paradigm in alcohol-dependent people have found that both the P3a and P3b components have a reduced amplitude compared to healthy controls (42, 79, 80, 82–84). A result that is replicated in healthy children of people with alcohol dependence (78, 85). This alteration of the P3 component both in people with alcohol dependence and in their offspring would reflect an attentional deficit from both the bottom-up and top-down processes, which seems to be produced by basal brain hyperexcitability (17, 76, 86). The literature proposes that this abnormal brain functioning is produced by an alteration in the mechanisms of cortical inhibition, and not by the consumption of alcohol per se (although it also contributes to the general impairment of brain functioning), being a previous vulnerability that they present (17, 76, 86). Because of this, the amplitude reduction of this component is proposed as an electrophysiological marker for alcohol addiction development. However, in other studies comparing this component between people with alcohol addiction and healthy controls, such as those performed by Bauer et al. (87), Fein et al. (83) and Malone et al. (88), these differences are not found when other variables such as the presence of a life history of major depression are taken into account. The discrepancy of results between studies may be reflecting the effect of the absence of differentiation between people who develop alcohol addiction due to the presence of different previous vulnerabilities (e.g., genetics), and people who develop it secondarily as a consequence of the presence of a particular set of symptoms (e.g., social anxiety) (81, 88, 89).

An example of this idea is reflected by Fein et al. (83). These authors study if there are differences in the P3b component generated by the oddball paradigm between people with alcohol addiction in abstinence with and without major depression. They found that those with alcohol addiction and major depression had no difference in the amplitude of the P3b component compared to the healthy controls group. They conclude that the absence of differences is due to the fact that the development of alcohol dependence, in this case, was caused by excessive consumption of the substance with self-medication as the main objective (83). Along the same lines, Malone et al. (88) propose that this does not only occur with the presence of concurrent major depression, but that this effect is also observed in people diagnosed with major depression throughout life. These two studies seem to reflect, by way of example, that the P3 component evoked during the performance of the oddball paradigm could help to identify those people with altered attentional functioning as a consequence of a previous vulnerability, having clear repercussions in the choice of the most appropriate therapeutic line (e.g., pharmacological and neuropsychological vs. neuropsychological) (90–94).

Besides the oddball paradigm, the Go-NoGo paradigm is another task that has also been quite studied with the aim of obtaining other types of electrophysiological markers. This task has been used in different versions, varying some of its parameters, such as the type of stimuli presented [e.g., geometric figures (74), neutral stimuli or alcohol-related stimuli (95)]. However, the basic design of the task consists of presenting a series of Go stimuli more frequently to which the participant has to give some kind of response, interspersing NoGo stimuli less frequently, where the participant has to inhibit his response. Although apparently both the oddball and the Go-NoGo paradigms are very similar, the main difference is that the Go-NoGo paradigm requires greater involvement in the task by the participant, while in the oddball paradigm the necessary involvement is lower. Precisely because of this, the cognitive process that allows us to evaluate this task is the inhibitory capacity and the influence of different stimulation conditions on it.

With the use of the Go-NoGo paradigm, and as with the oddball paradigm, the analysis of ERPs has been the most carried out, which has provided the most consistent results around the N2 and P3 components (96–99). On the one hand, the negative component N2 appears around 250 ms after the presentation of the NoGo stimulus with a front-central distribution, with greater amplitude in the frontal region (98). The literature proposes that this component reflects the subject's ability to recognize the need to inhibit the response to a stimulus of these characteristics, where a greater amplitude of the component would reflect a better recognition of this need to inhibit (98, 100). On the other hand, the positive component P3 appears between 300 and 600 ms after the presentation of the NoGo stimulus with a fronto-centro-parietal distribution, with greater amplitude in the central region (96). In this case, the literature proposes that this component reflects the subject's ability to carry out an effective inhibition of the motor response, where a greater amplitude of the component would reflect a better inhibition of the response (101). Specifically, in people with alcohol addiction, the literature shows that both N2 and P3 can be reduced compared to healthy participants, reflecting an inhibitory deficit at one or both levels even when behavioral outcomes do not reflect this deficit (96–99).

In summary, the tasks with more clinical evidence for the evaluation of the executive dimension with electrical brain activity are the oddball and Go-NoGo paradigms. In the case of the oddball paradigm, the cognitive process evaluated is the bottom-up and top-down attentional processing. The neuropsychophysiological components obtained with this task with greater clinical utility are P3a and P3b, allowing to identify those people with alterations of the attentional functioning as a consequence of a previous vulnerability. In the case of the Go-NoGo paradigm, the cognitive processes evaluated are both the inhibitory capacity and the ability to identify the need to inhibit.

In this case, the neuropsychophysiological components obtained with greater clinical utility are N2 and P3, allowing the assessment of this capacity with greater sensitivity than behavioral data.

EVIDENCE IN OTHER NEUROPSYCHOPHYSIOLOGICAL MEASURES

Research lines regarding the search for neuropsychophysiological markers of neurocognitive alterations of alcohol dependence are rising as the possibilities for signal analyses increase. This allows us to evaluate EEG signal obtained with more complex paradigms from the methodological and cognitive point of view. By employing different types of analysis of the brain electrical activity, such as frequency analysis methods (102), event-related oscillations (103, 104) and functional connectivity analysis (102).

Frequency-Based Analysis

In addition to electrical activity related to events, brain oscillations analysis has been used across the literature. Most frequency bands have been evaluated during resting-state recordings in alcohol dependence, although with more diffuse results than those found in ERP studies. As an example, results in resting delta (0, 1–4 Hz) are considered inconclusive (15). With respect to theta band (4–8 Hz), various outcomes have been found (15, 103), but it is fundamentally evidenced as an increase in tonic theta in frontal, central, and parietal areas (105), with greater values for those patients that experience relapse (106). Other studies, however, find a decrease of theta power related to greater cortical damage (107). Changes in theta could be indicating the cortical imbalance in the excitation-inhibition homeostasis (15, 105). Moreover, theta has been related to inhibitory and motor responses (108).

With respect to alpha band (8–12 Hz), that predominates through the resting-state, it seems to be reduced in alcohol-dependent individuals (15, 109) and it has been associated with cortical activations, alert mechanisms, and active inhibition (110, 111), possibly indicating the activation of compensation mechanisms.

For beta band (14–30 Hz), the most frequent result indicates that patients have a greater beta power in fronto-central areas, similar to their offspring, where this increase is produced prior to developing an alcohol use problem (112). There is even evidence of a greater desynchronization of beta in patients that relapse comparing to those that remain abstinent. Porjesz and colleagues support the theory that a greater beta power at frontal sources reflects the disbalance between excitatory and inhibitory neurons, that could underlie to AUDs vulnerability (112–114). In this manner, while findings in beta have been considered as a trait marker, outcomes in theta have been thought as a state marker.

Event-Related Oscillations

Evoked response oscillations analysis allows us to know in a detailed manner characteristic of brain rhythms during a task performance and their alterations. Within the study of the cognitive function, research groups have mainly found changes in theta band in several behavioral paradigms (34, 115–117).

In relation to incentive salience and motivational-related tasks, alcohol-dependent individuals show a decreased theta power during reward processing and weaker activity in prefrontal sources during the loss condition (34), the latter indicating difficulties in the risk evaluation process. Although a few number of studies are available regarding theta oscillations in substance-cue or reward processing, theta power is found altered in other ERP paradigms, namely visual oddball or conflict tasks in alcohol-dependent individuals (13, 118–120) and their offspring (103). Theta rhythm is thought to reflect frontal activity, implicated in attentional and monitoring processes (121) and it could be implicated in several cognitive operations, such as attentional control, inhibitory processes, and conflict responses.

Theta frequency band has been found to be modulated by stimuli valence. Aftanas et al. (122) measure theta synchronization and desynchronization while healthy subjects are exposed to stimuli with different emotional content, and find a time-locked synchronization theta at anterior and posteriors sites, at 200 to 500 ms post stimuli presentation. This frequency band is useful as a neuropsychophysiological marker of emotional processing. In particular, theta power at frontal areas during the processing of affective information can be useful to assess emotional regulation or control. While theta at occipital sites can be used as a measurement of early emotional assessment. Regarding alcohol effects on theta band in emotional tasks, binge drinkers seem to have an attenuated theta power in an affective appraisal task, during both early appraisal and later integrative processes (115). This lower theta responsitivity to emotions can suggest that binge drinkers already present some of the characteristic anhedonia of AUD patients.

In relation to the executive control dimension, for example, Kamarajan et al. (118) studied the evoked power of brain signals in alcoholics during the performance of a Go-NoGo task. The results showed that alcoholics had lower power in the delta and theta frequency bands compared to the control group in the NoGo condition (118). In this same line, Pandey et al. (117) found that alcoholics had lower power in the delta, theta, and alpha frequency bands compared to the control group, although behavioral differences were found only in the Go condition. Both studies reflect the presence of a neurocognitive deficit in both the execution and suppression of the motor response (117), where, in addition, Kamarajan et al. (118) suggest that oscillatory correlates during cognitive processing may be used as endophenotypic markers in alcoholism. Theta has already been suggested as indicatory of attentional and executive processes (121), hence, these results might indicate a role for this oscillatory activity in detecting and characterizing alterations of these processes during the course of AUDs.

In summary, we could think of brain oscillatory rhythms as possible markers for cognitive processes, such as attentional allocation and affective processing, as well as behavioral monitoring and risk-taking, and executive inhibitory processes,

deeply involved in the dependence course and vulnerability toward maladaptive behaviors. In particular, theta and beta rhythms at rest and during cognitive operations could be of interest in the search for clinical or stable biomarkers

Functional Connectivity of the Brain

Nowadays research focuses on more detailed and global characterization of brain functioning, functional connectivity (FC) and graph theory-based analysis bringing information upon the wiring and effective communication of the brain during several cognitive and emotional processes. In this way, fMRI studies find connectivity changes in the brain affected by chronic alcohol consumption, with an alteration of white matter tracts (123), and changes in attentional and salience networks, as well as reward-related and executive ones (124–126). Coherence analysis or phase-synchrony in several frequency bands between pair of brain regions are frequent ways of studying FC in neuropsychophysiological measures (127). There are several outcomes related to alcohol effects in the brain communication in resting-state. In this way, some studies show a reduced connectivity in theta and alpha, as well as beta band (109, 128). Others show an increase in theta (112, 129–131) and interhemispheric alpha and beta (132). Despite the quite diverse outcomes, there seems to be an agreement upon their role in the brain, and some of them have even been proposed as possible markers of alcohol effects in the brain or as vulnerability markers, such as changes in theta connectivity (131). In this manner, alterations in FC of theta might indicate changes in the inhibitory neuronal system, related to GABAergic and cholinergic neurotransmission (112), as well as emotional and motivational processing (133), whereas beta and alpha FC might reflect supervision and coordination processes involved in brain activation and deactivation systems (134).

Although reduced, there are some studies carried out in active states in EEG regarding alcohol or other substances' effects on functional connectivity study of the brain. In incentive salience, an fMRI study was carried out in a cue-exposure paradigm, as well as a resting-state EEG recording of alcohol-dependent individuals (130). Results showed fMRI changes in FC in frontal and limbic regions, highly implicated in motivated attention toward alcohol cues, as well as an increased connectivity in theta band in resting EEG in similar regions. Theta is considered to reflect emotional and motivational processes, and this result might underlie to the alterations present in the reward evaluation system in alcohol dependence. The authors of the mentioned study think that theta hyperconnectivity might have a relationship with craving, even hypothesizing the existence of a "central craving network", where different regions are in charge of appetitive and motivational aspects involved in incentive salience (130). Moreover, this idea is supported by a study with smokers, where theta coherence is increased toward nicotine cues in frontal and parieto-occipital sites, and it also predicts changes in craving (135). Alcohol-related cues could have a similar effect in brain connectivity, in fact, one of our studies (136) showed a relationship between resting-state beta connectivity and the approximation index toward aversive contexts related to the substance in a modified Alcohol Approach-Avoidance task (AAT). Specifically, a higher beta connectivity was related to a greater avoidance of aversive alcohol-related contexts, possibly indicating beta synchronization role in motivational and salience circuits.

Regarding reward-related processing, such as monetary tasks, no studies using EEG or MEG measures were found in FC in alcohol dependence. However, a study carried out in healthy population indicates changes in FC (133), reflected by increases in theta synchronization between frontal region and mostly parietal areas during the loss condition and greater theta FC within posterior regions during the gain condition. Moreover, this study is carried out in co-twins and they find a genetic heritability of fronto-parietal connectivity in theta during the loss condition, indicating that reward evaluation of negative outcomes could be genetically transmitted. Taking this into consideration, it would be interesting to evaluate theta connectivity in incentive or reward tasks and find out its possible role as a vulnerability marker for alterations in reward evaluation and its relationship with maladaptive behavior.

Neuropsychophysiological connectivity measures could also be applied to the assessment of negative emotionality. In healthy population, increased long-range connections between frontal parietal and temporal areas in beta and gamma bands are related to negative and positive emotional information processing (137). In this way, fast rhythms communication through the brain could play a role in emotional processing and could be of use in the research of neural markers of alterations in AUD.

In executive functioning, FC analysis can have a special relevance in the knowledge of electrical brain functioning in resting state and its relation to different executive aspects (e.g., impulsivity). Herrera-Diaz et al. found that alcohol-dependent individuals present, together with an increased beta power, a reduced FC at fronto-central and occipito-parietal regions in alpha and beta bands, comparing to healthy controls. What is more, resting FC in alpha band between anterior and central regions seems to have an inverse relationship with BIS-11 non-planned impulsivity scores (102). Authors propose that results reflect the existence of alterations in the brain's electrical signal at rest in alcohol-dependent individuals, indicating a possible association to psychopathological characteristics of the addictive behavior (102).

Brain Graph Characteristics

Research in brain connectivity and dynamics has been increasingly developed during the last years, revealing the importance of neural integration and segregation of communication between brain areas, as well as communication patterns, hubs, and efficiency of the information flow. These particular measures can be useful in different pathologies where functional brain alterations are evidenced (138, 139), as in addictions (140, 141). These measures rely on graph theory, a mathematical model used in several fields of science, particularly useful in neuroscience, more specifically in brain functional and effective connectivity. It comprehends brain functioning as a network, composed by vertices or nodes, e.g., brain areas or

channels and edges, e.g., connections (measured by statistical dependence) between pairs of areas (142). The functioning or communication between these nodes is characterized in terms of several elements, such a distance or path length between two areas, the number of connections received by a node (degree) or the number of connections forming a triangle around a node (clustering coefficient) (143). Moreover, the efficacy of this flow of communication depends on the distance between nodes, being inversely related to this index. These characteristics can be calculated at both local (segregation measures) and global (integration measures) levels (144). Segregation refers to the functioning of specialized areas and local networks in the brain, and it can be measured through parameters such as degree, local clustering or local efficiency. Whereas integration involves the coordination of neural populations giving rise to cognitive states, and it is generally measured by indexes such as the average shortest distance (characteristic path length) between nodes, global efficiency and global clustering level. These two principles create diverse and complex patterns of communication in the brain, allowing a balance between flow efficiency and costs, at both resting and during active states. In this way, brain connectivity tends toward a balance in energetic cost and to a maximization of the network (145). This is known as the "small-worldness" of a network and it implies an optimal functioning of local and global communication, characterized by a high global efficiency and clustering level, as well as a short characteristic path length (146).

So, how can these brain graph measures help us in the search for neuroscience-based clinical tools in alcohol dependence? Small-world attributes and characterization of neuropsychophysiological activity patterns can bring answers upon the mapping of functional connections in the altered networks in alcohol dependence. Information processing can be put together with a precise characterization and mapping of the communication flow through the brain, by detecting key nodes or hubs of connections, as well as possible changes in the network organization (147). In other words, we could detect local and global network deficiencies as well as possible compensation mechanisms, with extended communication to other areas or networks. In fact, the available literature upon this theme is still short, but it points toward this type of results. For example, in a fMRI study, individuals with policonsumption show a lower efficiency and a reduced small-worldness of the brain network (140), reflecting a loss of interregional communication in the brain. In alcohol dependence, a smaller global efficiency and clustering level have been related to a greater consumption severity (148), indicating their possible role in identifying neural markers for clinical severity. There is also data in EEG studies, low doses of alcohol in social consumers producing higher global efficiency and an increased density in resting alpha, as well as a short characteristic path (12). Similar observations were made in alcohol-dependent individuals during a working memory task (149), where smaller characteristic paths, reduced clustering, and an increased global efficiency were found in low beta band. These results might be indicating an altered functioning of network efficiency under the effects of alcohol, as well as a compensating mechanism in response to task demands, in order to carry out cognitive operations. Hence, graph-based measures of brain connectivity using neuropsychophysiological measures could help explain in detail neural communication alterations that happen through the course of dependence.

DISCUSSION

Brain electrical activity has been used with some degree of evidence to evaluate several of the cognitive processes that are contemplated in the dimensions of ANA. These cognitive components may describe alcohol dependence as an active process, in which event-related potentials or oscillations serve as a measure of the progression of the disorder. For instance, in relation to stimuli relevance evaluation, a greater alcohol salience can be observed in early visual ERP components, reflecting motivational processes. A common finding is a greater amplitude and sometimes latency of P1, N1, or P2 components, reflecting a preferential attention toward alcohol and related cues. This preferential attention can affect other cognitive processes, such as the inhibitory capacity, reflected by greater N2 amplitudes when alcohol-dependent individuals carry out an inhibitory process in presence of alcohol cues, dampening behavioral control. Moreover, incentive salience is also evidenced through higher P3 amplitudes in frontal regions and a reduced power of theta band, during loss and gain conditions in decision-making tasks. This supports the behavioral results that indicate difficulties within the proper evaluation of behavioral alternatives in AUDs. In the clinical context, patients with more altered values in this dimension put alcohol at the center of their thinking, without attending to other things in the environment. As a consequence, the risk of relapse in situations where alcohol is present is greater.

