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ORIGINAL RESEARCH

Identification of Plasma Inflammatory Markers of Adolescent Depression Using the Olink Proteomics Platform

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Purpose: The quality of life of worldwide adolescents has been seriously affected by depression. Notably, the inflammatory response is closely associated with the pathophysiology of depression. The present study applied a novel targeted proteomics technology, Olink proximity extension assay (PEA), to profile circulating immune-related proteins in adolescents with depression.

Methods: In the present study, the expression levels of 92 inflammation-related proteins were compared between adolescents with depression (ADs) (n=15) and healthy controls (HCs) (n=15), using the OLINK PEA inflammation panel. We further validated 5 top proteins that were identified through KEGG and GO analyses between 40 HCs and 50 ADs, including CCL4, CXCL5, CXCL6, CXCL11, and IL-18 using enzyme linked immunosorbent assay (ELISA).

Results: We identified 13 differentially expressed proteins between the two cohorts, including 5 up-regulated and 8 down-regulated proteins. Among them, the TRAIL protein levels were significantly negatively correlated with the HAMA-14 score ($r=-0.538$, $p=0.038$), and the levels of transforming growth factor α (TGF-α) were significantly associated with a change in appetite ($r=-0.658$, $p=0.008$). After validation by ELISA, CCL4, CXCL5, CXCL11, and IL-18 showed significant changes between ADs and HCs ($p<0.05$), while CXCL6 showed an up-regulated tendency in ADs ($p=0.0673$). The pooled diagnostic efficacy (area under the curve [AUC]) of these five inflammation markers in clinical diagnosis for adolescent depression was 0.819 (95% CI: 0.735–0.904).

Conclusion: We report a number of inflammation-related plasma biomarkers, which uncover a potential involvement of chemokines, cytokines, and cytokine receptors in adolescent depression. Their roles in the pathophysiology of depression need to be further elucidated.

Keywords: olink proximity extension assay, biomarkers, adolescent depression, inflammation

Introduction

Depression is a serious mental disorder that affects about 350 million people worldwide and is a leading contributor to the global disease burden.[1] In recent years, its incidence has increased in people aged 11 to 19 years.[2] Adolescents with depression (AD) have increased risks of social function impairment, substance abuse, suicide attempt, and actual suicide.[3] However, recent meta-analyses revealed the controversial efficacy of antidepressant drugs for the treatment of adolescent depression.[4,5] A better understanding of the specific biological and pathophysiological alterations related to this disease is needed. Such understanding is critical to achieving more effective approaches for the diagnosis and treatment of adolescent depression.

There is an extensive body of data showing that depression is associated with activated inflammatory response.[6] Altered levels of pro-inflammatory cytokines are likely highly associated with depression.[7] Indeed, the immunoregulatory response is a complex process involved in many immune factors. With the exception of several proinflammatory cytokines,[8] the

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peripheral immune response to stress stimuli remains largely unknown in depression. In addition, vegetative-depressive symptoms could be increased by cytokine-induced immune activation.[9] In an analysis of differences between adolescent and adult depression, Rice et al showed that adolescent depression was often characterized by vegetative symptoms, while adult depression had the loss of interest as a core symptom. Several studies also reported a link between vegetative symptoms and pro-inflammatory cytokines.[10] In cancer patients, IL-6 levels were potentially associated with vegetative depressive symptoms,[11,12] while IL-8, IL-10, and TNF-α were not.[11] Hence, the profiling of circulating immune-related proteins would help to better understand the role of the immune system in adolescent depression.

Proteomics is an established and widely used methodology for biomarker screening in human diseases.[13] Using proteomics methods, we have previously identified plasma markers in adult patients with major depressive disorder (MDD).[14,15] Olink proximity extension assay (PEA), a novel advanced proteomics technology, allows high-throughput and precise proteome profiling.[16] Through the Olink PEA, hundreds of designed proteins can be simultaneously detected in one human sample. This technology has already been shown to enable the targeted detection of proteins in body fluids with excellent reproducibility and stability. For example, researchers have discovered a set of promising blood biomarkers for central nervous system (CNS) diseases, such as bipolar disorder,[17] Alzheimer's disease,[18] and ischemic stroke.[19] Based on these findings, in our study, the Olink PEA inflammation panel was applied to identify alterations in distinct inflammation-related proteins between adolescents with and without depression.

Materials and Methods

The workflow of this study is shown in Figure 1.

Ethical Statement

All the procedures were reviewed and approved by the Ethical Committee of the Yongchuan Hospital of the Chongqing Medical University, Chongqing, China (Approval code: No.2022-KeLunShen-107). This study was conducted according to the Helsinki Declaration. Written informed consent was obtained from all participants over 18 years old. Concerning participants aged between 12 to 18 years, parental informed consent was provided.

