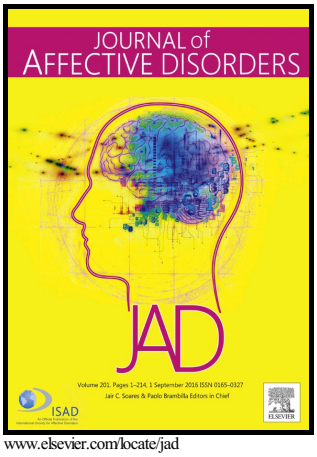

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Peripheral proinflammatory cytokines in Chinese patients with generalised anxiety disorder

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Abstract

Background: Inflammatory responses and inflammatory cytokines have been implicated in the pathogenesis of affective disorders, particularly major depression. Given the limited evidence relating to the potential role of proinflammatory cytokines in generalised anxiety disorder (GAD), we aimed to examine peripheral proinflammatory cytokines in Chinese patients with GAD.

Methods: A case-controlled cross-sectional study design, with recruitment of 48 patients with first episode GAD and 48 matched healthy controls. All participants completed measures of anxiety using well-established questionnaires, and serum levels of pro-inflammatory cytokines were measured using multiplex technology.

Results: Serum levels of CRP, IL-1α, IL-2, IL-6, IL-8, IL-12, IFN-γ, and GM-CSF were significantly higher in the GAD group in comparison to the control group ($p<0.05$). Pearson correlation revealed significant positive correlations between

anxiety measures and serum levels of CRP, IL-1α, IL-6, IL-8, IFN-γ, and GM-CSF ($p<0.05$).

Limitations: The cross-sectional study design does not permit definite conclusions on causal directions between inflammation and GAD. The study was limited to a panel of 8 cytokines and does not exclude the possibility of other important cytokines being involved.

Conclusions: These findings indicate an elevated peripheral proinflammatory response, and provide further support for low grade inflammation in GAD. Further research may identify an 'inflammatory signature' for diagnosis and treatment response, and guide the search for novel pharmacological interventions.

Key words:

Generalised anxiety disorder; Inflammation; Cytokine; Proinflammatory cytokines

1. Introduction

Generalised anxiety disorder (GAD) is a common, impairing and often chronic condition characterized by excessive and uncontrollable worrying. Systematic reviews of epidemiological studies within Europe have found a 12-month prevalence of 1.7% to 3.4 % and a lifetime prevalence of 4.3% to 5.9% (Wittchen and Jacobi 2005; Wittchen et al. 2011).

Recent advances in understanding of the role of cytokines in communications between the central nervous system and the immune system have led to integrative and explanatory models for various neuropsychiatric disorders. Inflammatory responses and inflammatory cytokines have been implicated in the pathogenesis of affective disorders, particularly major depression. Apart from the high comorbidity of GAD and major depression, similar treatment effects with antidepressants suggest possible common or similar neurobiological substrates. Camacho (2013) proposed

that anxious-depression should be considered as a chronic inflammatory phenomenon, and the pronounced response of central and peripheral cytokines to stress has prompted further interest in the potential role of cytokines in the pathogenesis of anxiety disorders. A recent case-control study indicates a relatively increased pro-inflammatory response, decreased anti-inflammatory response, and an altered cytokine balance in patients with GAD (Hou et al. 2017). A large cohort study examined the association between anxiety disorders (including GAD, social phobia, panic disorder, and agoraphobia) and inflammation (Vogelzangs et al. 2013), and found elevated CRP levels in male patients with current anxiety disorders and immune dysregulation in patients with a late-onset anxiety disorder. Evidence for adopting a neuroimmunological perspective on anxiety disorders has been extensively reviewed by Hou and Baldwin (2013). Due to a reliance on single cytokine measures, small sample sizes, the lack of standardized measurements, and high co-morbidity with other psychiatric conditions, findings in anxiety disorders are not consistently reported.

The aim of the current study was to investigate peripheral proinflammatory cytokine expression in Chinese patients with first episode GAD in comparison to heathy controls, and to explore possible associations with clinical characteristics, with the goal of identifying both state and trait inflammatory markers. The main predictions were as follows: Hypothesis 1: There will be an elevated serum proinflammatory cytokine levels in the GAD group in comparison to the control group; Hypothesis 2: The elevated cytokine levels will be associated with measures of anxiety.