Patients are not always aware of this attentional bias, so a good therapeutic proposal would be the training in attentional control and avoidance of risky situations at least at the beginning of treatment. As treatment progresses, work through associative learning could be targeted to establish new contingencies, new stimulus-response-reinforcement relationships. At this point the patient could address a re-evaluation of the way he makes decisions, to value the new reinforcements and train the delay of the reward (see **Table 1**).

Although the evidence in negative emotionality is scarcer and at the time being is still far from being used as a possible endophenotype for AUDs, it could give rise to promising results. In this manner, we could consider neuropsychophysiological activity under affective processes as markers for processes we know as altered in alcohol dependence. To this point, the bibliography considers the use of P1, N1, P2 and LPP components as evidence for the deficient processing of affective content. In addition, there is a promising line in which there is evidence for theta frequency band. Theta seems to be less modulated by affective content in binge drinkers (115), possibly indicating a reduced sensitivity to negative and

TABLE 1 | Behavioral and ERP/ERO paradigms within ANA framework (Incentive salience).

ANA dimension	Paradigm	Psychophysiogical variable	Cognitive processes assessed	Population	Treatment target	Vulnerability	Severity	Outcome	Course
Incentive salience	*Cue reactivity task*	N1: higher amplitude	Preferential attentional processing of substance cues	Alcohol dependence	Attentional bias/ Associative learning	?	+++	?	?
		P1: higher latency	Faster early attentional processing of alcohol cues	Social drinkers		?	+++	?	?
	Dot-probe attentional bias task	P1: higher amplitude and latency	Preferential early attention orientation	Individuals at risk (low alcohol sensitivity)	Attentional bias/ Associative learning	+	?	?	?
		Larger negativity between 220 and 280 ms	Difficulties reorienting attention away from alcohol cues	Individuals at risk (low alcohol sensitivity)		+	?	?	?
	Monetary incentive delay task	P300: decreased amplitude	Task demands processing and context updating Attentional resource allocation Decision making	Alcohol dependence and offspring	Cognitive evaluation process and decision making	+	+	+	++
		Theta: reduced power	Reward processing	Alcohol dependence		+	+	+	?

This table contains a brief summary of main ERP components, ERO activity and their alterations related to alcohol effects, within ANA's domain Incentive salience. It also makes a small mention to main cognitive functions that can be targeted inside psychotherapeutic programs. Shadowed columns refer to the evidence gathered by each neuropsychophysiological marker in the evaluation of cognitive and motivational processes of alcohol dependence in four steps: vulnerability toward alcohol use disorders, dependence severity, outcome and course. "<+++> indicates consistent outcomes in the literature, <++> indicates the presence of some studies, although inconclusive, <+> implies an incipient line of study, with preliminary results and finally, <?> indicates a future line of work.
Potential clinical use and treatment targets.

positive affect. In this way, those people with less recognition of the emotional content, and less adjustment to it, evidenced by the electrical activity, will have greater difficulties in interpersonal relationships (59). Difficulty in recognizing one's own emotions and those of others can lead on the one hand to interpersonal conflicts, which are considered to be one of the main risk factors for relapses (150, 151). There is also evidence of how the presence of negative affect can impair performance in other areas. For example escape drinkers, that is, individuals motivated to drink in order to avoid negative emotions, show greater N2 amplitudes, implicated in more controlled attentional operations, indicating a greater attentional bias toward alcohol cues (39). Hence, the presence of negative affect seems to lead to greater attention being paid to alcohol-related stimuli. Moreover, it could lead to the use of alcohol as the most effective mechanism for emotional self-regulation. Thus, a treatment goal for this patient profile should first impact on the identification of both negative and positive emotions and the acceptance that negative emotions are adaptive. Once the patient is aware of his or her emotional state, the therapeutic objective could be emotional regulation and adaptive coping (see **Table 2**).

TABLE 2 | Behavioral and ERP/ERO paradigms within ANA framework (Negative emotionality).

ANA dimension	Paradigm	Psychophysiogical variable	Cognitive processes assessed	Population	Treatment target	Vulnerability	Severity	Outcome	Course
Negative emotionality	*Affective images processing*	LPP: decreased amplitude to negative images.	Emotional cues classification and processing	Acute alcohol intake Binge drinkers	Affect regulation and coping	+	?	?	?
	Appraisal	Theta: decreased power	Emotional processing	Binge drinkers	Re-appraisal	+	?	?	?
	Emotional Face expressions	P100, N100, and N170: decreased amplitude.	Recognizing emotional face expressions	Alcohol dependence	Conscious affect processing	?	?	?	?

This table contains a brief summary of main ERP components, ERO activity and their alterations related to alcohol effects, within ANA's domain negative emotionality. It also makes a small mention to main cognitive functions that can be targeted inside psychotherapeutic programs. Shadowed columns refer to the evidence gathered by each neuropsychophysiological marker in the evaluation of cognitive and motivational processes of alcohol dependence in four steps: vulnerability toward alcohol use disorders, dependence severity, outcome and course. "<+++> indicates consistent outcomes in the literature, <++> indicates the presence of some studies, although inconclusive, <+> implies an incipient line of study, with preliminary results and finally, <?> indicates a future line of work.
Potential clinical use and treatment targets.

TABLE 3 | Behavioral and ERP/ERO paradigms within ANA framework (Executive dimension).

ANA dimension	Paradigm	Psychophysiogical variable	Cognitive processes assessed	Population	Treatment target	Vulnerability	Severity	Outcome	Course
Executive Dimension	*Oddball Paradigm*	P3a and P3b: reduced amplitude. P3: reduced Delta, theta: decreased power	Attentional resource allocation	Alcohol dependence Offspring Offspring	Attentional control	+++	+++	+++	+
	Go-NoGo	N2: reduced amplitude	Inhibition of the motor response	Alcohol dependence	Executive behavior: Identification of high-risk situations. Generation of alternative responses	+	++	++	+
		P300: reduced amplitude	Inhibition of the motor response	Alcohol dependence and offspring		++	+++	+++	?
		Delta	Attention and task demand			+	?	?	?
		Theta	Attention and inhibition	Alcohol dependence		++	?	?	?
		Alpha	Inhibition			+	?	?	?

This table contains a brief summary of main ERP components, ERO activity and their alterations related to alcohol effects, within ANA's domain executive function. It also makes a small mention to main cognitive functions that can be targeted inside psychotherapeutic programs. Shadowed columns refer to the evidence gathered by each neuropsychophysiological marker in the evaluation of cognitive and motivational processes of alcohol dependence in four steps: vulnerability toward alcohol use disorders, dependence severity, outcome and course. "<+++> indicates consistent outcomes in the literature, <++> indicates the presence of some studies, although inconclusive, <+> implies an incipient line of study, with preliminary results and finally, <?> indicates a future line of work.

Potential clinical use and treatment targets.

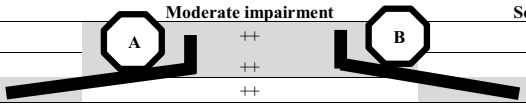
Finally, the most conclusive results are in the P3 component (see **Table 3**), where both alcohol-dependent patients and their offspring show alterations. Which represents a clear example of a possible endophenotype. This component reflects controlled processes of conscious attention, context updating processing and executive attention (15, 152), that are clearly comprised inside the executive dimension of ANA. In this dimension, there is also evidence in N2, and the delta, theta and alpha frequency bands, reflecting alterations in inhibitory control. The patient with alterations in this cognitive domain will be especially vulnerable to relapses, as it will be difficult to stop automatic behaviors in benefit of more controlled processes, such as rejecting the tendency to consume alcohol one they are inside a context of risk (153).

Taking into account what has been reviewed so far, the combination of information from neuropsychology, clinical psychology, and neuropsychophysiological markers could lead to differentiated clinical profiles, based not on the final behavior of pathological consumption, but on the underlying cognitive processes. The objective for a future line of research would be to adjust the treatment to the deficient processes and to the preserved ones. In addition, the development of this line of work, perhaps can lead us to explain the clinical heterogeneity that we find in daily practice. Thus, as an example for illustrative purpose, a patient with an A profile (see **Table 4**), who manifests both moderate levels of incentive salience and negative emotionality, and a mild affectation of the executive component, could find himself in situations of risk when exposed to the context of consumption, or in moments of emotional conflict. But he would only relapse when his executive control capacity would fail. Possibly his relapse would be characterized by a consumption not necessarily intense and with a determined duration until he could recover the executive control. However, the patient with a B profile (see **Table 4**), under the same risk conditions, but with severe impairment of the executive function, would hypothetically relapse more easily, leading to a relapse of a greater intensity and prolonged in time.

This future line of work would help design long-term treatment strategies, which considers the starting point, but also the evolution of these cognitive processes within the process of change caused by treatment and abstinence, and therefore it would be individualized (1, 2). Thus, within the framework of techniques that already have proven efficacy, such as contingency management, relapse prevention, motivational interviewing, or pharmacotherapy, the A-profile patient could follow a standardized treatment as a basis,

TABLE 4 | Examples of cognitive profiles in AUD based on cognitive and ERP/ERO evidence.

Domain	Low impairment	Moderate impairment	Severe impairment
Incentive Salience	+	++	+++
Negative emotionality	+	++	+++
Executive control	+	++	+++



Hypothetical deterioration profiles in the three dimensions of the ANA framework. The crosses indicate the severity of the alteration in each dimension analyzed. <+> indicates low impairment, <++> moderate impairment, and <+++> severe impairment.

but with an emphasis on recognizing risk situations and formulating alternative plans for consumption. While the B-profile patient, in addition to following the standardized treatment, could reinforce the therapy, by carrying out in the initial moments of the treatment a good management of contingencies, avoiding the situations related to alcohol, and then working explicitly with techniques of compensation or substitution of the inhibitory control.

This line of work has results on specific components such as the attention bias, approximation trends toward alcohol, and the underlying neuropsychophysiological measures such as alpha power or P3 amplitude, which seem to change after treatment (136, 154, 155), being good markers of interindividual change. Therefore, there could be evidence of greater homogeneity when used to assess the clinical course of patients. Since they show a great sensitivity to change along the treatment process (155, 156). Possibly because they reflect the integrity of some cognitive components that we could consider as intermediate endophenotypes, or as "stepping stones" to the disorder, but also toward the clinical recovery (8). This is a very promising line of work.

Notwithstanding, given the variability of the revised measures, the applicability of this proposal is still developing, in order to be accessible for the different health care professionals. Patrick et al. revise the specific needs for implementing these types of measures in our daily clinical practice (10), highlighting several of them. The first one has to do with the need for technological resources: although EEG equipment is habitual in hospital units, they are not always prepared for a complex event-related activity measurement. Additionally, this evaluation supposes an extensive methodological effort, this is probably the greatest difficulty encountered for results replicability and their employment. The second important need has to do with the generalization of the results and their consistency. A great diversity of cognitive and behavioral paradigms is available for cognitive and EEG evaluation, with different types of stimuli, diverse presentation times and even required responses from individuals. This could explain part of the variability found in research, thus a need for studies that verify validity and reliability of the proposed measures is becoming relevant. In this way, standardization and validation processes of behavioral event-related potentials paradigms would be enabled, similar to those carried out to develop neuropsychological assessment (10).

Despite the variability, most of the groups working in this line of research agree in highlighting several advantages of including electrophysiological variables as objective measures of the cognitive process, since their alteration may be present in patients whether or not the final behavior is successful, reflecting the lack of efficiency of the process (10). In this way, the results of neuropsychological and behavioral evaluation sometimes do not identify the high effort that the patient makes to reach successful behavior. Therefore, they do not identify either the lack of resources to cope with more demanding situations, since resources are destined to frequent events. Leaving the patient unable to adjust to more difficult or novel situations. That may make more difficult reinsertion into the different life spheres for the patient with alcohol dependence and in the final recovery achievement (11, 157). Thus, the employment of these types of measures brings a certain type of information that would otherwise pass unnoticed in the clinical context and that could be relevant in order to obtain a more objective profile of the three dimensions proposed by ANA. In the end, this would help into the development of adequate individualized treatment designs (1).

In conclusion, the neuropsychophysiological evaluation through event-related potentials is already set for being employed in the clinical context, although a more extensive diffusion and a process of standardization of paradigms are still necessary, so that professionals can acknowledge its use and for a greater applicability inside personalized interventional programs.

Future lines of work can focus on the use of novel measures of brain communication in AUD characterization. Functional and effective connectivity analyses of the brain can bring light upon the synchronization and coordination between regions and networks that take place during cognitive processing. Moreover, brain graph measures can help us observe specific brain areas that may be acting as hubs of communication, as well as network efficiency at local and global levels in several cognitive functions. In this way, we could observe specific network functioning and interacting from preattentional processes to more controlled and complex mental operations. This is interesting for AUD alterations, since several cognitive domains present deficiencies, considering the early salience of alcohol cues and problems in the affective processing and the executive function. To this date, functional connectivity studies in psychiatric conditions are mostly exploratory and have the purpose of characterizing the neural functioning of the brain. In AUD, functional connectivity studies using EEG have found alterations in brain synchronization in several frequency bands (alpha, beta or gamma) (132, 149, 158) and they seem to present a reduced efficiency of communication, although results are somehow scarce and diverse. Nonetheless, prospective studies should be able to offer clearer hypotheses on brain alterations in psychiatric patients, such as AUD, and to help find out if they persist in time or are modulated by different clinical factors.

AUTHOR CONTRIBUTIONS

RJ-B, JP-M, and GR performed the *Introduction* and the *Discussion* sections. AS performed the *Incentive Salience* section. ID-C performed the *Negative Emotionality* section. AM-M performed the *Executive Dimension*. RJ-B, GR, AS, ID-C, and AM-M contributed to the performance of the *Other Psychophysiological Evidences, Brain Functional Connectivity*, and *Network Characteristics* sections. FN, MG-G, and JM have collaborated in the manuscript planning and reviewing, as well as the writing supervision in terms of relevance for the reader. JM contributed by selecting the most relevant information for the aim of the publication. FN and MG-G have made a revision of the English version of the manuscript.

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Predicting Treatment Outcome in Major Depressive Disorder Using Serotonin 4 Receptor PET Brain Imaging, Functional MRI, Cognitive-, EEG-Based, and Peripheral Biomarkers: A NeuroPharm Open Label Clinical Trial Protocol

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Background: Between 30 and 50% of patients with major depressive disorder (MDD) do not respond sufficiently to antidepressant regimens. The conventional pharmacological treatments predominantly target serotonergic brain signaling but better tools to predict treatment response and identify relevant subgroups of MDD are needed to support individualized and mechanistically targeted treatment strategies. The aim of this study is to investigate antidepressant-free patients with MDD using neuroimaging, electrophysiological, molecular, cognitive, and clinical examinations and evaluate their ability to predict clinical response to SSRI treatment as individual or combined predictors.

Methods: We will include 100 untreated patients with moderate to severe depression (>17 on the Hamilton Depression Rating Scale 17) in a non-randomized open clinical trial. We will collect data from serotonin 4 receptor positron emission tomography (PET) brain scans, functional magnetic resonance imaging (fMRI), electroencephalogram (EEG), cognitive tests, psychometry, and peripheral biomarkers, before (at baseline), during, and after 12 weeks of standard antidepressant treatment. Patients will be treated with escitalopram, and in case of non-response at week 4 or intolerable side effects, offered to switch to a second line treatment with duloxetine. Our primary outcome (treatment

response) is assessed using the Hamilton depression rating subscale 6-item scores at week 8, compared to baseline. In a subset of the patients (n = ~40), we will re-assess the neurobiological response (using PET, fMRI, and EEG) 8 weeks after initiated pharmacological antidepressant treatment, to map neurobiological signatures of treatment responses. Data from matched controls will either be collected or is already available from other cohorts.

Discussion: The extensive investigational program with follow-up in this large cohort of participants provides a unique possibility to (a) uncover potential biomarkers for antidepressant treatment response, (b) apply the findings for future stratification of MDD, (c) advance the understanding of pathophysiological underpinnings of MDD, and (d) uncover how putative biomarkers change in response to 8 weeks of pharmacological antidepressant treatment. Our data can pave the way for a precision medicine approach for optimized treatment of MDD and also provides a resource for future research and data sharing.