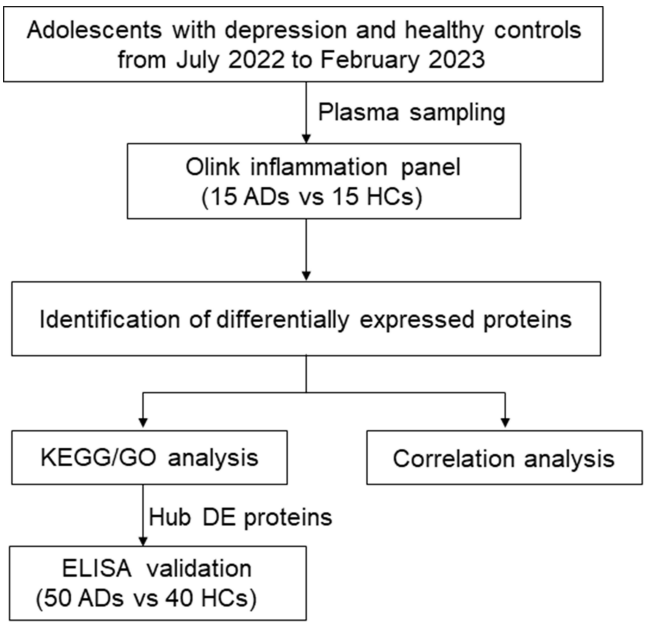

Figure 1 Flow-chart of inflammation-related markers identification in adolescents with depression.

Participants

In this study, all participants were recruited at the Yongchuan Hospital of the Chongqing Medical University, from July 2022 to February 2023. The inclusion criteria were the following: i) adolescents with or without depression, aged between 12 to 19 years; ii) confirmation of the depression diagnosis by trained psychiatrists fulfilling the criteria on the Diagnosis and Statistics Manual of Mental Disorders—version 4 (DSM-IV). According to Rice's work,[10] depression symptoms were described and classified into core symptoms, vegetative symptoms, and cognitive symptoms. The Hamilton Depression Scale-24 (HAMD-24; 24-Items) was used to assess symptom severity. The Hamilton Anxiety Scale-14 (HAMA-14) was used for anxiety assessment. iii) Healthy controls (HCs) with no previous neurological, DSM-IV Axis I/II, or medical illness, were also recruited during the same study period. Adolescents with any comorbidity, including physical, neurological, or psychiatric disorders and/or illicit drug use, were excluded from the study. Furthermore, to avoid interference with any immune response to inflammatory diseases or medication, subjects who were likely under an immune or inflammatory condition, or taking drugs with anti-inflammatory and immunosuppressive effects, were also excluded.

Plasma Collection and Storage

For all participants, peripheral venous blood samples were collected in vacutainer tubes containing the chelating agent ethylenediaminetetraacetic acid (EDTA). Plasma was obtained by blood centrifugation at 3000 rpm for 15 minutes, and was stored at –80 °C for future use.

Inflammation-Related Biomarkers Screening

The Olink PEA inflammation panel (Olink proteomics, Uppsala, Sweden), which contains 92 target proteins, was used to analyze the samples from 15 adolescents with depression (ADs) and 15 healthy controls (HCs). Each target protein was recognized by double antibody labeling and coupled with its complementary DNA barcode, which was subsequently quantified using a high-throughput microfluidic real-time PCR instrument, Biomark HD (Fluidigm, South San Francisco, CA). The final assay readout was presented as normalized protein expression values, which were further log2-transformed.

Bioinformatics Analysis

To uncover the main biological functions and signaling pathways in which they were involved, differentially expressed proteins (DEPs) were subjected to gene enrichment analysis. The Gene Ontology (GO) analysis was performed using Blast2Go (https://www.blast2go.com/). Statistical significance was assessed using the Fisher's Exact Test. The biological process, molecular function, and cellular component terms were enriched by DEP profiling between ADs and HCs. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to explore the genomic, chemical, and system functions of robust DEPs.

ELISA Validation

Plasma samples from ADs (n=50) and 40 HCs (n=40) were used for further validation by enzyme-linked immunosorbent assay (ELISA). DEPs with at least 1.3-fold changes (FCs) and a hub role in the top KEGG pathway were selected. The plasma levels of CCL4, CXCL5, CXCL6, CXCL11, and IL-18 were quantified using a solid-phase sandwich ELISA kit (Wuhan Fine Biotech Co., Ltd., China), according to the manufacturer's specifications.

Statistical Analysis

Statistical analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). Statistically significant differences between ADs and HCs were analyzed using the unpaired Student's *t*-test or the chi square test, as appropriate. The results were corrected for multiple comparisons using the false discovery rate (FDR) approach. Binary logistic regression analysis was used to construct a discriminative model using the identified molecules. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of the model. Sensitivity and specificity were assigned under the maximal Youden's Index (sensitivity+specificity−1). Associations between expression

levels of DEPs, the HAMD-24 and HAMA-14 scores, and DSM-IV depressive symptoms were assessed using Pearson correlation analysis. A p-value < 0.05 was considered statistically significant, and a p-value < 0.01 was considered highly statistically significant.

Results

Baseline Characteristics

This study enrolled a total of 90 participants, including 40 ADs and 50 HCs. The general clinical characteristics of all subjects are shown in Table 1. No significant differences regarding sex, age, and body mass index (BMI) values were found between the two groups ($p > 0.05$). All adolescents in the ADs group were naïve to antidepressant drugs. A significant difference in the HAMD-24 and HAMA-14 scores was found between the two groups ($p < 0.001$). In comparison with HCs, the average HAMD-24 score of 32.44 indicated severe depressive symptoms in ADs.