2. Methods

2.1. Participants

GAD patient group: Following referrals from consultant psychiatrists in the outpatient clinic at Suzhou Psychiatric Hospital, 73 patients, aged 18-60 years, with a BMI between 18-30, with 6 or more years of education, and a primary diagnosis of first episode GAD based on the International Classification of Diseases 10th Revision (ICD-10), were initially approached by researchers. 67 patients completed a pre-test screening interview comprising a structured diagnostic Mini International Neuropsychiatric Interview - MINI (Sheehan et al. 1998) and the 7-item Generalised Anxiety Disorder Questionnaire (GAD-7) with a threshold score of 10 points (Spitzer et al. 2006). Patients were excluded if they had comorbid psychiatric disorders. All GAD patients were medication naive and had no history of any antidepressant or anxiolytic intake. Participants were excluded if they reported any inflammatory events or had any intake of any medication with known immune-modulating effects, such as glucocorticoids, within 2 weeks prior to their testing session. 48 GAD patients were recruited (see GAD patient recruitment flow chart in Figure 1).

Healthy control group: 48 age-, gender-, and BMI-matched controls were recruited through advertising in local communities in Suzhou: they were healthy volunteers aged between 18-60 years, had a BMI between 18-30, had no physical illness or mental disorder, had 6 or more years of education and were not taking any medication. Participants who experienced any inflammatory event or taking any medication with known immune regulating effects within two weeks before the testing were excluded.

The study was approved by the Clinical Research Ethics Committee in Suzhou Psychiatric Hospital.

2.2. Measures

2.2.1. Measure of inflammatory cytokines

A sample of 10ml venous blood was taken from all participants at approximately the same time of day (9:00-10:00AM) and centrifuged for 15 min at 2500rpm. The cell free-serum was pipetted and aliquoted in 2 ml standard freezer vials which were then stored within 2 hours at -80° C until further analysis. The following inflammatory cytokines were measured by enzyme linked-immuno-sorbent assay (ELISA) including IL-1α, IL-2, IL-5, IL-6, IL-8, IL-12p70, GM-CSF and IFN-γ. The ELISA kits were manufactured by Wuhan Boshide Biotechnology Limited Company. The concentration of C-reactive protein (CRP) was measured by immunological transmission turbidity and the kits were produced by Shenzhen Mindray Bio-Medical Electronics Limited Company. Selection of cytokines was based on a recent review of inflammation and anxiety (Hou and Baldwin, 2013). Processing of blood samples was based on a protocol provided for human multiplex assays and recommendations for clinical trials (de Jager et al. 2009).

2.2.2. Questionnaire measures of anxiety

Generalized Anxiety Disorder Scale (GAD-7) (Spitzer et al 2006)

The GAD-7 is a self-report questionnaire for screening and severity measuring of GAD. The seven items assess severity of key symptoms of GAD according to reported response categories with assigned points. The sensitivity of the Chinese version was 86.2% and the specificity was 95.5% with a Kappa value 0.825 which indicates its good reliability and validity (He et al. 2010).

State Trait Anxiety Inventory (STAI) (Spielberger et al. 1993)

The STAI is a well-established self-report instrument that clearly differentiates between the temporary condition of state anxiety and the longstanding quality of trait

anxiety. The scale includes State Anxiety Inventory (SAI) and Trait Anxiety Inventory (TAI), which assess the severity of state anxiety and trait anxiety respectively. For the Chinese version of STAI, the Cronbach's alpha of SAI is 0.90 and the Cronbach's alpha of TAI is 0.73, and their correlation coefficient r was 0.59～0.75. The test-retest reliability coefficients of SAI and TAI were 0.88 and 0.90 respectively (Wang et al. 2000).

2.3. Study design and procedure

A case-controlled, cross-sectional cohort design was employed. All eligible participants provided written informed consent before taking part in the study. Participants who attended the laboratory were asked to rest for 5 minutes before their testing session. After blood samples were taken, participants were instructed to complete a questionnaire booklet.

2.4 Data analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS version 21). All variables were tested for normality, and transformation to symmetry using normal scores was undertaken when necessary. Group differences in sociodemographic characteristics between the GAD and healthy control groups were assessed by independent t-tests for continuous measures, and by Chi-square tests for categorical variables. Pearson correlational analysis was used to assess associations between measures of anxiety and serum levels of proinflammatory cytokines.