Clinical Trial Registration: The study was registered at clinicaltrials.gov prior to initiation (NCT02869035; 08.16.2016, URL: https://clinicaltrials.gov/ct2/results?cond=&term=NCT02869035&cntry=&state=&city=&dist=)

Keywords: major depressive disorder, biomarker, treatment response, serotonin 4 receptor, positron emission tomography, functional magnetic resonance imaging, electroencephalogram, cognition

INTRODUCTION

Major Depressive Disorder (MDD) is one of the most severe and common brain disorders worldwide with a huge impact on life quality and socioeconomic status (1, 2). It has been linked to serotonergic dysfunction, cognitive disturbances, brain network dysfunction, vulnerability to stress, neuro-inflammation, and gene by environment factors. Still, the understanding of the pathogenesis remains limited. Guidelines for MDD treatment selection are still predominantly based on simple clinical observations about overall MDD severity, and in the case of recurrent depressive episodes, it is also based on personal patient history of treatment responses. Conventional medical treatment is mainly based on intervention of the monoaminergic system in the brain, in particular the serotonin (5-HT) system. Selective serotonin reuptake inhibitors (SSRIs) act through blockage and subsequent downregulation of the serotonin transporter (SERT) (3), which presumably induces increased extracellular 5-HT levels. However, robust evidence for a central 5-HT hypofunction in patients with MDD *in vivo* is lacking (4). Roughly one third of patients suffering from MDD do not respond sufficiently to 5-HT acting drugs (5, 6), suggesting a diverse pathophysiology. The diagnostic criteria for MDD may cover a heterogenous collection of various biological entities and consequently, it is not surprising that a "one size fits all" treatment strategy is suboptimal (7). Currently, the time from starting to administer a potentially efficacious drug until it can be determined if the clinical response is satisfactory is, at best, 4–6 weeks. In clinical practice, the lack of convenient and accurate tools (e.g., quantitative and/or biological) to predict treatment response prolongs the delay from diagnosis to effective treatment and constitutes a major challenge for both clinicians and patients. Therefore, stratification of subtypes and a shift toward precision medicine, e.g., through identification of predictors of treatment response, so-called biomarker(s) that can help optimize treatment choice, is of paramount importance. Candidate biomarkers could be related to neurotransmission, specific neural networks or structural alterations in specific brain regions that can be detected by brain imaging modalities such as positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and electroencephalogram (EEG) or altered biophysiological or cognitive functions (4, 8). It has also been suggested that rather than a single biomarker, an algorithm involving a set of biomarkers may prove useful to subgroup patients and predict their response to certain treatment strategies in MDD (9). Several biomarkers derived from prior large studies such as iSPOT-D, EMBARC, and CANBIND for prediction of drug response in MDD have been proposed (10–12). Here, we use multimodalities (PET, fMRI, and EEG, cognitive testing, psychometrics, and peripheral biomarkers) as part of a deep phenotyping and as a unique feature to our trial, we study modes of action in the brain on a neurotransmitter level. Thus, our trial contributes with novel insights as well as

Abbreviations: MDD, Major Depressive Disorder; 5-HT: 5-hydroxytryptamine (Serotonin); SSRI, Selective serotonin reuptake inhibitor; SERT, Serotonin transporter; PET, Positron emission tomography; fMRI, Functional magnetic resonance imaging; EEG, Electroencephalogram; $5\text{-HT}_4\text{R}$, Serotonin 4 receptor; rs-fMRI, Resting state functional magnetic resonance imaging; ERP, Event-related potential; LDAEP, Loudness dependence of auditory evoked potentials; CAR, Cortisol awakening response; HPA-axis, Hypothalamic-pituitary-adrenal axis; HAMD_{17}, 17-item Hamilton Depression Rating Scale; HAMD_6, 6-item Hamilton Depression Rating Scale.

provide a dataset for cross-validation of other identified predictors of psychopharmacological antidepressant treatment response. In a non-randomized, longitudinal, open clinical trial, patients with moderate to severe depression will be treated with SSRI following Danish guidelines. In order to map neurobiological signatures of treatment, we will re-examine a subset of the cohort with neuroimaging and EEG after 8 weeks of SSRI treatment and assess cognitive changes after 12 weeks. This clinical trial is part of a larger research initiative, "NeuroPharm", which addresses pertinent and basic questions regarding human brain disease mechanisms and seeks to predict brain responses to categories of neuro-modulatory interventions as well as treatment efficacy (www.np.nru.dk). We anticipate that this study will critically advance and inform future stratification strategies, further uncover pathophysiological and treatment mechanisms and, hopefully, guide future precision medicine approaches to optimize treatment strategies for patients suffering from MDD.

Imaging techniques have vastly increased our understanding of the underpinning cerebral mechanisms involved in MDD (13). Serotonergic dysfunction is considered a central mechanism in depression, and a recent review points at the 5-HT 4 receptor (5-HT_4R) as highly implicated in MDD (14). For example, 5-HT_4R agonism has shown rapid antidepressant -like behavioral effects in rodents (15), and experimental models suggest that cerebral 5-HT_4R levels are sensitive to central 5-HT modulation in rodents (16, 17). Subsequent clinical studies from our group demonstrated that cerebral 5-HT levels can be indexed in an inverse manner through molecular brain imaging of the 5-HT_4R by using the PET-ligand 11C-SB207145 *in vivo* (18). We here aim to evaluate 5-HT_4R binding as a candidate predictor of antidepressant response to drugs targeting the 5-HT system in the hitherto largest cohort of MDD patients with PET brain imaging of serotonergic markers. We hypothesize that 1) patients with MDD differ in cerebral [^{11}C]SB207145 binding at baseline compared to healthy controls; 2) [^{11}C]SB207145 binding at baseline in patients with MDD predicts remission after 8 weeks of pharmacological serotonergic intervention; 3) After 8 weeks of serotonergic intervention, patients with remitter status have a significantly greater reduction in cerebral [^{11}C]SB207145 binding than non-responders. For an overview of primary hypotheses for other modalities, see **Appendix 1**.

fMRI can be used to assess regional activity and resting state functional networks in MDD. One systematic review found abnormal (negative bias) reactivity in amygdala responsiveness to facial expressions and emotional stimulation in patients with MDD versus healthy controls (19), and pre-treatment low amygdala reactivity has shown to be predictive for antidepressant treatment response (20). A study with 70 patients with MDD was able to predict treatment recovery with ~80%, by investigating amygdala reactivity to facial emotions and its interaction with history of early life stress (21). Another study from our group showed that amygdala reactivity was associated with brain 5-HT_4R binding and hence putatively extra synaptic 5-HT levels in healthy individuals. This established a plausible connection between 5-HT levels and amygdala activation, both involved in emotional cognitive processes (22). This exemplifies how a multimodal PET and fMRI strategy can highlight molecular mechanisms mediating drug effects on brain function (23). Resting state fMRI (rs-fMRI) measures fluctuations in fMRI signal during the absence of an explicit task and is widely used to assess distributed intrinsic networks such as the "default mode network" (24). Alterations in rs-fMRI connectivity have been described in MDD (25) and a recent study suggested that rs-fMRI can define subtypes of MDD and predict antidepressant treatment response (26), but this has been contested by others (27). Although promising, brain imaging studies have in general been inconclusive and with small sample sizes (9, 28). In the current trial, we will use task-based and rs-fMRI in a large cohort of patients with MDD and investigate the association between 5-HT_4R levels (as a proxy for brain serotonin levels) and the clinical outcome of SSRI treatment.

EEG, a monitoring technique for direct ongoing neural activity, has been reported to be associated with treatment response in MDD [see, e.g., review (29)]. Prior studies have found that treatment responders have higher cortical alpha activity (30) and higher theta activity at rostral anterior cingulate cortex compared to treatment non-responders (31, 32). Of note, these biomarkers were derived from the resting EEG data, which is relatively easy to implement in the clinic. Furthermore, earlier evidence from event-related-potential (ERP) studies have suggested that ERP biomarkers such as auditory P300 (a positive waveform around 300 ms after stimulus onset) and loudness-dependence of auditory evoked potentials (LDAEP) can be predict drug treatment response (33, 34), and are linked to the serotonergic transmitter system (35). In the current trial, the predictive values of pretreatment EEG/ERP biomarkers will be examined.

Disturbances in cognitive processes including memory, attention, and executive functions are commonly reported in MDD (36) and contribute to psychosocial impairment and workforce disability (37). In addition, affective bias in information processing (i.e., favoring negative information over positive information at different levels of information processing) has been proposed as a central mechanism in the development and maintenance of depressive symptoms (38) which is also predictive of later treatment response to antidepressant drugs (39). Notably, cognitive disturbances do not always resolve with the remission of a depressive episode, suggesting a dissociation between core mood and cognitive symptoms in MDD (40). Combined with the low cost and relative ease of testing in a clinical setting, this distinguishes cognitive disturbances as a promising marker for stratification of depression subtypes as well as an important target for antidepressant treatment. In the present study, we therefore aim to map a broad range of cognitive disturbances in MDD, including both cold (non-emotional) and hot (emotional) cognitive processes, and explore whether they may be used to characterize clinically relevant subgroups in MDD. Based on earlier observations in healthy individuals, we expect memory performance to map onto hippocampal 5-HT_4R availability (41) and possibly affective bias in verbal memory in MDD (42).

Evidence of inflammation-associated MDD has emerged over the years (43). Patients with MDD show elevated levels of inflammatory markers in peripheral blood (44) which may

affect treatment response such that higher levels are associated with worse response (45). It has also been suggested that patients with MDD have higher levels of activated microglia, as illuminated with PET (46). Proinflammatory cytokines may influence the 5-HT homeostasis in the brain by acutely upregulate SERT through intercellular pathways (i.e., linked to p38 mitogen-activated protein kinase) and presumably thereby reduce synaptic 5-HT levels (47). Interestingly, cognitive dysfunction, a prevalent symptom in depression, also appear to be linked to an inflammatory response (48). We here aim to determine if higher levels of systemic inflammatory markers are associated with 5-HT_4R brain binding, depression status at baseline and clinical treatment response.

Another area of interest is the association between MDD and signatures of early aging. There is an increased mortality and prevalence of age-related diseases in recurrent depression (49, 50). Oxidative stress on nucleic acids is a general element of aging and has been suggested to be an underlying biological mechanism of the accelerated aging observed in depression (51). Previous research from our group has found evidence for such a link, both in studies of psychological/biological stress and oxidative stress in patients and in rodent models of depression (52–54). Earlier findings indicate alterations in levels of oxidative stress during antidepressant treatment and it is hypothesized that treatment response is related to a transient increase in oxidative stress levels, perhaps due to neurotrophic processes and/or peripheral changes in energy metabolism (55–57). Urinary 8-oxodG and 8-oxoGuo are sensitive and specific markers for systemic DNA/RNA damage from oxidation (58). We here aim to investigate urinary 8-oxodG and 8-oxoGuo as a predictive biomarker for antidepressant treatment response, its association with changes in psychopathology, structural and functional brain changes, and markers of psychological and biological stress. Additionally, we will investigate whether hormonal [estradiol, testosterone, progesterone and follicle-stimulating hormone (in females)] and metabolic status can predict antidepressant treatment response and explore whether these associations are related to genetic make-up (specified below), psychopathology and the occurrence of early life stress using self-reported childhood adverse events and parental bonding quality questionnaires, which also may interact with the 5-HT system (59, 60).

Sexual dysfunction (e.g., low sexual desire, arousal difficulties, and anorgasmia) is a prominent feature of MDD, which often leads to a decline in quality of life (61, 62). Lack of interest to what is usually pleasurable, i.e., anhedonia, is a core symptom in MDD and may also be reflected in reduced sexual desire/interest. Sexual dysfunction, in particular anorgasmia and sexual arousal difficulties may further be linked to serotonergic dysfunctions (63) as seen in MDD. As a complicating factor, impaired sexual health related to MDD may worsen with antidepressant treatments targeting the 5-HT system. For example, in a group of 704 patients with MDD treated with an antidepressant drug (SSRI) or serotonin-norepinephrine reuptake inhibitor, about half of them developed or experienced worsening in their decreased sexual desire as a side-effect, which was also associated with reduced quality of life, lower self-esteem and adverse effects on mood and partner relations (62). We currently do not know which patient characteristics predict sexual dysfunction in response to SSRI treatment. However, differences in individual serotonergic brain architecture and/or serotonergic response to antidepressant treatment (e.g., SSRI) may play a role. In this study, we aim to map the frequency and predictors of SSRI induced sexual dysfunction and determine if serotonergic tonus (measured by 5-HT_4R PET binding) pre-treatment, or changes in response to SRRI treatment, is associated with sexual desire and/or development of SSRI-induced sexual dysfunction in MDD.

Previous findings from our group have repeatedly demonstrated a coupling between key features of the 5-HT system and hypothalamus- pituitary- adrenal axis (HPA-axis), which regulates the release of the stress-hormone cortisol (64). Such HPA-axis dynamics can be measured by the cortisol awakening response. Our results support that both serotonin transporter availability (65), and serotonergic tone or direct capacity for 5-HT_4R agonism (64) support a healthy cortisol response to HPA-axis stimuli. A well-functioning and dynamic HPA-axis is critical for coping with everyday life stressors, and HPA-axis dysregulation is a prominent feature of MDD. Although heightened CAR is associated with relapse of depressive episodes in patients with a history of depression (66), in the more advanced depressed stages, i.e., chronic depression, HPA-axis dynamics are blunted as opposed to recent-onset depression (67). Notably, normalization of the HPA-axis in response to SSRI treatment appears to protect against relapse (68). Thus, the SSRI treatment response is likely to depend on restoring HPA-axis dynamics at least in a subgroup of MDD patients. In this trial, we will assess CAR in patients with MDD, the effect of SSRIs on CAR, investigate its association with baseline 5-HT_4R distribution, as well as evaluate CAR as a predictor of antidepressant treatment outcome.

MATERIALS AND METHODS

Figure 1 shows a flowchart of the scheduled data collection over the 12 weeks of pharmacological drug treatment of patients with MDD. Healthy controls (HC) will be recruited as specified below. Patients will be examined before (at baseline; week 0) and after 1, 2, 4, 8 and 12 weeks of SSRI treatment has been initiated. Depression-severity will be monitored by the Hamilton Depression Rating scale 17 items ($HAMD_{17}$) and its subscale of 6 items ($HAMD_6$) (69). A subset of patients will be offered re-examination with PET, fMRI, and EEG after 8 weeks of treatment, to assess changes from baseline and its association to treatment response. Patients from the whole spectrum of treatment responses (from poor to excellent) will be invited in a continuous fashion for this part of the study until allotted re-examinations are completed. All patients will also repeat cognitive testing at week 12. The power analysis in preparation of the study was primarily anchored to the PET modality. We estimated that we needed to include 100 patients to reach a

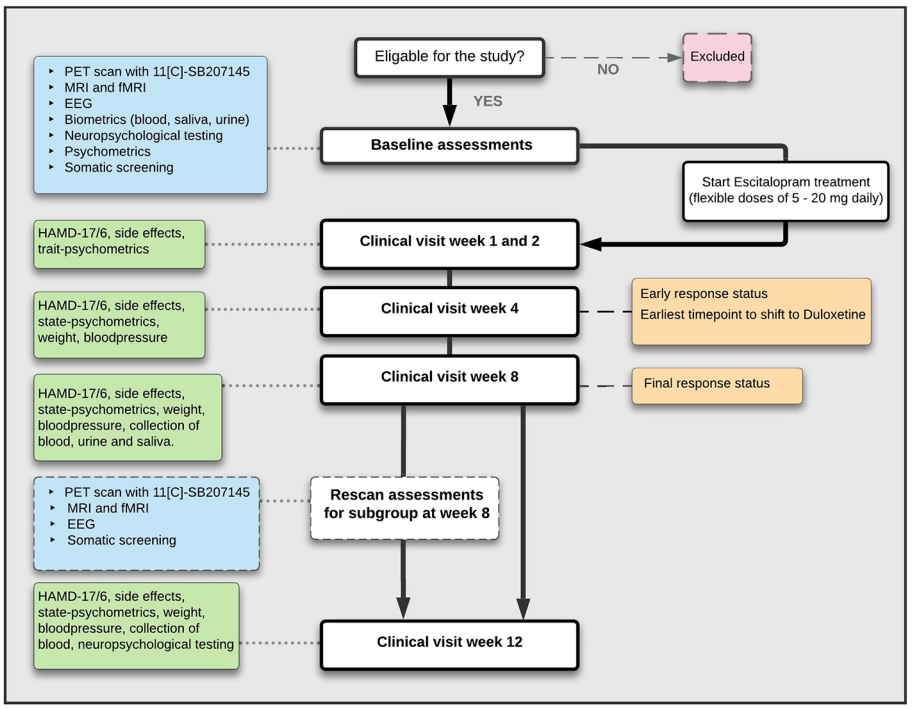

FIGURE 1 | Flowchart of study trial assessments for patients with MDD.

statistical power of 80% to detect an association between treatment response (binary classification, i.e., remitters vs non-responders, see response-definition below) and baseline 5-HT_4R non-displaceable binding potential (BP_{ND}). These calculations were based on an expected 20% maximum drop-out, ~50% remission rate after 8 weeks of treatment (5, 6) and an expected difference of 8% in 5-HT_4R binding between remitters and non-responders, corresponding to the previously found effect sizes on 5-HT_4R change in BP_{ND} after fluoxetine treatment (18). Calculations were further based on an average BP_{ND} of 0.71 and a standard deviation of 0.073 (18, 70). With a rescan subgroup of approximately 40 patients, and a Gaussian distribution of change in BP_{ND} with an SD of 0.08 (log scale), we had an expected power of 80% to identify a significant association between longitudinal changes in BP_{ND} and changes in $HAMD_6$ (i.e., secondary clinical outcome, continuous scale).

Participants

Patients are recruited from a central referral center within the mental health services in the Capital region of Denmark or directly referred from one of five general practitioners in collaborations with the study group (see **Figure 2** (CONSORT) for details). Data from healthy controls for the purpose of baseline comparisons to patients with MDD are available from a pre-existing database on site (71). The healthy control reference population will be supplemented with newly recruited healthy controls from a local volunteer database (www.nru.dk), as necessary.

Inclusion Criteria for Patients

Patients between 18 and 65 years of age with a moderate to severe, single, or recurrent episode of MDD consistent with the Diagnostic and Statistical Manual of Mental Disorders -5 (DSM-5) and International Statistical Classification of Diseases and Related Health Problems -10 (ICD-10) criteria will be recruited by a trained clinician. Inclusion requires a total score of >17 on $HAMD_{17}$ at baseline and the diagnose is confirmed by using the diagnostic tool Mini International Neuropsychiatric Interview (72). In addition, all patients are diagnostically verified by a specialist in psychiatry before final inclusion.