Depressive Symptoms in Adolescents with Depression

Three depressive symptom classes were identified in ADs, including core depressive, vegetative, and cognitive symptoms. We calculated the frequency of the detected depression symptoms in ADs (Table 2). All included ADs exhibited both depressed moods and loss of interest/anhedonia as core depressive symptoms. Four vegetative symptoms were more common in ADs, which were the loss of energy (96%), psychomotor retardation (94%), insomnia (94%), and change in

Table 1 Baseline Characteristics of Included Study Participants

Baseline Characteristics	Cohorts for Olink Inflammatory Assay			Cohorts for ELISA Validation		
	Control Group	ADs Group	p value	Control Group	ADs Group	p value
Sample Size	N = 15	N=15		N = 40	N=50	
Female/Male	8/7	8/7	1.000	22/18	34/16	0.206
Age (years)	15.3±1.8	15.3±2.0	1.000	15.7±1.4	15.2±2.0	0.138
BMI (kg/m2)	20.6±1.4	21.4±2.1	0.22	21.2±1.3	20.7±2.4	0.207
24-HAMD	2±0.93	34.13±9.83	<0.001	2.72±1.32	32.44±10.69	<0.001
14-HAMA	1.53±1.3	28.6±7.11	<0.001	1.23±1.2	28.13±8.16	<0.001

Abbreviation: Ads, Adolescents with depression.

Table 2 DSM-IV Depressive Symptoms of Adolescents with Depression

Depressive symptoms	Numbers	Frequency %
Core symptoms		
Depressed mood	50	100
Loss of interest/anhedonia	50	100
Irritability	37	74
Vegetative symptoms		
Change in appetite	45	90
Weight gain	4	8
Weight loss	29	58
Hypersomnia	3	6
Insomnia	47	94
Psychomotor retardation	47	94
Loss of energy	48	96
Cognitive symptoms		
Worthlessness/guilt	50	100
Loss of concentration	50	100
Suicidality	22	44

appetite (90%). In terms of cognitive symptoms, loss of concentration, suicidality, as well as worthlessness/guilt were the most common cognitive symptoms in ADs. 44% of the studied ADs had suicidal manifestations.

Olink Inflammation-Related Biomarker Identification

We evaluated and compared the expression levels of 92 inflammation-related proteins, between the AD and HC groups. In total, 13 inflammation-related proteins were identified as differentially expressed between the two groups. Among them, the plasma levels of C-X-C motif chemokine 6 (CXCL6), C-X-C motif chemokine 11 (CXCL11), interleukin-18 (IL-18), C-X-C motif chemokine 5 (CXCL5), and C-C motif chemokine 4 (CCL4) were significantly up-regulated. 8 proteins were significantly down-regulated in the AD group, including oncostatin-M (OSM), vascular endothelial growth factor A (VEGF-A), tumor necrosis factor ligand superfamily member 10 (TRAIL), transforming growth factor α (TGFα), tumor necrosis factor ligand superfamily member 14 (TNFSF14), interleukin-10 receptor subunit β (IL-10RB), Fms-related tyrosine kinase 3 ligand (Flt3L), and hepatocyte growth factor (HGF) (Figure 2A). These results remained statistically significant after FDR correction. A heatmap of these differentially expressed inflammation-related proteins is shown in Figure 2B.

Bioinformatic Analysis of Differentially Expressed Inflammation-Related Biomarkers

To investigate the potential functions of the 13 DEPs, we conducted GO and KEGG enrichment analyses. From the background of all annotated proteins, results indicated that these 13 proteins were enriched in several GO terms. As shown in Figure 3A–C, the top five enriched GO terms included biological processes, cellular components, and molecular functions. The KEGG enrichment analysis showed that several pathways, including the cytokine–cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and chemokine signaling pathways, were mainly involved in adolescent depression (Figure 3D).

Association Between DEPs and Depression in Adolescents

The correlation analysis among DEPs, the HAMD-24 and HAMA-14 scores was performed to assess the relationship between DEP levels and depression/anxiety degree. Results showed that the TRAIL protein levels were significantly negatively correlated with the HAMA-14 score ($r=-0.538$, $p= 0.038$), indicating that reduced TRAIL levels might be linked to a higher anxiety degree in ADs. No additional relative relationship was found among the other DEGs, HAMD-24, and HAMA-14 (Table 3).

We next evaluated the expression levels of DEPs linked to each depressive symptom. All patients in the AD group had a loss of interest, depressed moods, worthlessness/guilt, loss of concentration, psychomotor retardation, and insomnia. 96% of them displayed energy loss. Thus, correlations among these symptoms and 18 DEP levels were not analyzed. As shown in Figure 4, levels of TGF-α were significantly associated with a change in appetite ($r = -0.658$, $p = 0.008$). Moreover, a correlation trend was observed between suicidality and OSM levels ($r = -0.502$, $p = 0.056$), as well as irritability and HGF levels ($r=0.471$, $p= 0.076$).