3. Results

3.1. Demographic characteristics

Table 1 shows the demographic characteristics of the patients and controls. There were no significant group differences in terms of age ($t=0.52$, $p>0.05$), gender ($X^2=0.17$, $p>0.05$), BMI ($t=-0.24$, $p>0.05$), educational level ($t=-2.50$, $p>0.05$), or smoking and alcohol consumption ($p>0.05$ in both cases).

3.2. Comparisons of questionnaire measures of anxiety and serum proinflammatory cytokine levels between groups

Table 2 shows comparisons of questionnaire measures of anxiety and serum proinflammatory cytokine levels between the two groups. The level of clinical anxiety of the GAD group ranged from moderate to severe. Levels of state anxiety and trait anxiety measured by the STAI were significantly higher than in the healthy control group ($p<0.001$). Levels of proinflammatory cytokines including IL-1α, IL-2, IL-6, IL-8, IL-12p70, IFN-γ, GM-CSF, and CRP, were all significantly higher in the GAD group than in the control group ($p<0.05$), with IL-1α, IL-2, IL-6 showing greater group difference ($p<0.001$), whereas there was no group difference in terms of IL-5 ($p>0.05$).

3.3. Correlations between levels of inflammatory cytokines and measures of anxiety

Pearson correlation analyses were used to investigate possible associations between inflammatory cytokines and total anxiety, state and trait anxiety in the GAD patient group. There were significant positive correlations between total anxiety measured by the GAD-7 and serum levels of CRP, IL-1α, IL-6, IL-8, IFN-γ, and GM-CSF. State anxiety measured by the SAI and trait anxiety measured by the TAI were both positively correlated with IL-1α, IL-2, IL-6, IL-8, IL-12p70, IFN-γ and GM-CSF ($p<0.05$, see Table 3).

4. Discussion

We examined peripheral proinflammatory cytokine expression in Chinese patients with first episode GAD, and explored their possible associations with clinical characteristics of anxiety. The study presented a multi-panel of 8 proinflammatory cytokines and CRP measured simultaneously in well-characterized groups. The findings demonstrated increased levels of serum proinflammatory cytokines including IL-1α, IL-2, IL-6, IL-8, IL-12p70, IFN-γ, GM-CSF, and CRP in GAD patients when compared to well-matched healthy controls. In addition, serum proinflammatory cytokine levels were associated with measures of anxiety.

To our knowledge this is the first study to examine a spectrum of pro-inflammatory cytokines in Chinese patients with GAD. The elevated cellular immune response indicated by increases in IL-1α, IL-6, IL-12p70, IFN-γ and GM-CSF are consistent with findings from another GAD study in predominantly European patients (Hou et al. 2017) in which an elevated proinflammatory response was also found. The findings of the current study accord with findings from studies in posttraumatic stress disorder and panic disorder (Hoge et al. 2009; Baker et al. 2012; Passos et al. 2015) in which elevated peripheral cytokine levels for 18 of 20 different cytokines were reported compared to age and gender matched healthy controls as well as a generalized proinflammatory state. The increased CRP level in GAD is consistent with the findings from a large cohort study (Vogelzangs et al. 2013). IL-6 appears to be an important molecule in a range of signaling pathways within the central nervous system, and is involved in activities, ranging from neuronal physiology to neurodevelopment, neuroprotection and neurotoxicity. An elevated serum level of IL-6 has been reported in depression and acute psychosis and there are currently

ongoing therapeutic trials with antibodies targeting IL-6 in both depression and schizophrenia. The elevated level of IL-6 found in the current study is consistent with the finding of O'Donovan et al. (2010) indicating a distinct increase in IL-6 in clinical anxiety. The elevated IL-8 level aligns with findings from a study by Zhang et al. (2004). Our study also demonstrated an elevation in GM-CSF which, to our knowledge, has not previously been reported in GAD.

Evidence from experimental and clinical research shows the pivotal roles of cytokine signalling to the brain to produce neurochemical, neuroendocrine, neuroimmune, and behavioural changes (Kronfol and Remick 2000; Maier 2003; Loftis et al. 2010; Dantzer et al. 2008; Müller and Schwarz 2007). Four potential pathways are proposed in an extensive review by Capuron and Miller (2011): 1) by altering the metabolism of neurotransmitters such as serotonin, dopamine, and glutamate; 2) by altering the function of the HPA axis by having a stimulant effect on the expression and release of hormones such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol; 3) by affecting growth and development of nervous tissue via activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex that controls the transcription of DNA; and 4) by targeting neurocircuits in the brain that regulate motivation, reward, as well as anxiety, arousal and alarm, which leading to behavioural changes. Although the cross-sectional design does not allow us to investigate the causal direction between inflammation and anxiety, we further examined how state and trait anxiety were associated with proinflammatory cytokines and found that both state and trait anxiety were positively correlated with proinflammatory cytokines including IL-1α, IL-2, IL-6, IL-8, IL-12p70, IFN-γ and GM-CSF in patients with GAD.