Exclusion Criteria for Patients

Patients with a duration of their present depressive episode exceeding two years are not included. No more than one antidepressant treatment attempt in the current episode prior to inclusion is allowed and only patients with no antidepressant medication within the last two months are eligible. Patients with known contraindications or previous non-response to an SSRI drug after an adequate trial as well as a prior or present history of other primary axis I psychiatric disorders are not included, i.e., MDD must be the primary diagnosis. Other exclusion criteria are: severe somatic illness; substance or alcohol use disorder; insufficient language skills to undergo clinical assessments; acute suicidal ideation or psychosis; patients who are deemed by a psychiatrist to require other forms of antidepressant treatments; pregnancy or breast feeding; use of any CNS drug that cannot be washed out prior

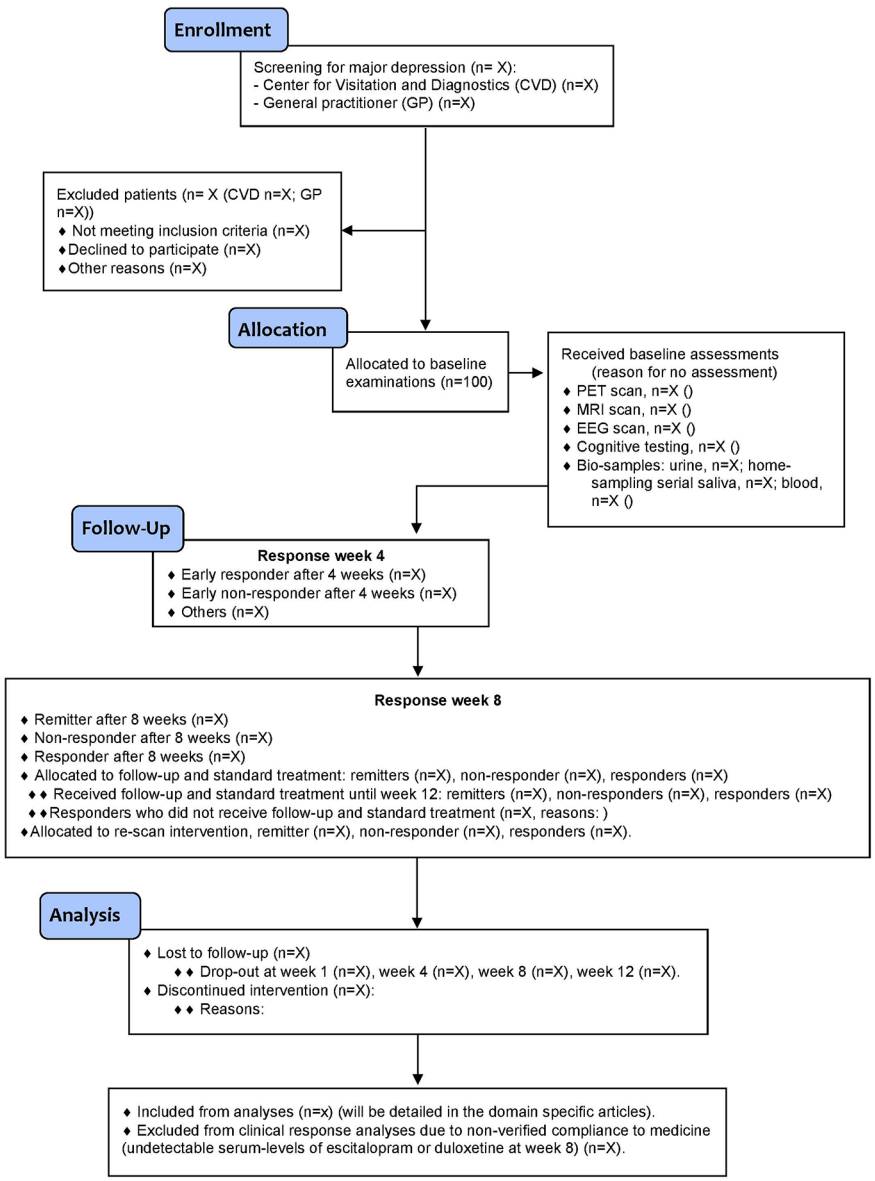

FIGURE 2 | Flow diagram (CONSORT) of the NeuroPharm trial.

to participation (e.g., metoclopramide, ondansetron, serotonergic migraine medicine, clonidine); medical conditions interfering with measurements, contraindications for PET and/or MRI scans; exposure to radioactivity >10 mSv within the last year; severe sensory or intellectual impediments interfering with comprehension of procedures or assessments and lastly any history of brain injury (i.e., loss of consciousness and amnesia or symptoms of concussion disorder).

Inclusion and Exclusion Criteria for Healthy Controls

Enrolled HC will be sought to match the patient population by gender and age distribution. All HC will be screened for MDD using a self-reporting questionnaire (major depression inventory) (73). The HC meet the same inclusion and exclusion criteria as required for patients apart from psychiatry related issues (e.g., no current or history of mental illness or unstable somatic condition).

Treatment and Investigation Program

Baseline Assessments Before Treatment

Each patient will receive a basic physical screening including somatic status, routine blood samples, electrocardiogram including QTc interval and collection of toxicology urine tests [The Rapid ResponseTM Multi-Drug Test Panel (Urine)] for detection of drug abuse within the last month. Women are screened for pregnancy through self-reported use of contraceptives and a pregnancy urinary

test if relevant. All study-participants will undergo baseline assessments of brain imaging with 11C-SB207145 PET and fMRI; EEG-examination; cognitive testing, collection of questionnaires and biological material (venous blood, urine, and saliva) as specified below. All HC will receive corresponding baseline assessments.

Clinical Procedure After Treatment Initiation

After completion of baseline examinations, patients will receive flexible doses of the SSRI drug escitalopram, initially 5 mg for 3–5 days depending on side effects (e.g., nausea), followed by 10 mg daily until their first follow-up visit and further adjusted individually to a maximum dose of 20 mg. Escitalopram was chosen as it binds with high selectivity to the 5-HT transporter and has minimal affinity for other receptors (74). Patients are allowed short-time treatment with cyclopyrrolone (a nonbenzodiazepine hypnotic agent) or oxazepam (a benzodiazepine) to reduce anxiety and sleep disturbances which may be prominent in the initial treatment phase and have shown not to influence treatment continuation (75), but all are requested to avoid use 3 days prior to brain scans. Clinical follow-up sessions with a study physician or trained research assistant are scheduled in an out-patient clinical setting after 1, 2, 4, 8 and 12 weeks of treatment to evaluate treatment response and side effects. Visits can deviate a maximum of one week from the original time scheduled. No cognitive behavioral therapy or other psychotherapy program is provided during clinical visits. No treatment (pharmacological or psychotherapeutically) other than the medical monotherapy provided in this study is allowed elsewhere during the trial. At week 4, early non-responders (see definition below) or patients with unacceptable side effects are offered to switch to a standard second line antidepressant treatment; duloxetine (individually adjusted doses of 30–120 mg per day), which is a serotonin-norepinephrine reuptake inhibitor. Duloxetine was chosen according to clinical guidelines for second line antidepressant treatments. Cerebral 5-HT_4R binding in humans is unaltered by injection of escitalopram (76). No prior *in vivo* studies have investigated the effect of duloxetine on 5-HT_4R binding in the human brain, but *in vitro* work has shown that duloxetine has negligible affinity for the 5-HT_4R (77). That is, none of the pharmacological compounds directly target 5-HT_4R's. The week 4 timepoint is in line with national guidelines in Denmark for switching to a second-line antidepressant treatment (4–6 weeks). Since our cohort receives frequent clinical follow-up sessions, patients can reach max dose of escitalopram (20 mg daily) already after 2 weeks. As such, switching after 4 weeks is considered appropriate for early non-responders in this trial set-up. All antidepressant medicine will be provided for free to improve compliance. Compliance will be assessed by serum escitalopram/duloxetine blood tests after eight weeks of treatment as well as tablet count at each follow-up. At each visit, depressive symptoms are rated using the $HAMD_{17}$ and the $HAMD_6$ subscale. $HAMD_6$ captures core symptoms of depression more directly (and disregards sleeping quality), and has been found to be sensitive to antidepressant treatment response (69). Potential side effects due to intervention will be monitored at each visit using the "Udvalg for Kliniske Undersøgelser" scale (78). To ensure agreement and allow alignment of ratings, $HAMD_{17}$/$HAMD_6$ co-ratings between all the clinical investigators will be performed regularly during data collection. A maximum of 20% deviation from the "gold-standard" chief psychiatrist is allowed, or else a new satisfactory co-rating is needed before independent rating of study participants.

Clinical Response Status

Primary Clinical Outcome Measure. The primary outcome measure is categorical and built to capture patients with an early as well as sustained, either excellent or poor response to treatment. Patients are classified as either "remitters", "non-responders", or "intermediate responders" after 8 weeks of treatment. These categories are based on percentage changes of depressive symptoms from baseline, as measured by $HAMD_6$. Remitters must have ≥50% reduction in $HAMD_6$ at 4 weeks (early responders) and a $HAMD_6$ score <5 after 8 weeks of treatment. Non-responders have <25% reduction in $HAMD_6$ after 4 weeks (early non-responder) and <50% reduction in $HAMD_6$ after 8 weeks of treatment. Patients who do not meet the criteria above are defined as "intermediate responders" at week 8. The primary predictor analyses are directed to predict treatment response in a binary fashion (either remitter or non-responder (see **Figure 3**).

Secondary Clinical Outcome Measure. As a secondary outcome, we use a continuous response measure, i.e., $HAMD_6$ changes from baseline at week 8 divided by $HAMD_6$ at baseline, to allow analyses of the association between antidepressant treatment response and baseline characteristics or treatment-induced changes in the neurobiological modalities of interest.

Examination Modalities

PET Imaging and Quantification of 5-HT_4R Brain Binding

PET scans are conducted using a high-resolution research tomography Siemens PET scanner (CTI/Siemens, Knoxville, TN, USA) (256 × 256 × 207 voxels; 1.22 × 1.22 × 1.22 mm). Participants are positioned uniformly in spine position and a specialized head holder is applied to reduce head motion during the scan. All participants undergo a 6 min transmission scan and are given an intravenous bolus of approximately 600 MBq of the PET tracer ligand [^{11}C]SB207145. The bolus is administered over 20 s followed by a 120-min dynamic PET data acquisition. The radioligand is synthesized immediately prior to injection as described elsewhere (79).

Preprocessing and PET Quantification

The 120 min dynamic PET acquisitions are reconstructed into 38 time frames (6 × 5 s, 10 × 15 s, 4 × 30 s, 5 × 2 min, 5 × 5 min, and 8 × 10 min) using a 3D-OSEM PSF algorithm (16 subsets and 10 iterations) (80) and TXTV-based attenuation correction (81). For motion correction, the AIR 5.2.5 software will be used (82), aligning each PET frame to the first 5-min frame. Structural 3-Tesla MRI scans will be used for co-registration of the PET

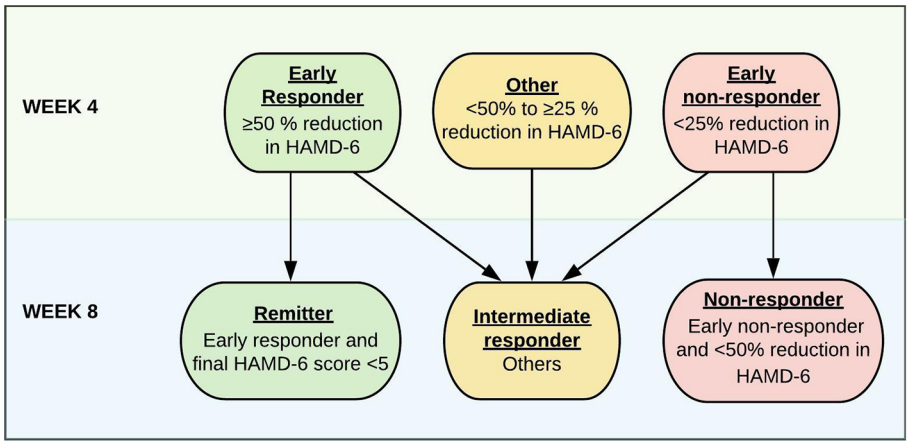

FIGURE 3 | Response categorization for patients with MDD after 4 and 8 weeks of antidepressant treatment based on changes in $HAMD_6$ score.

images with SPM8 software. Automatic delineation will be carried out in a user-independent manner in PVElab software (83) and mean tissue time activity curves for grey matter volumes will be extracted for kinetic modeling. No partial volume correction will be performed because of the high resolution of the scanner. Regions of interest (ROI) have been chosen due to their known relevance in mood disorders and abundance of 5-HT_4R density (84). The selected ROIs for the primary analyses are neocortex, putamen, caudate nucleus and hippocampus. Co-registration and correct ROI placement for all subjects will be inspected in three planes by a trained investigator. PMOD version 3.0 (PMOD, Zurich, Switzerland) will be used for kinetic modeling and quantification of the 5-HT_4R binding is performed using non-displaceable binding potential (BP_{ND}) as the final outcome measure. The simplified reference tissue model will be used with cerebellum (excluding vermis) as reference region which previously has been validated in humans (76). BP_{ND} is defined as:

$$BP_{ND} = \frac{f_{ND} \times \text{Bavail}}{KD}$$

where f_{ND} is the tissue free fraction of non-protein bound ^{11}C-SB207145, Bavail is the concentration of available 5-HT_4R and K_D is the dissociation constant for the tracer at equilibrium. Thus, BP_{ND} is proportional to the density of 5-HT_4R.

MRI and fMRI Imaging

All participants are screened for MR-compatibility and thoroughly instructed how to perform the fMRI paradigms by a trained study assistant who uses standardized instructions. All MRI scans for patients will be acquired using the same Siemens 3-Tesla Prisma scanner with a 64-channel head coil. High-resolution structural T1- and T2-weighted MR images will be acquired. Blood oxygenation level dependent fMRI scans will be obtained during a commonly used emotional faces paradigm (85, 86), reward-related guessing paradigm (87, 88) and a 10-min rs-fMRI scan. During the rs-fMRI scan, participants are asked to close their eyes, let their mind wander and to not fall asleep. All structural scans of patients will be screened for pathological abnormalities by a medical specialist in radiology.

EEG

EEG data is recorded using a 256-channel HydroCel Sensor Net system (EGI, Inc., Eugene, OR) at 1,000 Hz with 0.1–100 Hz analog filtering where vertex electrode serve as the reference. Impedances across all electrodes are kept below 50 kΩ. EEG/ERP recording at baseline included: resting EEG (with eyes closed and open), two-tone auditory oddball and the LDAEP tasks. The same EEG/ERP recording will be re-tested in a subgroup of patients after 8 weeks of treatment.

Resting EEG

Resting EEG is recorded during four 3-min periods with a counterbalanced order of OCOC (O for eyes open, C for eyes closed) or COCO between subjects. Participants are instructed to remain still and relax, avoid eye-blinks and movements and to relax chin muscles during recording. Absolute and relative powers are computed using the following frequency bands: δ (1–4 Hz), θ (4–8 Hz), α (8–12 Hz), and beta (8–30 Hz). In addition, alpha peak frequency (APF) is identified by the frequency at maximal absolute power from the spectral range of 7–13 Hz. Frontal alpha asymmetry will be calculated using alpha power with the formula of (F4 – F3)/(F4 + F3) (Arns et al., 2015). Furthermore, theta activity will be extracted from anterior cingulate cortex with exact low-resolution electromagnetic tomography (eLoreta).

Task Elicited ERPs

The two-tone auditory oddball paradigm consists of two acoustic stimuli with different frequencies. Participants are presented with a series of standard tones (500 Hz) and deviant tones (1,000 Hz) binaurally through inserted earphones (Etymotic Research Inc., ER 3C). They are instructed to press a button when the deviant tones are presented while ignoring the standard tones. ERP components

such as N1 and P3 will be computed, both peak latency and amplitude (baseline to peak) will be extracted by the averaged trials. Participants are presented with five acoustic stimuli with different intensities (60, 70, 80, 90, and 100 dB SPL) in the same frequency of 1,000 Hz. No response is needed. The primary outcome is the slopes of peak-to-peak N1/P2 amplitudes extracted from the average trials at each intensity. A more comprehensive description of the EEG data will be presented in the subsequent reports.

Cognitive Testing

All participants undergo cognitive testing using selected tasks from the novel test battery EMOTICOM, assessing affective and social cognition including emotional face recognition, emotional threshold detection, theory of mind, and moral emotions (89). In addition, affective memory (90), working memory, reaction time and IQ will also be assessed. Testing is planned and conducted by trained neuropsychologists prior to start of drug intervention and again after 12 weeks of treatment.

Psychometrics

Apart from clinical visits including $HAMD_{17/6}$ ratings, patients will apply self-monitoring during the study period and fill out Danish versions of online questionnaires throughout the study. All questionnaires will be imported directly to an internal database through LimeSurvey, a free and open source software. Before EEG scans and cognitive testing, all participants will report their current mood state using an in-house Likert-scale. An adjusted Likert-scale will be filled out after each MR-scan. During visits at week 4, week 8, and week 12, patients are also asked to fill out a comprehensive set of self-rating state questionnaires (see **Table 1** for a full overview). Healthy controls will be asked to fill out selected state questionnaires as part of their baseline assessments.

TABLE 1 | Table over questionnaires obtained throughout the study.

Questionnaires	Time point					
	Baseline	Week1	Week 2	Week 4	Week 8	Week 12
MINI	X					
HAMD-17/6	X	X	X	X	X	X
UKU		X	X	X	X	X
NEO-PIR	X					
CATS	X					
EHI	X					
OS-FHAM	X					
PBI-mother/ father	X					
POMS*	X				X	X
Likert-scale*	X				X	X
BDI-II	X			X	X	X
MDI	X			X	X	X
PSS	X			X	X	X
SHAPS	X			X	X	X
RRS	X			X	X	X
CSFQ_F_C	X			X	X	X
SUSY item 32	X			X	X	X
Activity	X			X	X	X
GAD-10	X			X	X	X

*Trait questionnaires at baseline includes personality traits with NEO-PIR (91); Child Abuse and Trauma Scale (CATS) (92) a survey about early life stress which has shown to be able to modulate the serotonin system in the brain (93); handedness with Edinburgh Handedness Inventory (EHI) (94); an in-house version of the Family History Assessment module (FHAM) questionnaire, i.e., "Online Stimulant" (OS)-FHAM; Parental Bonding Inventory (PBI) (both mother and father) (95). State conditions included a self-rating questionnaire of Profile of Mood States (POMS) (96); an in-house Likert-scale; Beck's Depression Inventory-II (BDI-II) (97); Major Depression inventory (MDI) (98); Cohen's Perceived Stress Scale (PSS) (99, 100); Snaith-Hamilton Pleasure Scale (SHAPS) (101); Rumination Response Scale (RSS) 102; Changes in Sexual Functioning Questionnaire (CSFQ) (103); "Sundhed og Sygelighed" Sex Quality Questionnaire item 32 (SUSY-item 32) (104); an in-house questionnaire about daily physical activity (71); and Generalized Anxiety Disorder-10 (GAD-10) (105). * Collected in immediate extension to EEG and MR examinations or cognitive testing.*

Biomaterials

Blood

At baseline, all participants will be screened for basic somatic status to exclude somatic conditions with possible influence on depressive symptoms. Blood samples will be collected throughout the study (see **Table 2** for a full overview) for determination of inflammatory status (high sensitivity C-reactive protein, tumor necrosis factor-α, Interleukin-6, -18, and -10) (106–109); epigenetic variations (SERT, FKBP Prolyl Isomerase 5, Catechol-O-Methyltransferase (COMT), monoamine oxidase-A, glucocorticoid-, estrogen-, oxytocin receptor and oxytocin gene-methylation); extraction of DNA for genotypes of relevance (rs41271330 (110), serotonin-transporter-linked polymorphic region (5-HTTLPR) (70), COMT, Brain-Derived Neurotrophic Factor val66met) and ABCB1, FZD7, and WNT2B (that presumably influence responsiveness to pharmacological antidepressant treatment (111)). At week 8, serum samples of the antidepressant drug (i.e., escitalopram or duloxetine) are collected as trough concentrations in steady state, with primary purpose of monitoring compliance. The samples will be stored at −20°C (or −80°C for plasma EDTA samples) until analyzation in batches at completion of the trial. Quantification of escitalopram and duloxetine in serum will be performed at the Laboratory of the Danish Epilepsy Centre, Filadelfia, using a routine UPLC-MS/MS method developed in-house. Standard operating procedure instructions have been established before trial initiation and will be followed during the assessment of all biomaterial.