Validation of Top Differentially Expressed Proteins in AD Plasma

Among the 13 DEPs, CXCL6, OSM, CXCL11, IL18, CXCL5, and CCL4 displayed fold changes over 1.3 in ADs, in comparison with HCs. Hence, based on the largest fold changes and the bioinformatics analyses, CCL4, CXCL11, CXCL5, CXCL6, and IL-18 were selected and validated by ELISA. According to the ELISA results (Figure 5), CCL4, CXCL11, CXCL5, and IL-18 expression levels were significantly higher in the plasma of ADs, in comparison with those of HCs ($P < 0.05$); the expression levels of CXCL6 had an increasing tendency to significance in adolescents with depression, in comparison with HCs ($p=0.0673$). This finding was consistent with the trend observed in the Olink PEA data. The Figure 6 present the AUC-ROC of CCL4, CXCL11, CXCL5, CXCL6, IL-18, and the pooled AUC-ROC for five protein combination with 0.819 (95% CI:0.735–0.904). However, the levels of five proteins in 50 ADs did not exhibit any correlation with either the HAMD-24 or the HAMA-14 scores (data not shown).

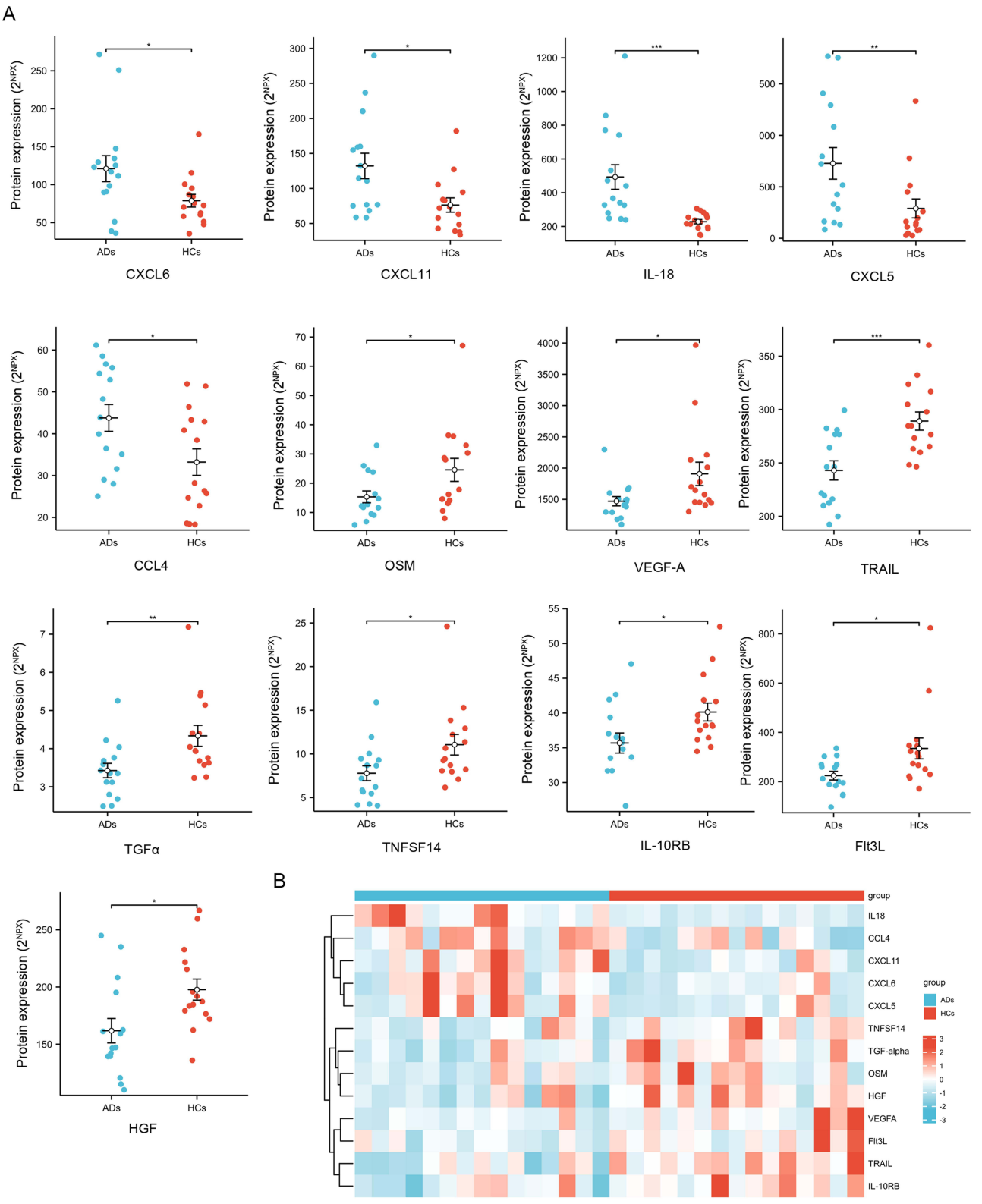

Figure 2 Differential expressed protein levels between adolescents with depression (ADs) and healthy controls (HCs). (**A**) Scatter plot of the 13 differentially expressed proteins. (**B**) Heatmap of 13 differentially expressed proteins. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001.