The strengths of the current study include the measurement of multiple proinflammatory cytokines, a well-matched study population without medication use, and consideration of potential confounding factors. However, the findings must be interpreted in light of several limitations. First, the cross-sectional design of the study does not allow for definite conclusions on causal directions in the observed associations. Second, serum cytokine levels are unstable and can be affected by biological circadian rhythms, although we tried to control for this by taking blood between 8am and 10am. Third, our study was limited to a panel of 8 cytokines and does not exclude the possibility that other cytokines or chemokines may also be important. Fourth, GAD patients with comorbid depression were excluded this study but depressive symptoms were not measured, which limits the examination of whether the anxiety cytokine phenotype is independent of depression symptom as Hou et al. (2017) investigated in a previous similar cohort study. Finally, future studies are required to identify the cellular source of the increased proinflammatory cytokines identified in the current study and the underlying mechanisms that drive these changes.

While extensive research has been conducted to investigate the link between inflammation and psychiatric disorders in western countries, there has been growing research interest in the link between inflammation and depression in China recently. A meta-analysis (including 9 case-control studies in China involving 432 patients with depression and 277 healthy controls) conducted by Zhang et al. (2014) reveals significantly increased IL-6 in patients with depression in comparison to healthy controls, which is consistent with the trends reported in western literature. Due to different environmental risk factors between the east and the west, such as diet, smoking and alcohol use, antibiotic use, hygiene status, microbial exposures, and

pollution, we would expect to see a difference of inflammatory cytokine levels between the east and the west psychiatric population. However it is difficult to compare the existing data in the literature due to different study designs and different methodological measures of inflammatory markers employed. Therefore, more research is warranted to explore how the change of inflammatory cytokine levels in Chinese psychiatric population differs from that in the west and identify relevant environmental risk factors.

Cytokine patterns have shown promise in predicting treatment response in depression, which could provide the key to treatment resistance (Gimeno et al. 2009; Au et al. 2015). Anti-inflammatory drugs demonstrate some antidepressant effects and can enhance responsiveness to antidepressants in a subgroup who show evidence of increased inflammation (Benros et al. 2013; Raison et al. 2013; Köhler et al. 2014; Kappelmann et al. 2016). With supporting data accumulating in depression (Miller and Raison 2016), more evidence is needed to investigate whether dysregulated immune systems may contribute to treatment resistance in anxiety disorders, and can provide new treatment targets, in particular, for anxiety patients with increased systemic inflammation. Enhanced understanding of how cytokines mediate interactions between the central nervous system and the immune system could reveal biomarkers for treatment resistance and predictors for treatment response, provide new targets for developing novel anxiolytic agents, and help improve clinical outcomes in anxiety disorders.

Various natural products from traditional Chinese medicine have shown to safely suppress proinflammatory pathways and control inflammation-associated disease. In vivo and/or in vitro, evidence have shown that anti-inflammatory effects of traditional Chinese medicine occur by (1) modulation of inflammatory signal transduction

pathways linked to nuclear factor-κB, activator protein 1, mitogen-activated protein kinase; (2) induction of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase; (3) reduction of inflammatory molecule production including nitric oxide synthase, cyclooxygenase 2, prostaglandin E2, and nitric oxide; (4) reduced activity of inflammatory cells; (5) altered regulation of cellular function; and (f) changes in the balance of proinflammatory and antiinflammatory cytokines (Pan et al. 2011). Clinical trials have also demonstrated the efficacy of traditional Chinese medicine for the prevention and treatment of many chronic inflammatory diseases. Therefore, if the role of inflammation in anxiety disorders is confirmed in further larger studies, traditional Chinese medicine may create new opportunities for innovation in pharmacological treatment for anxiety disorders.

5. Conclusions

Data from the current study demonstrates an elevated peripheral proinflammatory cytokine phenotype in patients with first episode GAD. Growing understanding of cytokines in GAD may help identify an inflammatory signature for diagnosis and treatment response, and guide the search for new pharmacological interventions that selectively target and modulate specific immune phenotypes related to GAD. The safety and antiinflammatory efficacy of traditional Chinese medicine may create new opportunities for innovation in pharmacological treatment for anxiety disorders.