Saliva

Saliva will be collected to determine the total cortisol output across one day as well as dynamics of the HPA-axis, as indexed by CAR. Serial saliva samples will be sampled at home and collected at baseline and at week 8 (see **Table 2**). Those visits will be placed as close to the PET-scan day or week 8 visit as possible, and patients are instructed to take samples immediately after awakening and again after 15, 30, 45, and 60 min, at 12, 6, and 11 pm. Participants are also instructed to collect saliva samples preferably during weekdays, not perform strenuous exercise <2 h and not to have any oral intake or brush their teeth <1 h prior to sampling. Cohen's Perceived Stress Scale and basic information about sleep and food intake will be filled out in conjunction with the home-sampling. All participants receive careful training in saliva collection, instructions of home-sampling procedures; cold storage of samples and fast delivery either by mail or personal delivery to the laboratory facility for preparation. When received, salivary test-tubes are centrifuged and stored at −80°C until later single-batch analysis.

TABLE 2 | Somatic status and biomaterial assessed at various timepoints throughout the study.

Analysis	Sample	Timepoint		
		Baseline	Week 8	Week 12
Somatic blood-sample screening	Hemoglobin, white blood cell count, metamyelo.+myelo.+promyelocytes. C-reactive protein.	X	X	X
	Na+, K+, Creatinine	X		
	ASAT, ALAT, GGT, LDH, BAP	X		
	Albumin, Coagulation factors II+VI+X, thrombocytes	X		
	B12, Folate	X		
	25-OH-vitamin D	X		
	Blood sugar, HbA1c	X		
	Triglycerides, total-cholesterol, HDL, LDL	X		
	TSH, Ionized Calcium	X		
	Estradiol, testosterone, progesterone, FSH (females)	X		
Somatic examination	Electro Cardiogram (ECG)	X		
	Neurological status	X		
	Somatic status	X		
Compliance to medicine control	S -escitalopram or S -duloxetine		X	
Biobank	Inflammation and cytokines (hsCRP, TNF-α, IL-6, IL-18 and IL-10)	X	X	X
Biobank	Epigenetics (5-HTT, glucocortocoid-, FKBP5, COMT, MAO-A, estrogen-, oxytocin receptor and oxytocin gene-methylation)	X	X	X
Biobank	Genotypes (rs41271330, 5-HTTLPR, COMT, BDNFval66met)	X		
Biobank	Gene transcription profiles (mRNA and microRNA, ABCB1, FZD7 and WNT2B)	X	X	X
Oxidative stress	Urine (8-oxo-dG and 8-oxo-Guo)	X	X	
Biobank	Saliva (Cortisol awakening response)	X	X	

Urine

Spot-urine samples will be collected at baseline and week 8 visits for patients (see **Table 2**) in 2-ml Eppendorf tubes and will be stored at −20°C for later single-batch analysis. Apart from pregnancy and drug-screening (see *Baseline Assessments Before Treatment*), all urine samples will be analyzed for 8-oxodG and 8-oxoGuo markers for systemic DNA/RNA damage with ultra-performance liquid chromatography with tandem mass spectrometry and normalized to urinary creatinine (112).

Statistical Analyses

Evaluating Associations Between Baseline Measures, Changes From Baseline Measures, and Clinical Outcomes

Baseline data from each modality of interest, i.e., PET, EEG, fMRI, MRI, cognitive measures, peripheral molecular markers, and clinical/demographic patient profiles, will be available for evaluating associations with the clinical outcomes for the entire group (n = 100 included). Changes from baseline data will be available for the subgroup (around n = 40 invited), who will be re-examined with brain scans and EEG for evaluating an association between changed measures and clinical outcomes. Similarly, cognitive follow-up data will be collected for all patients after 12 weeks of treatment. Primary analyses will test mean differences in baseline measures of the biomarkers from each modality between healthy controls and patients as well as response groups (remitter vs. non-responder at week 8, i.e., primary clinical outcome) using multiple linear regressions. This analysis focuses on the two extreme outcome groups. Secondary analyses will test the association between baseline measures of the biomarkers from each modality and antidepressant treatment response on a continuous scale, i.e., relative change in $HAMD_6$, using linear multiple regression. This analysis incorporates the full spectrum of clinical outcomes. Similar analyses will be performed to study the association between the change from baseline measures of the biomarkers and the clinical outcomes. Regression models will be adjusted for age and sex, as well as modality-specific relevant covariates. For instance, 5-HTTLPR status is predictive of 5-HT_4R binding (70) and will be adjusted for in the analyses concerning 5-HT_4R. When relevant, interactions will be evaluated, e.g., we will test if the association between the clinical outcome and 5-HT_4R is moderated by inflammatory status. Diagnostic regression tools will be used to assess model's assumptions (e.g., linearity of the effects, normality assumption for residuals). When violated, corrective procedures will be used (e.g., splines and bootstrap resampling) (113). As appropriate, adjustments for multiple comparisons will be performed within each modality. In the analysis of the PET data, we will instead use a Latent Variable Model relating the 5-HT_4R binding in several brain regions (neocortex, caudate nucleus, putamen and hippocampus) to treatment outcome *via* a latent variable (114). This allows us to assess the association between 5-HT_4R binding and clinical outcome with a single test. Patients are considered with un-verified compliance if they have taken less than 2/3 of their antidepressant medicine, missed their week 8 visit, or have undetectable serum drug levels at week 8 (i.e., <10 nM for escitalopram and <15 nM for duloxetine). Patients with un-verified compliance will not be included in primary longitudinal analyses of treatment response. Missing data will therefore be handled using complete case analysis which in regression models is valid when the probability of dropping out of the study is, conditional on the covariates, independent of the outcome. If any participants were to be excluded during the study because of their clinical outcome, a sensitivity analysis will be performed.

Evaluation of the Predictive Value of the Biomarkers Within Modality

Logistic regression models for the primary clinical outcome will be used to obtain the probability of each patient to be a remitter (vs. a non-responder) based on its clinical data and the value of a modality-specific biomarker. Given a threshold (e.g., 0.5),

patients with an estimated probability greater than the threshold will be predicted to be remitters, otherwise to be non-responders. The receiver operating characteristic (ROC) curve will be used to assess the compromise between sensitivity and specificity of this classification across thresholds. Since a 33% remission rate is expected in treatment regimen comparable to ours (5), we will focus on the ROC curve with high-specificity. The AUC (area under the curve) of the relevant part of the ROC curve will be reported as a summary of the predictive performance of each biomarker. The classification performance (accuracy, positive predictive value, negative predictive value) at the threshold optimizing the sum specificity and specificity will also be reported. To limit optimistic biases, these measures will be estimated using five-fold cross-validation (115). A permutation procedure will be used to obtain the null distribution of the predictive performance, against which the observed performance will be compared. Additional classification schemes may be considered (e.g., responder status as defined by ≥50% reduction in $HAMD_6$ at week 8), with appropriate adjustment for inflated type-I error, to facilitate comparison of the current data with other relevant clinical trials. The predictor performance will be evaluated in a modality specific fashion and at a next stage, combined predictors will be evaluated.

Predictive Value of the Biomarkers Across Modalities

Two strategies will be considered to optimize prediction of treatment response using biomarkers measured at baseline across modalities. In the first strategy, we will combine the specific biomarker-candidates across all modalities (as predefined in **Appendix 1**), which will generate around 50 candidate biomarkers. A dimension reduction step will be used to define a small number of predictors (roughly 5–10) that will be used in a logistic regression model. The second strategy will use an algorithm to (i) identify, in a data-driven way, biomarkers with a predictive value among all the existing biomarkers (roughly 5,000–10,000) and (ii) predict treatment response based on the identified subset of informative biomarkers. We will investigate the use of machine learning methods (e.g., random forest, neural networks) as well as ensemble methods [e.g., Super Learner (116)]. The assessment of the predictive performance of these strategies will be carried out as described in the previous section.

Ethics Approval and Consent to Participate

The study protocol complies with the Declaration of Helsinki II and data collected during the trial will be monitored throughout the study period (for every 10^{th} patient included) by an independent Good Clinical Practice unit in the capital region of Denmark (www.gcp-enhed.dk/en). The study has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (reference number: H-15017713), the Danish Data Protection Agency (04711/RH-2016-163), and Danish Medicines Agency (protocol number: NeuroPharm-NP1, EudraCT-number 2016-001626-34). All potential participants will receive oral and written information about the study by the enrolling clinician, and all enrolled participants will provide written informed consent prior to inclusion. Adverse events have been scheduled to be reported annually to the Danish Medicines Agency. The study was registered at clinicaltrials.gov prior to initiation (NCT02869035), date: 08.16.2016.

Availability of Data and Materials

Data management and monitoring during the study agrees to the rules on protection of personal data. To protect confidentiality, paper-based material (e.g., cognitive test results) will be stored in a secured archive, while electronical data files that are identifiable will be stored in password secured files behind firewall in accordance to regulations. To promote data quality, the primary outcome measurement ($HAMD_{17/6}$ scores) will be obtained during interviews on paper, manually transferred into the local database through LimeSurvey and cross-checked twice before used in analyses. Biological material will be coded with a unique identification-number and access to de-identification keys is restricted to authorized personnel only and stored in a temporary biobank located in secured areas in the laboratory facility. The biomaterial will later be analyzed in batches to reduce noise, and potential extra material after the end of the clinical trial will be transferred to the CIMBI biobank (71). All biological material will ultimately be anonymized after 15 years after the end of trial.

Progress to Date

The study opened for inclusion of patients in August 2016. To date, the remaining biological data including genetic status of healthy controls are planned to be collected. Obtained biological material is currently being analyzed and processing of imaging data is on-going. Results from the trial are planned to be communicated to the participants and public through publication in international medical journals.

DISCUSSION

The main purpose of the present study is to identify individual or combined predictors (biomarkers) of standard pharmacological antidepressant treatment outcome in MDD, by using multiple modalities such as brain imaging (PET, fMRI), EEG, cognitive tools, and clinical and molecular markers. Special emphasis in the study design has been given to evaluate the biomarker 5-HT_4R PET as a promising clinically relevant tool since the $5HT_4$R availability is of interest in the pathophysiology and as a therapeutic target in MDD, and also as an index of serotonin tonus. The aim of this trial is not to investigate the specific treatment efficacy but to investigate biomarkers for response to standard treatment in a naturalistic setting, e.g., similar to the STAR*D trial (117). The study includes multiple cross-sectional and longitudinal measures in a large number of patients and controls, which

offers a unique opportunity to (a) uncover potential biomarkers or clusters of biomarkers of treatment prediction, (b) apply the findings for stratification of MDD, (c) advance the understanding of pathophysiological underpinnings of MDD, (d) map neurobiological signatures of antidepressant treatment response, and lastly (e) to ideally pave a way for a precision medicine approach for optimized treatment of MDD.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committees on Health Research Ethics in the Capital Region of Denmark (reference number: H-15017713). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GK, MJ, and VF conceived the concept of the study, with help and support from AJ and KK-F. KK-F, C-TI, VD, DS, PF, MG, HP, AG, and BO have supervised study design and contributed to drafting of 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.2020.00641/full#supplementary-material

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The Diagnostic Value of the Combination of Serum Brain-Derived Neurotrophic Factor and Insulin-Like Growth Factor-1 for Major Depressive Disorder Diagnosis and Treatment Efficacy

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Background: Last decades of psychiatric investigations have been marked by a search for biological markers that can clarify etiology and pathogenesis, confirm the diagnosis, screen individuals at risk, define the severity, and predict the course of mental disorders. In our study, we aimed to evaluate if BDNF and IGF-1 serum concentrations separately and in combination might be used as biomarkers for major depressive disorder (MDD) diagnosis and treatment efficacy and to evaluate the relationships among those proteins and clinical parameters of MDD.

Methods: Forty-one MDD patients (according to DSM-5) and 32 healthy controls (HC) were included in this study. BDNF and IGF-1 serum concentrations, psychopathological (MADRS, CGI) and neuropsychological parameters (PDQ-5, RAVLT, TMT-B, DSST), functioning according to Sheehan Disability Scale were analyzed in all subjects at admission and 30 MDD patients after 8 weeks of vortioxetine treatment. Correlational analyses were performed to explore relationships between BDNF and IGF-1 and clinical characteristics. AUC-ROCs were calculated to determine if the value of serum BDNF and IGF-1 levels could serve for MDD diagnosis.

Results: MDD patients had significantly lower serum BDNF (727.6 ± 87.9 pg/ml vs. 853.0 ± 93.9 pg/ml) and higher serum IGF-1 levels (289.15 ± 125.3 ng/ml vs. 170.2 ± 58.2 ng/ml) compared to HC. Significant correlations were obtained between BDNF levels and MDD status, depressive episode (DE) severity, precipitating factors, executive functions disruption (TMT-B, RAVLT immediate recall scores) and all subdomains of functioning. As for IGF-1, correlations were found between IGF-1 level and MDD status, DE severity, number and duration of DE, parameters of subjective and objective cognitive functioning (PDQ-5, RAVLT, TMT-B, DSST scores), and all subdomains of functioning. The associations between IGF-1 concentrations and cognitive tests' performance were stronger than those of BDNF. Separately both BDNF and IGF-1 demonstrated good

discriminating ability for MDD diagnosis with AUC of 0.840 and 0.824, respectively. However, the combination of those neurotrophins had excellent diagnostic power to discriminate MDD patients from HC, providing an AUC of 0.916. Vortioxetine treatment significantly increased BDNF and attenuated IGF-1 serum concentrations, improved all psychopathological and neuropsychological parameters and functioning.

Conclusions: The combination of IGF-1 and BDNF might be considered as a diagnostic combination for MDD.

Keywords: major depressive disorder, insulin-like growth factor-1, brain-derived neurotrophic factor, biomarkers, cognitive functions, vortioxetine

INTRODUCTION

The last decades of psychiatric investigations have been marked by a search for biological markers that can clarify etiology and pathogenesis, confirm a diagnosis, screen individuals at risk, define the severity, and predict the course of mental disorders (1–3). With respect to major depressive disorder (MDD), abnormal neuroplasticity in cerebral regions, responsible for emotional and cognitive processing, is considered to be one of the key pathogenic mechanisms (4, 5) that potentially have a biomarker value. It is associated with alterations in the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4/5, nerve growth factor, insulin-like growth factor (IGF-1), etc. (6–8). The vital role of the neurotrophins is explained by their involvement in the processes of neuronal growth, differentiation, maturation, and survival, synaptic transmission, an equilibrium between neuroregeneration and neurodegeneration, as well as memory formation (9). Therefore, there is reason to believe that two proteins—BDNF and IGF-1—can be used as marker molecules for the MDD diagnosis and therapy effectiveness (7, 8).

Recent studies have shown lower BDNF concentrations in the brains of suicidal MDD persons (10, 11). Further, postmortem studies of MDD individuals revealed an association between the diminished BDNF expression in hippocampal areas and a decrease in the volume of these anatomical structures (12). According to meta-analyses, serum and plasma BDNF reductions have been noted in antidepressant-free individuals with MDD as compared to healthy subjects (13–15). The decreased peripheral BDNF levels normalized in reprisal for several interventions [e.g., antidepressants (16, 17), electroconvulsive therapy (18, 19), aerobic exercise (20)]. Thus, we can reasonably assume that the decline in peripheral (plasma/serum) BDNF levels reflects its reduced expression in the brain and could serve as a neurobiological marker of impaired neuroplastic processes in MDD. In addition, the increase of the neurotrophin concentration could indicate therapy effectiveness.

According to our recent systematized data, there are discrepancies in IGF-1 levels in MDD patients across the studies, although the majority demonstrate higher levels of peripheral IGF-1 compared to healthy controls (HC) (8). The elevation of peripheral total IGF-1 concentrations and its decline after antidepressants` treatment were established in several studies in MDD patients (21–23), moreover, it was reported that only patients in remission had attenuated IGF-1 concentrations following treatment (21, 22). The enhancement of the peripheral IGF-1 expression in MDD may be a compensatory mechanism in response to its brain synthesis decrease (24) or diminished neurotrophin bioavailability due to the hyposensitivity of IGF-1 receptors under the neuroinflammatory stress (25). In our recent work, we assumed that the activity of the cerebral-hepatic axis rise in response to inadequate cerebral IGF-1 levels (26). As a consequence, the production of IGF-1 in the liver increases. When cerebral IGF-1 production is restored, the liver IGF-1 production and its blood concentration decrease.

However, the clinical value, sensitivity, specificity, and predictive significance of a single biomarker for MDD remain doubtful. To solve this problem, the approach that uses the cumulative sensitivity and specificity of biomarkers` combination might be applicable (27). Therefore, in our study we aimed: 1) to evaluate if BDNF and IGF-1 serum concentrations separately and in combination might be biomarkers for MDD diagnosis and treatment response; 2) to evaluate the relationships between clinical MDD parameters and serum levels of the above-mentioned neurotrophins.