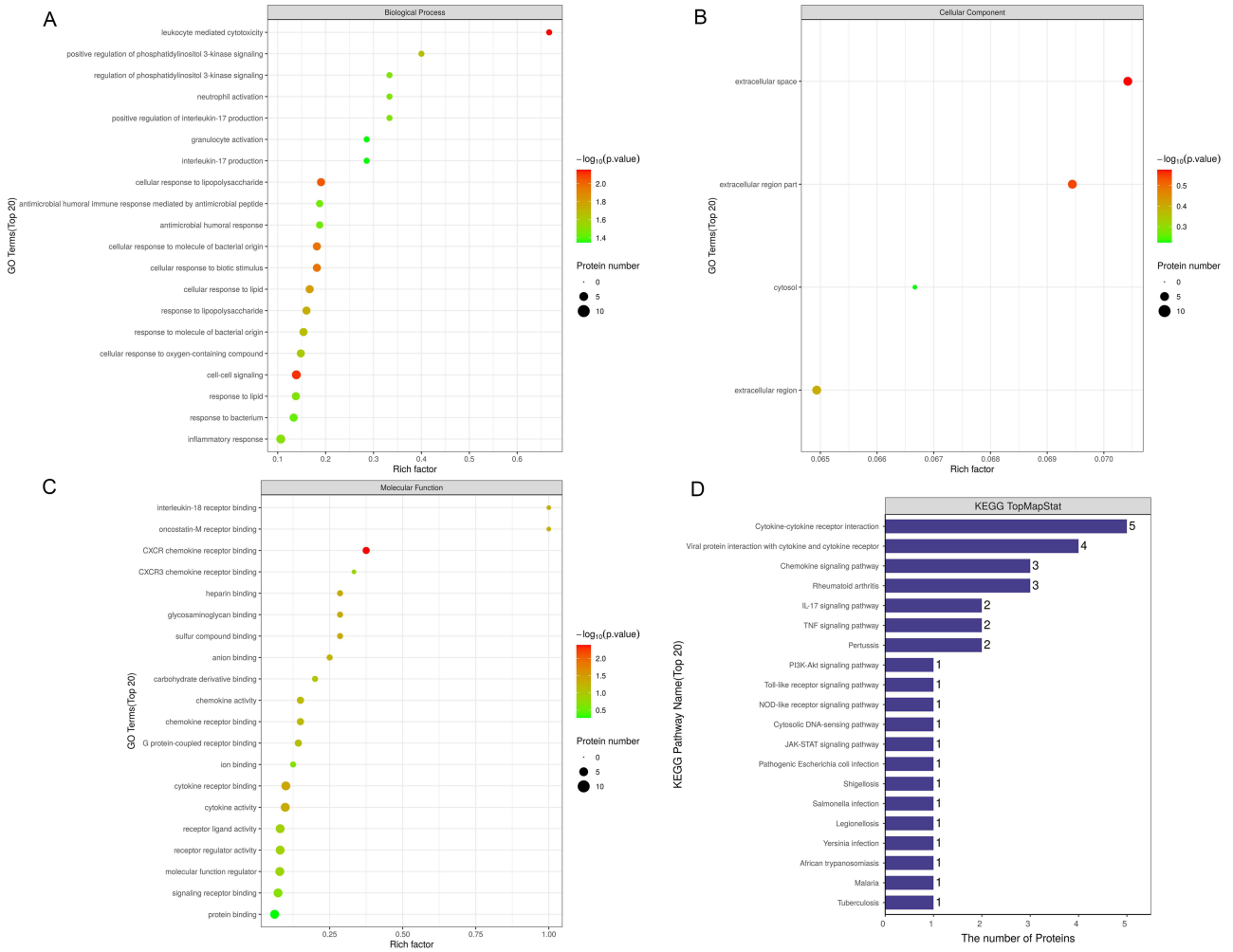

Figure 3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed proteins. GO enrichment analysis showed the most relevant (**A**) biological process, (**B**) cellular component biological process, and (**C**) molecular function terms related to the differentially expressed proteins. (**D**) KEGG enrichment analysis showed the top enriched pathway of the differentially expressed proteins.

Discussion

Depression is a common mental health disorder, whose worldwide prevalence has increased in adolescents and children. Clinicians diagnose and evaluate depression based on the clinical symptoms and scales. Nevertheless, objective methods remain absent and critically necessary for a more accurate diagnosis. Along with technological developments, multiple potential blood biomarkers have been discovered to be involved in major depression disorders, including changes in the levels of metabolites,[20] proteins,[21] or exosomal miRNAs.[22,23] As such, these classes of molecules are valuable for the diagnosis and treatment of depression.

In the present study, we applied the Olink PEA technology to analyze the levels of 92 inflammation-related proteins in ADs and HCs. To the best of our knowledge, our work is the first study using Olink PEA to profile inflammatory indicator differences between depressed and healthy adolescents. Levels of 13 proteins were significantly differentially expressed between ADs and HCs. While proteins including VEGFA and IL-18 showed consistent changes with previous studies, we focused on novel and important findings based on bioinformatics analyses.

T-cell mediated chemokines in adolescents with depression.