Contributors

Drs TZ and GY were involved in the design, conduct, data analysis and writing up of the study and contributed equally to the manuscript as first authors. Dr RH was involved in the design, data analysis and writing up of the study. Dr DB was involved

in the design and writing up of the study. Drs XC, MP, JF, QL, and ZG were involved in the recruitment and data collection in the study.

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Conflict of interest

Within three years of beginning the work submitted, Professor David S. Baldwin has received financial support from H. Lundbeck A/S (advisory board attendance), AstraZeneca, Janssen and Pfizer (lecture fees), and the UK Ministry of Defense (research ethics committee membership). All other authors report no biomedical financial interests or potential conflicts of interest.

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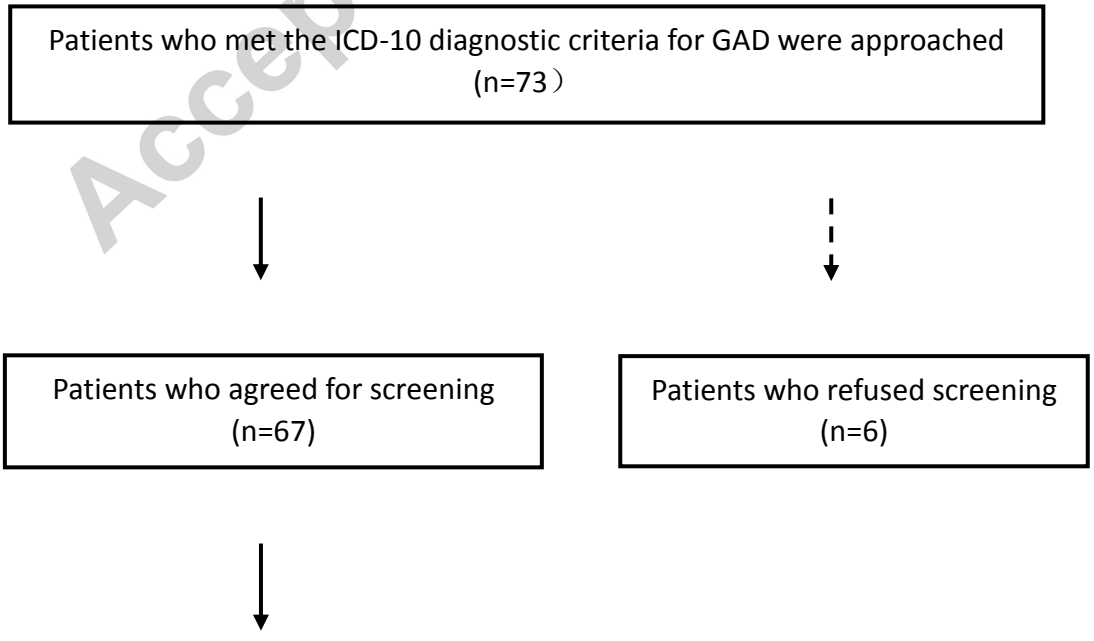