MATERIALS AND METHODS

Study Design

This was a case-control study, which included 73 participants aged 18 to 65 years. Outpatients (n=41) with MDD diagnosis according to DSM-5 criteria (28) were included through Zaporizhzhia Regional Clinical Psychiatric Hospital, Ukraine. Eligibility criteria for the study participants were described elsewhere (29). Before entering the study, all patients had received no actual antidepressant medication. Subjects were excluded if they had any other psychiatric diagnosis, high suicidal risk, substance dependence/abuse over the past year,

Abbreviations: AUC-ROC, areas under the receiver operating characteristic curves; BDNF, brain-derived neurotrophic factor; CGI-I, clinical global impression improvement; CGI-S, clinical global impression severity; DE, depressive episode; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; DSST, digit symbol substitution test; IGF-1, insulin-like growth factor-1; HC, healthy controls; MADRS, Montgomery-Asberg depression rating scale; MDD, major depressive disorder; PDQ-5, perceived deficit questionnaire-5; RAVLT, Rey auditory verbal learning test; ROC, receiver-operating characteristic; SD, standard deviation; SPSS, statistical package for the social sciences; TMT-B, trail making test B.

significant neurological disorders, head trauma, unstable medical conditions, history of endocrine diseases, psychotic symptoms, the risk for the hypomanic switch (29). Thirty-two healthy controls (HC) with no current psychiatric disorder were enrolled within the same period that the MDD patients were included. HC were excluded based on the use of medications and/or illicit drugs; the intake of alcohol within 48 h of the study visit; and the presence of an unstable medical condition, which could affect cognitive function (29).

All the participants gave written informed consent to take part in the study and attended a baseline visit to undergo the evaluations. The appraisal was reiterated in 30 patients after 8 weeks of vortioxetine treatment 10-20 mg per day.

The study was approved by the local ethics committee and performed following the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and registered at ClinicalTrials.gov (NCT03187093).

Clinical Assessments

The severity of depression at baseline and changes after treatment were assessed with MADRS (30), Clinical Global Impression Severity (CGI-S) and Improvement (CGI-I) scales (31). Perceived Deficit Questionnaire-5 (PDQ-5), which measures perceived difficulties of cognitive functioning, was used to evaluate subjective cognitive functioning (32). Functioning was rated using the Sheehan Disability Scale (SDS) (33).

Neuropsychological Assessments

To describe neurocognitive functioning, a battery of neuropsychological tests covering the most impaired cognitive domains in MDD was administered to all participants. The tests were managed using paper and pencil. The instruments included:

1. Rey Auditory Verbal Learning Test (RAVLT), to evaluate immediate verbal memory, retroactive and proactive interference effects, delayed recall, and recognition (34);
2. Trail Making Test B (TMT-B), to assess processing speed, executive function i.e., set-shifting (34);
3. Digit Symbol Substitution Test (DSST), to assess processing speed, executive function, learning, memory, attention, and concentration (34).

The procedures of the tests were described previously (29).

BDNF and IGF-1 Measurements

In MDD patients and HC, BDNF, and IGF-1 concentrations were evaluated in the morning. Blood was taken by a venous puncture between 8:00 and 11:00 a.m. within the first 2 days after clinical assessments in 73 participants at baseline and 30 patients after 8 weeks of vortioxetine (10-20 mg per day) treatment. Blood was centrifuged at 3000 g for 10 min, and the serum was stored at -20° C until further processing. IGF-1 was assessed with the chemiluminescence immunoassay Immulite 2500 (Siemens AG, Germany) and Human IGF-1 Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, United States). The measurement range for IGF-1 was from 20 to 1600 ng/ml. BDNF was measured using the chemiluminescence immunoassay «Sunrise» (TEKAN, Austria, GmbH) and BDNF Sandwich ELISA Kit («Millipore-ChemiKineTM»). The measurement range was from 15 to 1000 pg/ml.

Statistical Analysis

Data analyses were done with the SPSS for Windows, Version 20.0 (SPSS Inc., United States). The results were presented as median (interquartile range) or means (SDs) or percentages. The statistical significance of between-group comparisons was determined using non-parametric and parametric criteria when appropriate. The relationships between demographic and clinical parameters and BDNF or IGF-1 concentrations were assessed with Spearman's or Pearson's correlation coefficient. Thereafter, the areas under the receiver operating characteristic curves (AUC-ROC) were calculated to determine if the value of serum BDNF or IGF-1 level could discriminate patients with MDD from HC. A cutoff was derived from the ROC curve with empirical optimal sensitivity and specificity. In addition, a ROC curve for the combination of two biomarkers (BDNF and IGF-1) was built to assess if this combination has a higher value to discriminate patients with MDD from HC. For this purpose, we ran a binary logistic regression to get the probability and built a ROC curve using the probability as the test variable. The statistical threshold was set at $p < 0.05$.

RESULTS

Characteristics of Controls and MDD Patients

Table 1 demonstrates the main demographic, psychopathological, neuropsychological, and functional characteristics of MDD and HC groups. There were no differences in age, gender, and level of education between groups. Surveyed cohorts had a higher percentage of women than men. The mean years of education in HC and MDD patients was 15 years. Although the number of people with predisposing factors (those that put a person at risk of developing MDD, which included the presence of childhood psychotrauma, persistent stress, and MDD in relatives) was higher in MDD patients, only the presence of precipitating factors (specific events or triggers to the onset of the current DE) significantly differed MDD patients from HC.

Besides the expected statistical difference in MDD patients and HC on MADRS and CGI-S total scores, a significant distinction in neuropsychological test performance was found between the comparison groups. MDD participants were significantly worse ($p < 0.0001$) in executive functioning (DSST, TMT-B scores), processing speed (DSST, TMT-B scores), set-shifting (TMT-B), and all parameters of verbal memory (RAVLT subtests). MDD patients also had a significantly lower level of subjective cognitive functioning as compared to HC.

After 8 weeks of vortioxetine treatment in 30 MDD patients, we revealed that the intake of the antidepressant significantly improved the clinical parameters of patients (**Table 1**). Thus, we

TABLE 1 | Demographic, psychopathological, neuropsychological, functional characteristics, and serum BDNF and IGF-1 levels in healthy controls and MDD patients.

Variables	HC (n = 32)	MDD (n = 41)	*p*	Treatment group(n = 30)		*p*
				Pre-treatment	*Post-treatment*	
Demographic characteristics						
Women, n (%)	20 (62.5)[b]	27 (65.9)	0.77	20 (69)	–	
Age, years[§]	38.0 (12.2)[a]	36.4 (12.8)	0.60	35.1 (12.9)	–	
Education, years[§]	15.4 (1.9)[a]	14.7 (2.0)	0.16	14.6 (2.1)	–	
Childhood psychotrauma, n (%)	4 (12.5)[b]	13 (31.7)	0.05	10 (34.5)	–	
Persistent stress, n (%)	12 (37.5)[b]	22 (53.7)	0.17	14 (48.3)	–	
Precipitating factors, n (%)	3 (9.4)[b]	**26 (63.4)****	**<0.0001**	**20 (69.0)****		
Number of DE	–	1 (1-2)	–	1 (1-2.5)	–	
MDD in relatives, n (%)	4 (12.5)[b]	12 (29.3)	0.09	10 (34.5)	–	
History of DE, n (%)	–	14 (34.1)	–	11 (37.9)	–	
Clinical assessments						
Psychopathological						
MADRS total score	2 (0-3.5)[c]	**28 (21.5-31.5)****	**<0.0001**	29 (24.5-33)[d]	**5 (2.5-10)[##]**	**<0.0001**
CGI-S score	1 (1-1)[c]	**4 (4-4.5)****	**<0.0001**	4 (4-5)[d]	**2 (1-3)[##]**	**<0.0001**
Patient-reported cognitive symptoms						
PDQ-5 total score	1 (0-2)[c]	**6 (4-11.5)****	**<0.0001**	7 (4-12.5)[d]	**2 (1-4)[##]**	**<0.0001**
Neuropsychological						
RAVLT immediate recall total	65.5 (58.25-69)[c]	**50.5 (45-54.5)****	**<0.0001**	51 (45-54)[d]	**69 (65-72.5)[##]**	**<0.0001**
RAVLT proactive interference	8 (6-9)[c]	**6 (5-7)****	**<0.0001**	6 (5-7)[d]	**8 (6-9)[##]**	**<0.0001**
RAVLT retroactive interference	15 (13.25-15)[c]	**11 (9-13)****	**<0.0001**	11 (9-13)[d]	**15 (14-15)[##]**	**<0.0001**
RAVLT delayed recall	15 (13-15)[c]	**11 (9-13)****	**<0.0001**	11 (9-13)[d]	**15 (14-15)[##]**	**<0.0001**
RAVLT delayed recognition	15 (15-15)[c]	**15 (14-15)***	**0.003**	15 (13.5-15)[d]	**15 (15-15)[#]**	**0.001**
TMT-B (s) [§]	52.4 (12.8)[a]	**72.3 (21.2)****	**<0.0001**	72.4 (23.0)[e]	**45.4 (15.6)[##]**	**<0.0001**
DSST	63 (55.5-68)[c]	**50.5 (44.25-59.75)****	**<0.0001**	54 (44.5-60.5)[d]	**62 (52-73)[##]**	**<0.0001**
Patient-rated functioning						
SDS Work score	0 (0-0)[c]	**6 (5-8)****	**<0.0001**	6 (5-8)[d]	**1 (0-3)[##]**	**<0.0001**
SDS Social score	0 (0-0)[c]	**7 (3.75-9.25)****	**<0.0001**	7 (4.5-10)[d]	**1 (0-3)[##]**	**<0.0001**
SDS Family score	0 (0-0)[c]	**6 (4-8)****	**<0.0001**	6 (4.5-8)[d]	**1 (0-2.5)[##]**	**<0.0001**
SDS Total score	0 (0-1.75)[c]	**19 (12-24.5)****	**<0.0001**	19.5 (13.25-25)[d]	**2.5 (0-8)[##]**	**<0.0001**
SDS absenteeism, days	0 (0-0)[c]	**0 (0-1)****	**<0.0001**	0 (0-1)[d]	**0 (0-0)[#]**	**0.007**
SDS presenteism, days	0 (0-0)[c]	**3 (2-5)****	**<0.0001**	4 (2.25-5.75)[d]	**0 (0-1.5)[##]**	**<0.0001**
Serum protein levels						
BDNF (pg/ml)[§]	853.0 (93.9)[a]	**727.6 (87.9)****	**<0.0001**	737.3 (90.4)[e]	**905.3 (59.6)[##]***	**<0.0001**
IGF-1 serum level (ng/ml) [§]	170.2 (58.2)[a]	**289.2 (125.3)****	**<0.0001**	288.2 (132.6)[e]	**173.4 (71.2)[##]**	**<0.0001**

Data are presented as median (upper-lower quartile) unless otherwise stated; [§]data are presented as means (SD).

BDNF, brain-derived neurotrophic factor; CGI-S, clinical global impression—severity of illness; DE, depressive episode; DSST, digit symbol substitution test; HC, healthy controls; IGF-1, insulin-like growth factor; MADRS, Montgomery-Asberg depression rating scale; MDD, patients with major depressive disorder; PDQ-5, perceived deficits questionnaire—5 items; RAVLT, Rey auditory verbal learning test; SDS, Sheehan disability scale; TMT-B, trail making test part B.

[a]ANOVA (analysis of variance) analysis, controls vs. MDD patients.

[b]Chi-square test, controls vs. MDD patients.

[c]Mann–Whitney U-test, controls vs. MDD patients.

[d]Wilcoxon test (paired samples), "Pre-treatment" vs. "Post-treatment".

[e]Paired-samples t-test, "Pre-treatment" vs. "Post-treatment".

*Compared with controls, *p < 0.05, **p < 0.01. Compared with "Pre-treatment", [#]p < 0.05, [##]p < 0.0001.*

observed a significant decrease in depression severity (MADRS, CGI-S, SGI-I scores), improvement of cognitive impairment (measured as subjectively as objectively), and functioning.

Serum Concentrations of BDNF and IGF-1

Table 1 shows serum protein levels of BDNF and IGF-1 in HC and MDD patients. It was demonstrated that serum BDNF concentrations were significantly lower in MDD persons compared to HC ($p < 0.0001$), whereas the concentrations of IGF-1 were significantly higher in patients than HC ($p < 0.0001$). Although IGF-1 concentrations were slightly higher in women than in men as in MDD [299.4 (131.5) ng/ml vs. 271.5 (116.5)] as in HC group [174.1 (53.4) vs. 164.2 (67.2)], those differences did not reach a significant level.

After 8 weeks of treatment with vortioxetine, BDNF levels were significantly higher in post-treatment than pre-treatment ($p < 0.0001$), moreover, they were prominently higher than in HC ($F = 9.36$, $p = 0.003$). Whereas, IGF-1 concentrations in MDD group post-treatment were significantly lower than pretreatment ($p < 0.0001$) and not significantly different from HC ($F = 1.86$, $p = 0.18$).

Correlations of Serum BDNF and IGF-1 Levels With Clinical Variables

Next, we performed correlational analyses to determine possible associations between serum BDNF and IGF-1 concentrations and demographic and clinical parameters in the whole sample (**Table 2**). We established prominent inverse relationships between BDNF concentrations and MDD status ($r = -0.57$, $p <$

TABLE 2 | Spearman's/Pearson's correlations between demographic, psychopathological, neuropsychological characteristics, and serum BDNF and IGF-1 levels in MDD patients and healthy controls.

Variables	BDNF (pg/ml)	*p*	IGF-1 (ng/ml)	*p*
Demographic characteristics				
Age, years	0.02	0.9	-0.17	0.16
Gender	0.15	0.20	0.10	0.44
Depression status	**-0.57****	**<0.0001**	**0.50****	**<0.0001**
Persistent stress	0.06	0.59	0.01	0.93
Precipitating factors	**-0.33****	**0.004**	0.20	0.11
Recurrence of DE	-0.03	0.79	0.16	0.19
Number of DE	-0.04	0.75	**0.43****	**0.001**
Duration of DE, weeks	-0.02	0.85	**0.37****	**0.002**
MDD in relatives	-0.12	0.32	0.05	0.68
Clinical assessments				
CGI-S score	**-0.44****	**<0.0001**	**0.45****	**<0.0001**
PDQ-5 score	-0.19	0.16	**0.43****	**<0.0001**
MADRS total score	**-0.43****	**<0.0001**	**0.46****	**<0.0001**
RAVLT immediate recall total score	**0.33****	**0.007**	**-0.42****	**0.001**
RAVLT proactive interference score	0.19	0.11	**-0.40****	**0.001**
RAVLT retroactive interference score	0.17	0.16	**-0.42****	**0.001**
RAVLT delayed recall score	0.23	0.06	**-0.37****	**0.003**
RAVLT delayed recognition score	0.18	0.14	-0.19	0.15
TMT-B (s)	**-0.27***	**0.03**	**0.55****	**<0.0001**
DSST score	**0.24***	**0.048**	**-0.45****	**<0.0001**
SDS Work score	**-0.37****	**0.003**	**0.40****	**0.001**
SDS Social score	**-0.46****	**<0.0001**	**0.39****	**0.002**
SDS Family score	**-0.38****	**0.002**	**0.40****	**0.001**
SDS Total score	**-0.43****	**<0.0001**	**0.43****	**<0.0001**
SDS absenteism, days	-0.12	0.35	0.23	0.07
SDS presenteism, days	**-0.36****	**0.003**	**0.31***	**0.01**
Serum proteins` levels				
Serum BDNF level	1	–	-0.17	0.18
Serum IGF-1 level	-0.17	0.18	1	–

BDNF, brain-derived neurotrophic factor; CGI-S, clinical global impression—severity of illness; DE, depressive episode; DSST, digit symbol substitution test; HC, healthy controls; IGF-1, insulin-like growth factor; MADRS, Montgomery-Asberg depression rating scale; MDD, patients with major depressive disorder; PDQ-5, perceived deficits questionnaire—5 items; RAVLT, Rey auditory verbal learning test; SDS, Sheehan disability scale; TMT-B, trail making test part B.
**p < 0.05 and **p < 0.01.*

0.01), precipitating factors (r = -0.33, p < 0.01), CGI-S (r = -0.44, p < 0.01), MADRS (r = -0.43, p < 0.01), TMT-B score (r = -0.27, p < 0.05), all subdomains of functioning and positive correlations between serum BDNF levels and RAVLT immediate recall level (r = 0.33, p < 0.01).

As for IGF-1, positive correlations were found between IGF-1 level and MDD status (r = 0.50, p < 0.01), number (r = 0.43, p < 0.01) and duration of DE (r = 0.37, p < 0.01), CGI-S score (r = 0.45, p < 0.01), PDQ-5 score (r = 0.43, p < 0.01), MADRS score (r = 0.46, p < 0.01), TMT-B score (r = 0.55, p < 0.01) and all subdomains of functioning and negative associations between IGF-1 and the performance of RAVLT and DSST tests. Moreover, the correlations between IGF-1 concentrations and performance of cognitive tests were higher than that of BDNF.

Serum BDNF and IGF-1 Levels for MDD Diagnosis

The discriminating ability of serum proteins' level to separate MDD participants from HC was determined with ROC analysis. The diagnostic value of BDNF and IGF-1 is shown in **Figure 1**. Separately BDNF (cutoff < 763.17 pg/ml, sensitivity = 81%, specificity = 73%) and IGF-1 (cutoff > 178.00 ng/ml, sensitivity = 84%, specificity = 64%) demonstrated good diagnostic effectiveness, with AUC of 0.840 and 0.824, respectively. However, the combination of two neurotrophins showed excellent diagnostic value for MDD diagnosis, with an AUC of 0.916.