Four chemokines (CXCL5, CXCL6, CXCL11, and CCL4) were significantly elevated in ADs. In general, chemokines enable neutrophil recruitment to chronic inflammation sites, contributing to Th1 cell-mediated inflammatory reactions. As a downstream target of IL-17A, increased CXCL5 levels are involved in different inflammatory processes.[24–26]

Table 3 Correlation Analysis Between Depressive Symptoms Severity and DEGs in Adolescents with Depression

	CXCL6		OSM		CXCL11		IL18		CXCL5		VEGFA		TRAIL	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
HAMD-24	−0.440	0.101	0.230	0.409	−0.233	0.404	−0.040	0.887	−0.330	0.230	−0.037	0.897	−0.309	0.263
HAMA-14	−0.363	0.183	−0.034	0.903	−0.019	0.946	0.442	0.099	−0.251	0.368	0.032	0.910	−0.538*	0.038*
	CCL4		TGF-α		TNFSF14		IL-10RB		HGF		Flt3L			
	r	P	r	P	r	P	r	P	r	P	r	P		
HAMD-24	−0.229	0.411	−0.096	0.734	0.455	0.088	−0.134	0.635	0.338	0.218	−0.353	0.196		
HAMA-14	−0.035	0.901	−0.200	0.475	−0.203	0.468	−0.149	0.596	−0.075	0.789	−0.059	0.836		

Abbreviations: HAMD-24, Hamilton Depression Scale-24; HAMA-14, Hamilton Anxiety Scale-14.

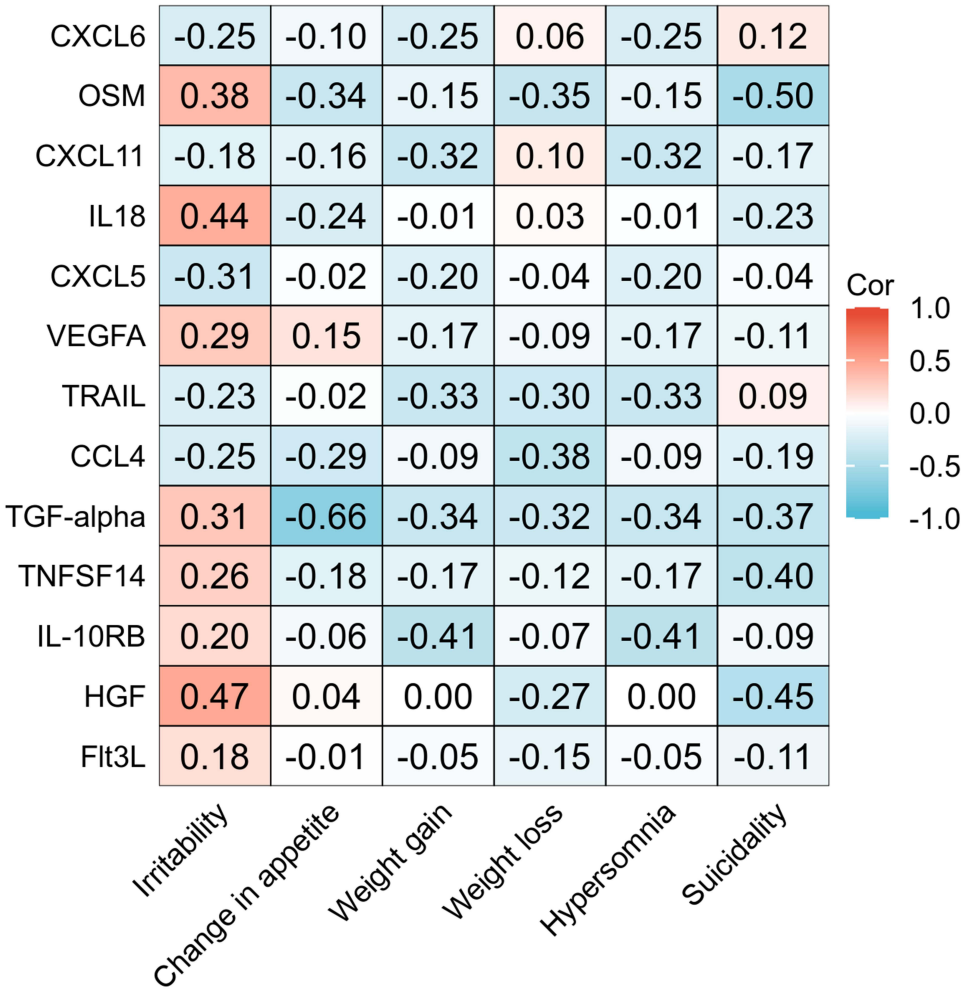

Figure 4 Relationship between DEP levels and the depressive symptoms in adolescents with depression.

Importantly, lipopolysaccharide (LPS) administration was able to enhance CXCL5 expression in microglia cells.[27] The plasma levels of LPS were reported to be higher in adolescents with MDD than in healthy individuals,[28] which possibly originated from the disturbed gut microbiota in MDD.[29] Although we did not quantify LPS levels in ADs, the increased CXCL5 expression levels might be induced by the overload of LPS in MDD. Consequently, the elevated CXCL5 levels may play an alarming role in the dysregulation of the gut microbiome in MDD. CXCL11 could bind to CXCR3 expressed on $CD8^+$ and $CD4^+$ T cells.[30] Elevated serum levels of CXCL11 were found to be related to multiple sclerosis[31] and traumatic brain injury.[32] It is speculated that increased CXCL11 levels could facilitate the recruitment of activated Th1 cells and initiate a protective response of the inflammation sites.