DISCUSSION

This is the first study to investigate the diagnostic power of the combination of two biomarkers—BDNF and IGF-1—for MDD diagnosis. In our study, we found significantly lower BDNF and higher IGF-1 serum concentrations in patients compared with HC. Following 8 weeks of vortioxetine therapy, serum BDNF

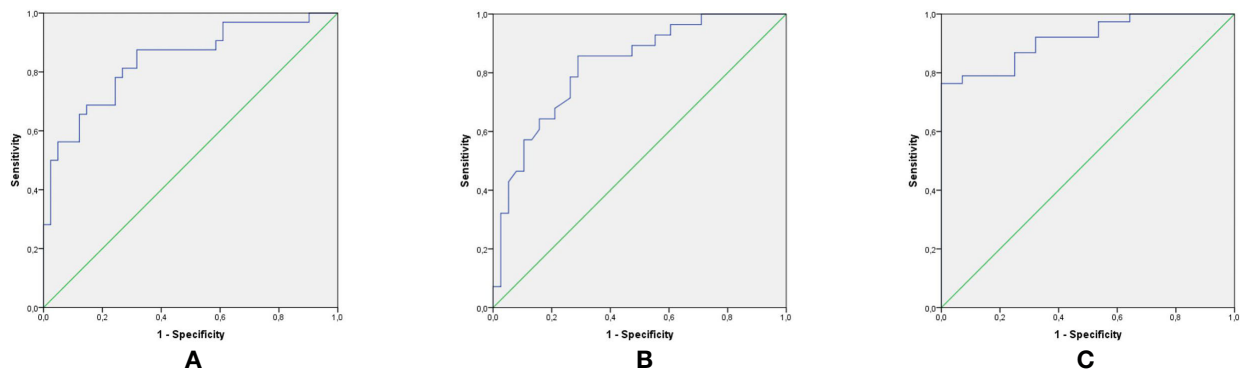

FIGURE 1 | Diagnostic value of serum BDNF and IGF-1 for MDD diagnosis. **(A)** ROC curve for BDNF to diagnose MDD (p < 0.0001, AUC: 0.840; sensitivity; 81% and specificity: 73% for a cutoff of < 763.17 pg/ml). **(B)** ROC curve for IGF-1 to diagnose MDD (p < 0.0001, AUC: 0.824; sensitivity; 84% and specificity: 64% for a cutoff of > 178.00 ng/ml). **(C)** ROC curve the combination of BDNF and IGF-1 to diagnose MDD (p < 0.0001, AUC: 0.916). AUC, Area Under the ROC Curve; BDNF, Brain-Derived Neurotrophic Factor; IGF-1, Insulin-Like Growth Factor; MDD, major depressive disorder; ROC, receiver-operating characteristic.

levels were significantly higher in post-treatment than pre-treatment and compared to the controls. IGF-1 concentrations in subjects post-treatment were significantly lower than pretreatment and not different from the controls. Significant correlations were obtained between serum BDNF levels and MDD status, the severity of the current DE, presence of precipitating factors, executive functions' disruption (TMT-B and RAVLT immediate recall scores), and all subdomains of functioning. As for IGF-1, correlations were found between IGF-1 level and MDD status, severity, number, and duration of current DE, subjective and objective cognitive functioning (PDQ-5, RAVLT subtests, TMT-B, DSST scores) and all subdomains of functioning. The associations between IGF-1 concentrations and the performance of cognitive tests were stronger than that of BDNF. Separately both BDNF and IGF-1 demonstrated good discriminating ability for MDD diagnosis; however, the combination of these two proteins had excellent diagnostic power to discriminate MDD patients from HC, providing the AUC of 0.916.

Firstly, we revealed that MDD patients had decreased BDNF concentrations and that these concentrations returned to normal after vortioxetine intake. These results are in line with previous works that found lower serum and plasma BDNF concentrations in depressed persons in comparison with healthy subjects (13, 14, 17, 27, 35–42). Several antidepressants were shown to increase serum/plasma BDNF levels in MDD persons compared to pre-treatment levels, including agomelatine (41), duloxetine (27), escitalopram (27, 43, 44), fluoxetine (41, 45), milnacipran (36), paroxetine (36, 38, 44), sertraline (44), venlafaxine (44), and vortioxetine (17, 46). However, recent works have shown that not all antidepressants increase BDNF concentrations (47), and antidepressant-induced rise in BDNF concentrations are more substantial in responders compared to non-responders (48–51). Moreover, it was demonstrated that high-intensity exercises (52), electroconvulsive therapy (15, 18, 19, 53), and deep brain stimulation (54) also increase BDNF levels. In animal studies, it has been revealed that vortioxetine enhances the behaviors of depressed rodents probably by influencing the cAMP/CREB/BDNF signal pathway and promoting the expression of BDNF in the dorsal and ventral hippocampus of the animals (55, 56). Moreover, it was shown that vortioxetine, but not fluoxetine, raised hippocampal BDNF concentrations in rats (57). Another study demonstrated that vortioxetine amplified the number of synapses and mitochondria substantially, together with increased BDNF levels, while fluoxetine showed no effect (58). It was suggested that fast changes in BDNF concentrations and synaptic/mitochondria plasticity of the hippocampus during vortioxetine treatment may be attributed to vortioxetine's modulation of 5-HT receptors (58). Longitudinal studies in humans revealed that it is more likely that BDNF is a biomarker for the state of MDD and treatment response rather than a risk factor for MDD (15, 40).

In our study, we obtained significant associations between serum BDNF levels and MDD severity, precipitating factors, executive functions` disruption (performance of TMT-B and RAVLT immediate recall), and all subdomains of functioning. Previous studies in MDD patients have found correlations between BDNF levels and the presence of melancholic features and psychomotor retardation (59), DE severity (36, 60) and duration (60), and cognitive decline during performances of TMT-B (61) and verbal delayed recall (62). Nevertheless, one study evidenced no correlations between patients' plasma BDNF levels and cognitive functioning (38).

Regarding peripheral IGF-1 increase in MDD patients and its decrease after vortioxetine treatment, our results are consistent with some studies (21, 22, 24, 25, 63–65). Data on a significant decrease of serum IGF-1 concentrations during antidepressant treatment are supported by previous investigations, in which amitriptyline, doxepin, fluoxetine, and paroxetine led to a substantial decline of peripheral IGF-1 levels (21, 22, 66). Nevertheless, one study showed that free IGF-1, not total, concentrations did not change after antidepressant treatment (22). The authors suggested that discrepancies between total and biologically active free IGF-1 levels could be due to adaptation mechanisms to changes typically found in major depression (22).

Although in our study we found no significant differences in IGF-1 levels between women and men in both MDD and HC groups, several previous studies have shown variability across the genders in IGF-1 levels in MDD patients (67–69) These differences between genders may be explained by variations of sex hormone or fluctuations of growth hormone and IGF-1-binding protein (67).

The reported here correlations between IGF-1 level and severity, number, and duration of DE, subjective and objective cognitive functioning (PDQ-5, RAVLT subtests, TMT-B, DSST), all subdomains of functioning are in line with our previous results (23). Nevertheless, here we pointed out that the associations for IGF-1 concentrations and the level of performance of cognitive tests were stronger than those for BDNF levels.

Lastly, the results of ROC-analysis demonstrated that BDNF and IGF-1 separately provided fairly good diagnostic power to separate MDD patients from HC, meanwhile the combination of these neurotrophins showed excellent diagnostic value. Therefore, serum BDNF and IGF-1 levels might be a potential biomarker combination as a diagnostic test for MDD. To the best of our knowledge, we were the first to combine those two serum proteins for MDD diagnosis. Previously it was shown that the arrangement of tPA, BDNF, TrkB, proBDNF, and p75NTR might be a diagnostic biomarker panel for MDD (27) and the combination of BDNF, FGF-2, TNF-α, and 5-HT may predict the efficacy of escitalopram therapy (70).

Limitations

Our study was limited by its small sample size, therefore, obtained data need to be replicated in larger samples to confirm our findings.

CONCLUSIONS

Patients with MDD had significantly lower BDNF and higher IGF-1 serum concentrations compared to controls. Vortioxetine

treatment normalized those disruptions, moreover, after 8 weeks of treatment BDNF concentrations in MDD individuals were significantly higher compared to HC. In the whole sample at baseline BDNF levels correlated with the presence of precipitating factors, meanwhile, IGF-1 levels—with the number and duration of DE. Therefore, we can suggest that BDNF is related to acute stress. As for cognition, IGF-1 had stronger associations than BDNF with the disturbances in different cognitive domains, also, it correlated with the subjective cognitive level. Both factors were associated with functioning in different spheres of life. As BDNF as IGF-1 were significantly associated with MDD status and severity of a current episode and separately demonstrated good discriminating ability for MDD diagnosis. However, their combination had excellent diagnostic power to discriminate MDD patients from HC. Thus, the combination of IGF-1 and BDNF might be considered as a biomarker panel for MDD diagnosis.

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 the local ethics committee of State Institution Zaporizhzhia Medical Academy of Postgraduate Education Ministry of Health of Ukraine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OL supervised the study. OL and AT were involved in the concept of the study and collection of the data. Both authors equally contributed to data preparation and literature search. OL and AT analyzed the data and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version. of the manuscript.

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CASE REPORT
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Case Report: PANDAS and Persistent Lyme Disease With Neuropsychiatric Symptoms: Treatment, Resolution, and Recovery

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This case report describes the diagnosis and treatment of a pre-pubertal (onset at age 7) Caucasian female with serological evidence of Lyme disease accompanied by multiple neuropsychiatric symptoms 6 months following a vacation in a tick endemic area of the United States. Prior to the diagnosis of Lyme disease, the patient also met the clinical diagnostic criteria for PANDAS (Pediatric Autoimmune Neuropsychiatric Disorder Associated with Strep), with serological evidence of three distinct episodes of streptococcal pharyngitis. All three episodes of strep occurred during the 6-months interval between suspected Lyme disease exposure and the onset of multiple neuropsychiatric symptoms. Her sometimes incapacitating symptoms followed a relapsing and remitting course that impacted her personal, family, social, and academic domains. Over a span of 31 consecutive months of treatment with various antimicrobials and three courses of intravenous immunoglobulins (IVIg) she experienced complete remission and remains symptom free at the time of this publication. Written permission was obtained from the minor patient's mother allowing the submission and publication of this case study.

Keywords: Lyme, PANDAS, Cunningham Panel, neuropsychiatric, IVIg, strep pharyngitis, basal ganglia encephalitis (BGE), autoimmune encephalitis

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BACKGROUND

In current medical practice, patients with co-occurring Lyme borreliosis and autoimmune encephalitidies secondary to strep infections, such as PANDAS (1–3), are met with a number of inherent challenges (4). The ability to obtain accurate serological testing results for Lyme disease and common co-infections is a challenge for patients and providers alike due to the varied reported accuracy of different Lyme tests (5). To complicate things further, Lyme disease testing is fraught with controversy regarding methodology and interpretation of test results. In 1980, the Centers for Disease Control and Prevention (CDC) began surveillance for Lyme disease, identifying only 10 states where Lyme disease was believed to occur. Currently, all 50 states have reported cases of Lyme disease (6). In 2017, the CDC received reports of a total of 42,743 confirmed and probable cases of Lyme disease, but they estimate that in the US ∼300,000 patients may contract Lyme disease annually (7). One clinical sign of Lyme disease exposure from a tick bite is an erythema migrans (EM) rash, but often patients with documented Lyme disease do not present with EM (8, 9).

Medical literature supports numerous cases of neuropsychiatric symptoms in children who have a diagnosis of Lyme disease as well as other tick-borne infections. For example, a 14-year-old boy experienced a sudden onset of psychotic behavior which persisted despite multiple hospitalizations and treatment with psychotropic medications. He was subsequently diagnosed with neurobartonellosis after he developed cutaneous lesions, which has been documented as common in individuals reporting neuropsychiatric symptoms and *Bartonella* spp. infection or exposure (10). He was treated with a combination of antimicrobials and experienced a gradual progressive decrease in neuropsychiatric symptoms and was able to discontinue all psychotropic drugs (11). Another well-documented case describes a 12-year-old boy who had a compulsion to pedal a stationary bicycle, unwilling to stop long enough to eat or go to school, resulting in a 30-pound weight loss, a skeletal appearance, and multiple hospitalizations. He was found to be infected with Borrelia and recovered after a course of intravenous penicillin (12).

An extensive review article documents increasing evidence and recognition that Lyme borreliosis can cause psychiatric symptoms (13). Drawing from databases and using search engines along with clinical experiences, the authors concluded that Borrelia can "cause immune and metabolic effects that result in a gradually developing spectrum of neuropsychiatric symptoms usually presenting with significant comorbidity which may include developmental disorders, autism spectrum disorders, schizoaffective disorders, bipolar disorder, depression, anxiety disorders (panic disorder, social anxiety disorder, generalized anxiety disorder, posttraumatic stress disorder, and intrusive symptoms), eating disorder, decreased libido, sleep disorder, addiction, opioid addiction, cognitive impairments, dementia, seizure disorders, suicide, violence, anhedonia, depersonalization, dissociative episodes, derealization, and other impairments" (13).

Data from an unpublished survey of over 1,000 parents of children with PANDAS and/or PANS, conducted by Moleculera Labs in 2018, "Economic and Psychosocial Costs of PANDAS and PANS," revealed that, on average, patients have seen up to 12 medical providers, requiring ∼3 years before receiving a diagnosis of PANDAS or its broader diagnostic category, PANS (Pediatric Acute-onset Neuropsychiatric Syndrome). The survey results also revealed that at least 20% of patients with PANDAS and/or PANS experience a delay of more than 12 months before receiving appropriate treatment even after being diagnosed with this type of autoimmune encephalopathy.

INTRODUCTION

This is a case report of a previously healthy 7-year-old girl who was conceived through artificial insemination resulting in an uncomplicated, full-term pregnancy and delivery by caesarian section. She weighed 8 pounds, 10 ounces at birth and was breast fed. Her family history was unremarkable for rheumatic fever, tics, OCD, autoimmune disorders, psychiatric illness, or allergies. 6 months prior to the onset of her symptoms, the patient and her family vacationed in a tick endemic area of the US, but the patient had no known tick attachment or erythema migrans (EM) rash. Quite abruptly, over a period of 3 weeks, the patient experienced dramatic declines in cognitive functioning, concentration, and ability to focus, a loss of math skills, the onset of dysgraphia and difficulty with social cues, decreased processing speed, word selection problems, anxiety, fatigue, nighttime awakening, chills, joint and muscle pain, moodiness, both general and separation anxiety, and panic attacks. She also experienced obsessions and compulsions and aggressive behavior which was completely out of character according to her mother. She said to her mother, "Mom, something happened to my brain." Previously, she enjoyed music and dance lessons but her overall activity was decreased. Because the patient had consistently been identified as an academically gifted child, a call from the patient's teacher came as a surprise when the mother was asked, "Did you drop your daughter on her head?" The patient regressed from being a year ahead of her class in math, to being unable to add beyond the number 10. She began having trouble comprehending more difficult reading. During a ride home with her mother, the patient asked, "Who are you? What's your name again?" And "I know you are mommy but what's your name?" Her mother began to think that her daughter may have experienced an accident or head injury, but there was no physical sign or history of any type of injury. An EEG was performed with normal findings.

CASE PRESENTATION

This previously asymptomatic, healthy 7-year-old girl experienced an abrupt onset of several physical, neurological, and psychiatric symptoms increasing in intensity over a 3-week period. The patient's mother reports that strep pharyngitis was diagnosed by a previous physician on three separate occasions with the first episode occurring 180 days prior to the onset of neuropsychiatric symptoms. The first course of treatment was a 10-day course of amoxicillin which resulted in no change in her behavior or functioning. The strep infection recurred, and amoxicillin was again prescribed with no behavioral improvement. The third episode of strep throat (Quest Labs DNase B results of 407), (reference range <376) was treated with a course of clindamycin with a notable improvement in the patient's symptoms (14). Neurologists at a university medical center referred the patient to the Psychiatry department. Instead, the patient's mother chose to have her evaluated by a developmental pediatrician who arrived at a diagnosis of Pediatric Autoimmune Neuropsychiatric Disorder Associated with Strep (PANDAS) (15, 16). Due to the patient's worsening symptoms, a second opinion was sought, and additional blood work performed including a CDC Lyme Western Blot which was positive, leading to the additional diagnosis of Lyme disease. Quest Laboratories' Lyme disease enzyme immunoassay (EIA) with reflex to Western Blot was positive at 1.25, (0.00–0.90 negative index value). The patient was referred to the university's infectious disease clinic where treatment to address the Lyme disease diagnosis began. A PICC line was inserted to facilitate

the administration of IV Rocephin. A lumbar puncture (LP) was performed as part of the infectious disease evaluation with normal results. At this point, because the patient had both a diagnosis of PANDAS and Lyme disease, her mother sought the opinion of a pediatric Lyme disease specialist who was also familiar with diagnosing and treating PANDAS. A graphic representation of the timeline from the initial suspected Lyme disease exposure through her complete course of treatment is shown in **Figure 1**.

On her first visit with the pediatric Lyme disease specialist, the patient presented with crying, anxiety, headache, joint pain, decreased cognitive functioning, fatigue, nighttime awakening and an extreme fear of sleeping alone. Her ASO titer was negative but a DNase B titer was elevated at 407 (<376). Her ESR was normal. The IV Rocephin 1.5 gm QD prescribed by the previous doctor was continued, but her PICC line became occluded requiring removal. Oral Omnicef 300 mg BID was initiated to replace the Rocephin. Zithromax 250 mg BID and Tindamax 250 mg QD (Saturdays and Sundays only) were initiated.

Due to complaints of right upper quadrant pain, an abdominal ultrasound was ordered which was unremarkable ruling out concerns of possible Rocephin cholelithiasis. Ursodiol 300 mg BID was ordered to help break down cholesterol that had possibly formed in the gall bladder. A CDC Lyme WB was negative although repeated Lyme testing through IGeneX Laboratory revealed a positive Lyme WB IgM with positive double starred bands 31 and 41 and indeterminate bands 39 and 83–93. Lyme WB IgG and *Bartonella henselae* testing showed negative IgG and IgM results. Babesia FISH (RNA) was negative. Her previously elevated DNase B was now normal as was her ASO titer. An elevated *Mycoplasma pneumoniae* IgG at 2.01 (reference range $\leq$ 0.90 ISR) indicated a previous infection and *M. pneumoniae* IgM was negative. A basic metabolic panel, CBC, urinalysis, ESR, and ANA were completely within normal limits. Genetic testing indicated that the patient was a carrier of the *HLA-DRB1*, *2,* and *4* genes which are reported to occur more frequently in patients with Lyme disease and rheumatoid arthritis (17–19). It is also been observed that *B. burgdorferi* can alter the repertoire of self-peptides bound to MHC class II molecules and influence the likelihood of autoreactive T-cells which could lead to infection-induced autoimmune illnesses (20).