CCL4 is an additional pro-inflammatory chemokine with potent chemoattractant activity. Under severe inflammatory conditions in the CNS, CCL4 produced by endothelial cells of the BBB binds CCR5 expressed by circulating mononuclear cells, facilitating the invasion of the CNS. In fact, the CCL4 role in depression remained unclear. A meta-analysis showed decreased CCL4 levels in depression; however, other studies did not.[33] In addition, higher levels of CCL4 are associated with poor cognitive performance[34,35] and are also involved in bipolar disorder.[35,36] As the DSM-IV results show, all the included ADs manifested cognitive decline, characterized by loss of concentration, and feelings of worthlessness or guilt. Since we did not record a detailed cognitive performance when the patients were admitted, we could not establish any correlation between cognitive performance and up-regulated CCL4 levels.

Cytokine and cytokines receptors in adolescents with depression.

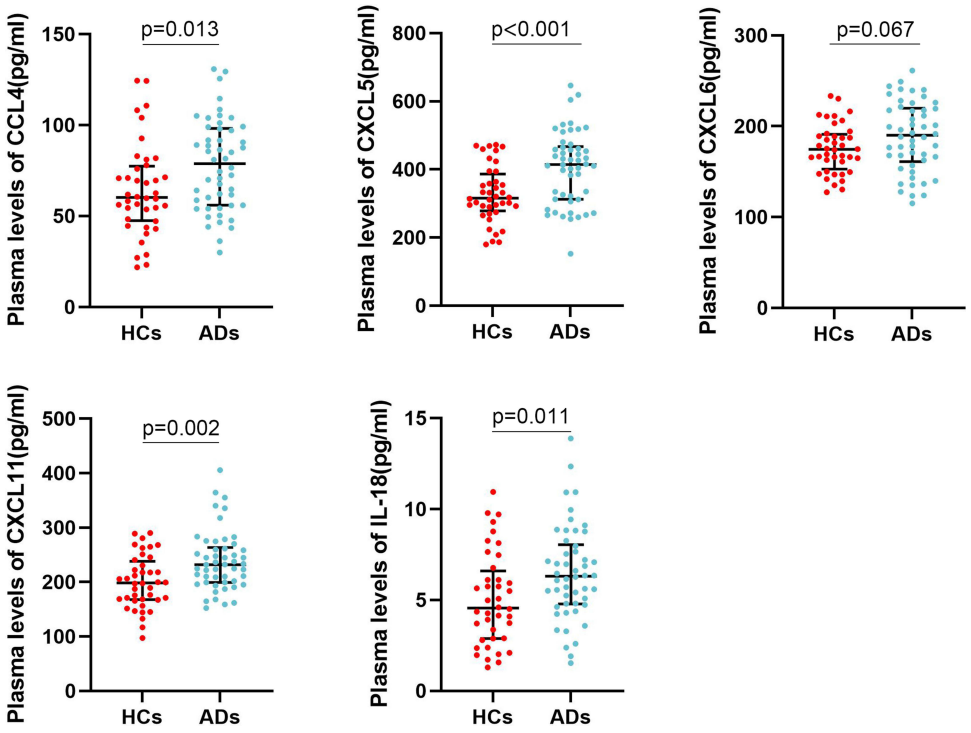

Figure 5 Elisa validation of plasma CCL4, CXCL5, CXCL6, CXCL11, and IL-18 levels in adolescents with depression (ADs) and healthy controls (HCs).

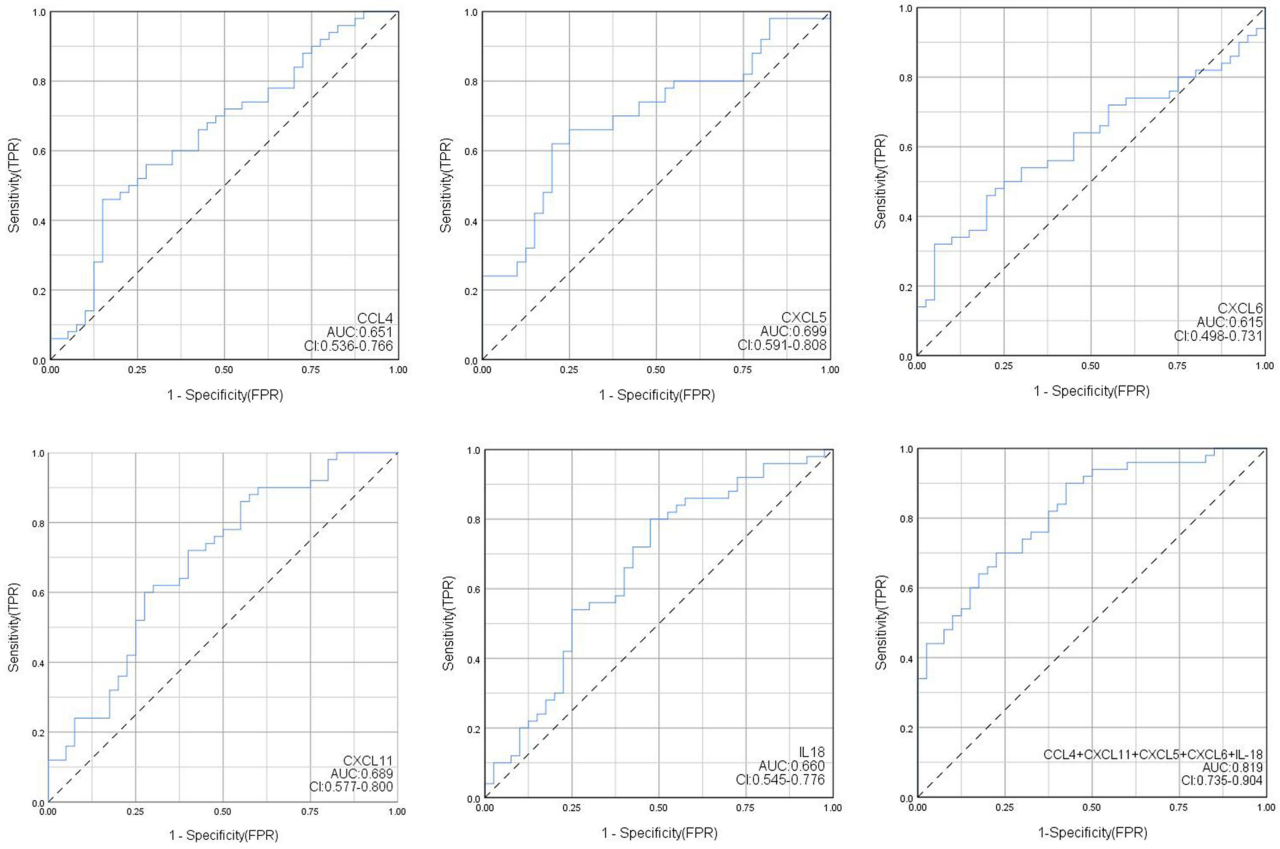

Figure 6 Receiver operating characteristic (ROC) curve for CCL4, CXCL5, CXCL6, CXCL11, IL-18, and the five-protein combination.

In agreement with most studies, we found that IL-18 levels were significantly increased in ADs. TRAIL, TNFSF14, and IL-10RB were significantly down-regulated in the plasma of ADs, which were seldom examined in the respective studies. TRAIL is a cytokine that inhibits T cell activation, cell cycle progression, and production of interferon γ and interleukin-4. Lower levels of TRAIL were found in patients with multiple sclerosis (MS), in comparison with HCs.[37] Blockade of TRAIL expressed in $CD4^+$ myelin-specific T cells reduced caspase-dependent neuronal cell death.[38] Therefore, TRAIL may have a protective role in the immunopathogenesis of depression.

It should be noted that IL-10RB is a transmembrane protein that plays important cell signaling roles. Although the origin of circulating IL-RB is unclear, IL-10RB detection in the plasma by Olink PEA has been previously reported.[39,40] As the IL-10 cytokine family receptor, IL-RB plays an important role in mucosal defenses,[41] and is related to the immune response against the resident bacterial flora of the intestine.[42] Potential intestine damage, also called leaky gut, has been previously reported in both adults and adolescents with depression.[28,43,44] It is speculated that IL-RB is released into the circulation from the intestine epithelial cells through the disrupted intestinal barrier. However, the intestinal origin of IL-RB has still to be confirmed.

Our study has several limitations. Firstly, the sample size of the Olink PEA inflammation assay was small. Although we applied an ELISA validation methodology with extended samples, further studies of larger cohorts are needed in order to confirm our findings. Secondly, some studies reported the effects of antidepressant treatment on blood cytokine levels in patients with MDD. However, we only assessed the inflammatory changes between healthy and drug-naïve depressed adolescents. The potential regulation of marker levels by antidepressant medicines needs to be further studied. Furthermore, the two most common cytokines associated with depression (TNFα and IL-6),[7] were not statistically significantly changed in ADs. In fact, elevated levels of TNF-α and IL-6 were detected in ADs (data not shown). If a larger sample size was used, a statistically significant difference might be uncovered. On the other hand, the levels of TNF-α and IL-6 might not be altered in the ADs studied here, due to ethnic and environmental differences. Finally, the ROC value of CCL4 was not of sufficiently high accuracy (>70%) for AD diagnosis. Since we did not perform an extended validation of other DEPs, the combination of CCL4 with other inflammatory indicators might be more valuable for diagnosis.

Conclusion

In conclusion, using the Olink PEA proteomics technology, we found 13 significantly altered proteins in ADs, unraveling possible roles of chemokines, cytokines, and cytokine receptors in adolescent depression. These proteins may affect the brain and peripheral systems, by attracting immune cells (especially T cells) to trigger immune reactions. Their roles in AD, by promoting either detrimental or anti-inflammatory effects, remain unclear and need to be further elucidated in the framework of the pathophysiology of adolescent depression.

Disclosure

The authors report no conflicts of interest in this work.

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