The patient's mother was given a Lyme disease checklist to complete prior to each appointment indicating symptoms and improvement or lack of improvement since the previous visit. The Lyme disease checklist asks the parent to rate the patient on 35 symptoms including (1) unexplained fevers, sweats, chills or flushing, (2) weight change (loss or gain), (3) fatigue, (4) hair loss, (5) swollen glands, (6) sore throat, (7) testicular/pelvic pain, (8) menstrual irregularity, (9) unexplained milk production/breast pain, (10) irritable bladder or dysfunction, (11) upset stomach/abdominal pain, (12) constipation/diarrhea, (13) chest pain/rib soreness, (14) shortness of breath or cough, (15) heart palpitations, (16) joint pain/swelling, (17) joint stiffness, (18) muscle pain/cramps, (19) muscle twitching, (20) headaches, (21) neck creaks/cracks/stiffness, (22) numbness/tingling/stabbing sensations, (23) facial paralysis, (24) double vision/floaters/loss of vision, (25) buzzing/ringing/ear pain/sensitivity, (26) vertigo/dizziness, (27) lightheadedness/poor balance, (28) tremors, (29) difficulty thinking, (30) difficulty concentrating, (31) forgetfulness/poor short term memory, (32) disorientation/getting lost, (33) difficulty with speech or writing, (34) mood swings/irritability/depression, and (35) too much/too little sleep. In addition, the parents were asked to rate symptom severity on a mild, moderate, or severe scale as well as symptom frequency on an occasional, often, or constant scale.

At a follow-up office visit 8 weeks later, her Lyme disease checklist indicated overall improvement with no headaches, but increased insomnia and scattered joint sensitivities. Range of motion, biometrics and vital signs were normal except for an oral temperature of 99.2° F. She had no "dark circles" under her eyes, no tremors and her balance was normal. 2 weeks later the patient experienced a sudden symptom recurrence. Omnicef was discontinued, Zithromax continued at the same dosage and Augmentin added at 500 mg BID. EMLA cream (Lidocaine 2.5% and Prilocaine 2.5%) was prescribed as a topical anesthetic to

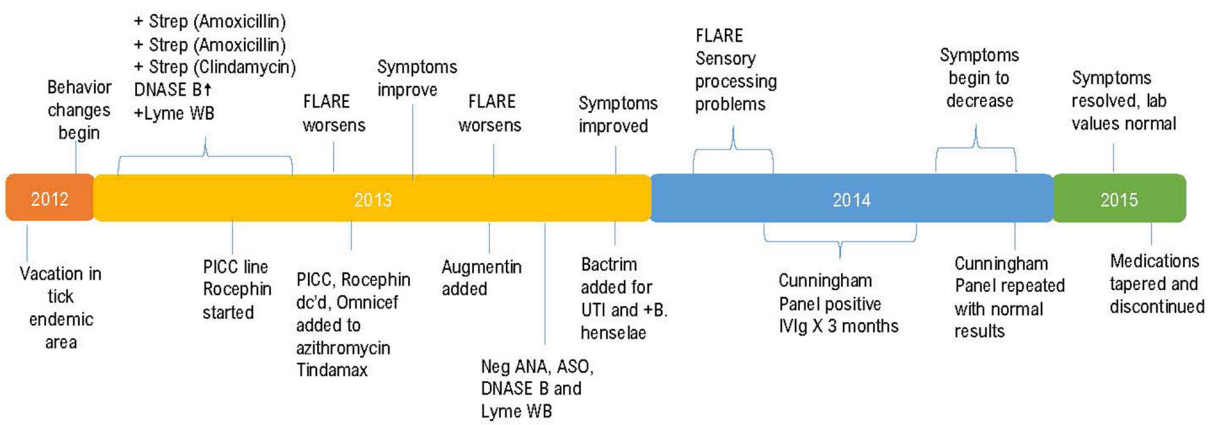

FIGURE 1 | Timeline between probable disease exposure and completion of treatment.

treat her joint sensitivities. Lab values at this visit were within normal limits.

10 weeks following the regression, at her next office visit, she had no major complaints and her Lyme disease checklist indicated overall symptom improvement. She denied having headaches and reported increased energy, improved cognitive function and enjoyment of her music lessons. Her physical examination was within normal limits. ANA, CRP, ASO, Streptozyme, and *M. pneumoniae* lab results were all within normal limits. The patient was attending day camp, Monday through Friday, all day, without problems. 2 weeks following this appointment, she suddenly became argumentative, hyperactive, and combative and experienced chills and headache. Bactrim SS BID was added due to a suspected urinary tract infection and to address new IGeneX laboratory results indicating the presence of Lyme borreliosis with positive double starred bands 31 and 41 and double starred bands 39, 83–93 indeterminate. Quest laboratory testing showed an elevated *B. henselae* IgG at 1:64. *B. henselae* IgM was within normal limits at <1:20. Both Ehrlichia and Anaplasma testing had negative results.

2 months later at her next office visit, the patient presented as unhappy and experiencing some facial "twitching." However, her appetite was good (no previous restrictive eating or food refusal was noted) and she was engaged in activities including dance and tennis.

Two additional months later, labs were repeated. Quest laboratory testing indicated negative results for *E. chaffeensis, A. phagocytophilum,* and *B. henselae* IgG and IgM. IGeneX testing showed an indeterminate Lyme IgM with double starred bands 31 and 41. Lyme IgG testing was interpreted as positive with double starred bands 31, 41, and 58 reactive. IGeneX interpretation guidance states the IgG WB is considered positive if two or more of the double starred bands are present from either Group 1 or Group 2. Group 1 includes bands 23–25, 31, 34, 39, 83–93. Group 2 includes bands 23–25, 34, 39, 41, 83–93. *B. henselae* IFA IgG and IgM were both reported as negative. *B. duncani* IgG was negative but IgM was reported as indicative of active infection at a level of 80 with values > 40 considered elevated. This was presumed to be a co-infection due to the original tick exposure as no additional tick exposure was identified.

CD45RA testing, which is a marker of naïve T cells, showed an elevated result of 38% (reference range 5–37%) which is an indication of the amount of time elapsed (∼10 days) since the most recent antigenic stimulation (21, 22). Although the patient's CD45RA was not significantly abnormal, as it was only slightly outside of the upper end of the normal range, it may be supportive of the confirmed presence of an additional co-infection with *B. duncani*. The presence of *B. duncani* prompted the addition of Mepron 375 mg BID to her medication regimen. Less than 5 days following this visit, she experienced an onset of severe stomach aches and "migraines" which caused her to leave school early 4 out of 5 days each week for several weeks. These symptoms slowly resolved, and her medication regimen was continued without additional changes.

4 months later at her sixth office visit, her mother reported that she was having panic attacks and was "terrified" to sleep alone. She had some residual combativeness, lack of focus, and cognitive interference. The patient's energy level was improved, and she was engaging in her normal activities. Physical examination and vital signs were normal. Sensory processing problems were evident with heightened sensitivity to lights and sounds. She appeared to manage well at home but struggled with these symptoms in her classroom resulting in the development and implementation of an Individual Educational Plan (IEP). Neuropsychiatric testing was ordered to assess this issue. Quantitative immunoglobulin values were all within normal limits. Quest laboratory testing at this visit revealed negative results for *B. henselae* IgG and IgM, *E. chaffeensis* IgG and IgM, and *Anaplasma Phagocytophilum* IgG and IgM. IGeneX testing showed negative results for *B. henselae* IgG and IgM, and Lyme WB IgG. Lyme WB IgM was indeterminate.

The Cunningham Panel™ of Tests (Moleculera Labs, Oklahoma City, OK) was ordered to assess the presence of antineuronal antibodies against specific neuronal receptors. The patient's Anti-Dopamine D1 Receptor (DRD1) and Anti-Dopamine D2L Receptor (DRD2L) were elevated, her Anti-Lysoganglioside-GM1 (LYSO-GM1) was normal, her Anti-Tubulin (TUB) was elevated, and her Calcium/calmodulin-dependent protein kinase II (CaMKII) was normal (23–25). Published studies demonstrated that the elevated presence of one or more of these antineuronal antibodies and antibody mediated stimulation of CamKII was strongly associated with autoimmune neuropsychiatric symptoms such as those present in PANDAS and PANS (26, 27). Based upon the patient's Cunningham Panel™ test results (See **Table 1**) the decision was made to prescribe intravenous immunoglobulin (IVIg) in accordance with established treatment guidelines for the patient's level of symptom severity (28, 29). She received IVIg at 2 gm/kg given over a 2-days period for a total of three treatments at 4-weeks intervals.

A significant evidence base exists for the use of IVIg in PANDAS patients, particularly those exhibiting a relapsing and remitting course of illness with moderate to severe symptoms, although additional randomized, double blind,

TABLE 1 | Cunningham Panel results—test 1.

	Dopamine D1 (titer)	Dopamine D2 (titer)	Lysoganglioside (titer)	Tubulin (titer)	CaM Kinase II (% of baseline)
Patient result	8,000	>32,000	320	4,000	119
Normal ranges	500–2,000	2,000–8,000	80–320	250–1,000	53–130
Normal mean	1,056	6,000	147	609	95
Interpretation	Elevated	Elevated	Borderline	Elevated	Normal

placebo control studies need to be performed (28). One well-known study, based on the hypothesis that PANDAS and Sydenham's chorea have similar group A strep etiology, proposed that immunomodulatory treatments could effectively treat neuropsychiatric symptoms. Comparing IVIg to therapeutic plasma exchange (TPE) showed that reassessment at 1-month following treatment, the TPE and IVIg groups both showed "striking improvements in obsessive-compulsive symptoms, anxiety, depression, emotional lability, and global functioning" (30).

Although controlled trials have only evaluated IVIg given as a single course, unpublished data based on the experiences of clinicians and researchers engaged in the PANS Consortium suggest that one to three repeated doses of IVIg may be helpful in children who exhibit a positive response to the initial dose but then experience a relapse as the exogenous antibodies are cleared (28).

In another randomized, controlled trial of IVIg for PANDAS, it was concluded that IVIg was safe and well-tolerated but differences between groups were smaller than anticipated and the double-blind comparison failed to demonstrate superiority of IVIg over placebo (31). It was proposed that the study design may have negated the potential observation of an improvement with IVIg by the administration of antibiotics to both groups prior to either IVIg treatment or placebo. An additional study concluded that children with PANDAS derive a favorable response to IVIg at 12-months follow up "consistent with its role in Ig replacement and immune modulation" (32).

At the conclusion of her IVIg treatments, the patient, now age 9 and in third grade, was normal in height and weight. She had no complaints and her activity level continued to improve with decreased fatigue. Her appetite was normal, and her Lyme disease checklist indicated overall improvement. She did have some residual obsessions and compulsions, intermittent hand tingling, slight facial tics, and a humming tic. Medications at this time included Mepron 375 mg BID, Omnicef 300 mg BID, Tindamax 250 mg QD (Saturdays and Sundays only), and Zithromax 250 mg QD, all of which were continued due to the presence of her residual symptoms. The protocol utilized regarding the length of time for which antibiotic treatments were administered was based upon on the patient's symptoms and laboratory results. Before discontinuance of antibiotic treatment, the patient must exhibit symptom resolution along with negative laboratory findings for a at least 2 consecutive months.

Now 21 months into her treatment, at her seventh office visit, her mother reported that she was doing "pretty well" despite a symptom flare the previous month which lasted ∼3 weeks but had completely resolved. Her Lyme disease checklist indicated that her status was stable. Vital signs were normal except for a slight elevation in her oral temperature at 99.3 F. The patient was doing well in her new school, had a normal activity level and appetite, and was enjoying her usual activities including music and dance lessons. Physical exam revealed no involuntary movements, no vocal or motor tics, clear lungs, and normal reflexes. IGeneX testing at this time showed indeterminate results for Lyme WB IgM with double starred bands 31 and 41 ++ and double starred bands 39 and 83–93 indeterminate. Lyme WB IgG was negative. *B. henselae* IFA IgG and IgM were negative. *B. duncani* IFA IgG was negative, however, *B. duncani* IgM was positive at 80 with normal values falling < 40. All findings were negative per CDC standards. A Cunningham Panel™ was repeated (Test 2) to assess the post-treatment status of antineuronal antibodies. The results of the five assays were all within normal ranges (27) (see **Table 2**).

At this point, it was determined that no further IVIg was needed. Because the family was unable to obtain insurance coverage for the IVIg treatments, they incurred the expense out-of-pocket which averaged $12,000 per treatment (33, 34). At her follow up visit 6 months later, 31 months after her initial visit, the patient's Lyme disease checklist indicated overall improvement. Her final laboratory testing for tick-borne diseases, strep antibodies, and *M. pneumoniae* were all within normal limits.

Outcome of Treatment

Currently this patient appears to be fully recovered and has been discharged from the care of the pediatric Lyme disease specialist. She is asymptomatic and performing academically at the "top" of her class according to her mother. A summary of the serological evidence of exposure to tick borne illness and streptococcal infection is included in **Table 3**.

DISCUSSION

This patient's case may be representative of numerous other cases of autoimmune neuropsychiatric illnesses where a patient may have concomitant infections and co-morbid diagnoses. What is known with growing certainty is that post-infectious neuropsychiatric illness appears to be increasing in frequency or, at least, in frequency of diagnosis. In cases such as the one presented here, it can be challenging for a patient to deal with controversy surrounding the diagnosis of PANDAS and the legitimacy of a diagnosis of Lyme disease with a neuropsychiatric presentation. Patients with chronic neuropsychiatric symptoms

TABLE 2 | Cunningham Panel results Post-treatment—test 2.

	Dopamine D1 (titer)	Dopamine D2 (titer)	Lysoganglioside (titer)	Tubulin (titer)	CaM Kinase II (% of baseline)
Patient result	1,000	8,000	40	500	108
Normal ranges	500–2,000	2,000–8,000	80–320	250–1,000	53–130
Normal mean	1,056	6,000	147	609	95
Interpretation	Normal	Borderline	Normal	Normal	Normal

TABLE 3 | Serological evidence of exposure to *B. burgdorferi, B. henselae*, *B. duncani* and streptococcal infections.

Serological evidence of exposure to *Borrelia burgdorferi*	
Testing 1	Lyme ELISA +1.25 (0.00-0.94); Lyme WB IgM + 23, 41
Testing 2	Lyme WB IgM + 31, 41
Testing 3	Lyme WB IgM + 31, 41 and 58
Serologic evidence of exposure to *B. henselae*	
B. henselae IgM neg; *B. henselae* IgG + 1:64 (<1:64)	
Serologic evidence of exposure to *B. duncani*	
B. duncani IgM 80 (IgM >40 indicates active infection)	
Serologic evidence of exposure to group A streptococcus	
DNASE B + 407 (<376 u/mL)	

who do not respond adequately to traditional psychotropic medications may have an underlying immune-mediated condition triggered by one or more infections as evidenced in this case report. In addition, patients with genetic susceptibility to immune dysregulation, such as identified in this patient, carriers of the *HLA-DRB1*, *2,* and *4* genes may increase the likelihood of an autoimmune encephalopathy indicated by the presence of antineuronal antibodies associated with neuropsychiatric symptoms. The *HLA-DRB1* gene plays a critical role in the immune system, where the HLA complex helps the immune system distinguish the body's own proteins from proteins made by foreign invaders such as viruses and bacteria. There is increasing evidence that IVIg and immunoglobulins are effective in treating autoimmune neuropsychiatric illness although the mechanism of action is uncertain.

It is often noted that patients diagnosed with PANDAS or PANS are frequently found to have multiple co-infections (35). Technically, since the initial treatment of the strep with amoxicillin did not initially result in improvement in the patient's neuropsychiatric symptoms, whereas with the addition of clindamycin the patient's symptoms began to improve, it could be concluded that Lyme may have been the pathogenic agent. However, in clinical experience it has been found that improvement in neuropsychiatric symptoms does not typically occur unless all co-infections are addressed and resolved. Another explanation may be that once the co-offending infection was also resolved, the patient's neuropsychiatric symptoms began to improve. This also brings up an issue with nomenclature; since this patient had a Lyme infection, she could be clinically diagnosed with PANS, or because of the strep diagnosis, clinically this patient could also be diagnosed with PANDAS. Because this patient had concurrent infections, we have referred to this case as a patient with PANDAS and Lyme co-infection. We suggest that the utilization of the term "Basal Ganglia Encephalitis (BGE)" may have more clinical utility when referring to the possible pathophysiology, where the type of infectious trigger may not be essential in the nomenclature of the clinical syndrome (26, 36).

CONCLUSION

The subject of this case report had a concomitant diagnosis of Lyme borreliosis and PANDAS, both of which are consistent with the neuropsychiatric symptoms she experienced. As evidenced by her recovery and resolution of symptoms, treating both the Lyme infection and streptococcal infection, as well as treating the underlying autoimmune etiology of her neuropsychiatric symptoms resulted in a successful outcome. This case report and treatment history reiterates the complex and challenging nature of infection-triggered autoimmune neuropsychiatric disorders such as PANDAS and PANS and that multiple concomitant infectious agents can frequently be identified in patients suffering from these complex neuropsychiatric disorders. The presence of elevated antineuronal antibodies identified by the Cunningham Panel™ provided an aid in the diagnosis and in directing immunomodulatory treatment. The post-treatment resolution of these autoantibodies provided pathophysiological support for addressing both the infection(s) and the underlying immune system dysfunction which resulted in a positive medical outcome for this patient.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent from the patient's mother was obtained allowing the submission and publication of this case study.

AUTHOR CONTRIBUTIONS

CJ: treating physician of subject of case study, provided oversight, and approval of manuscript. CS: provided oversight and technical assistance during the preparation of the manuscript. AC: provided case review and draft development of the manuscript. DB: treating physician and provided case review. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: AC and CS are employed by Moleculera Labs.

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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