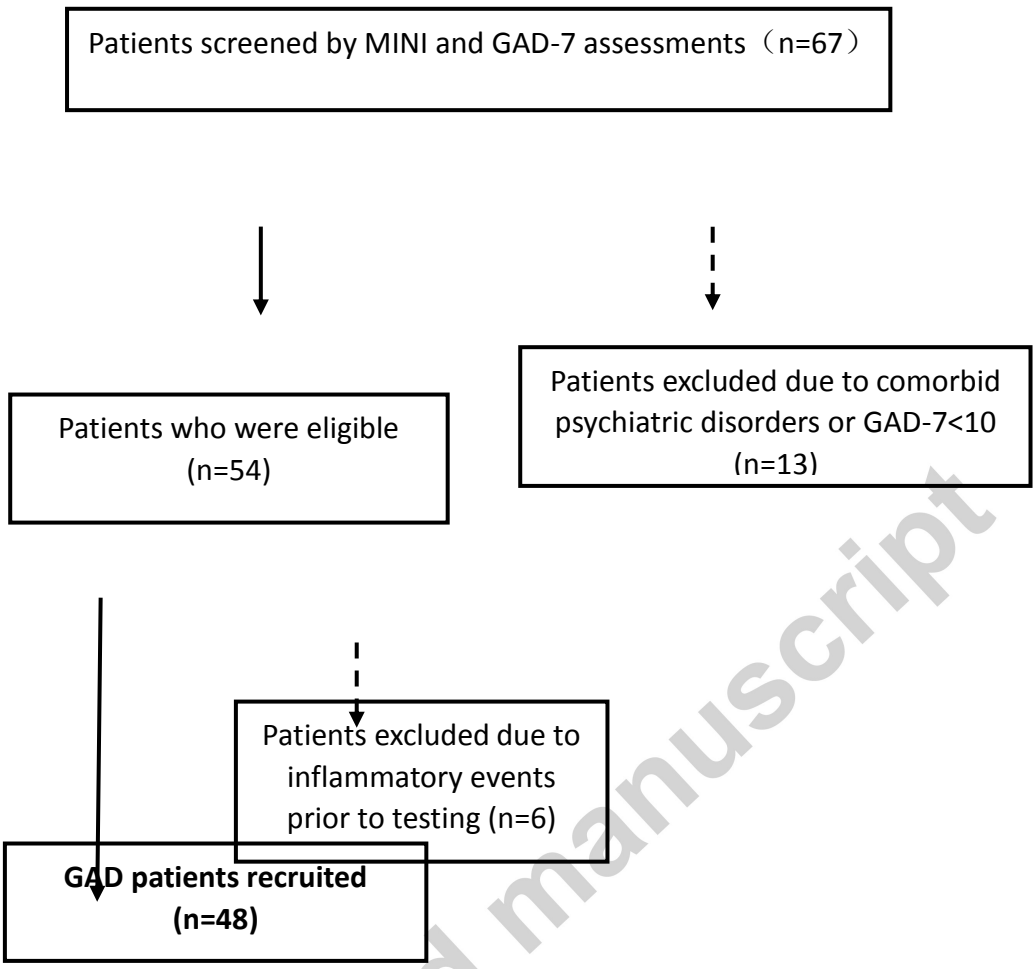

Figure 1 GAD patient recruitment flow chart

Tables

Table 1 Demographic characteristic of GAD patient group and healthy control group

	GAD (n=48)	HC (n=48)
Gender - male (n:%)	20 (41.67%)	22 (45.83%)
Age (years, Mean ± SD)	40.75±12.	39.56±10.06

	21		
BMI (kg/m^2, Mean ± SD)		22.56±2.7	22.69±2.63
	3		
Education (years, Mean ± SD)		10.35±2.5	11.71±2.76
Smoking	4		
Smoker (n:%)			11 (23%)
Non-smoker (n:%)		14 (29%)	37 (77%)
Alcohol consumption		34 (71%)	
Frequent user (more than 3 times/week) (n:%)			5 (10%)
Non-frequent users (less than 2 times/week)		9 (19%)	43 (90%)
(n:%)		39 (81%)	

Table 2 Comparisons of self-report questionnaire measures and inflammatory cytokine levels between groups

Variables	GAD group (n=48) (Mean±SD)	Control group (n=48) (mean±SD)	t	P
GAD-7	15.75±3.12	2.85±2.00	23.83	<0.001**
SAI	54.88±11.95	32.69±6.15	11.44	<0.001**
TAI	54.73±10.81	33.73±6.86	11.36	<0.001**
CRP（mg/L）	1.19±0.80	0.68±0.70	3.31	0.001**
IL-1α（pg/ml）	70.34±3.60	16.94±3.42	74.50	<0.001**
IL-2（pg/ml）	7.25±3.42	4.95±2.31	3.85	<0.001**
IL-5（pg/ml）	5.81±2.37	5.67±1.60	0.34	0.734
IL-6（pg/ml）	12.55±2.37	2.71±1.35	14.79	<0.001**

IL-8（pg/ml）	44.64±16.21	35.69±11.70	3.10	0.003*
IL-12p70（pg/ml）	18.16±24.17	10.82±4.72	2.06	0.042*
IFN-γ（pg/ml）	23.32±15.52	16.48±6.80	2.79	0.007*
GM-CSF（pg/ml）	19.07±11.12	13.40±8.54	2.80	0.006*

Note: GAD-7: Generalized Anxiety Disorder Scale; SAI: State Anxiety Inventory; TAI: Trait Anxiety Inventory; CRP: C-reactive protein; IL-1α: Interleukin-1α; IL-2: Interleukin-2; IL-5: Interleukin-5; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-12p70: Interleukin-12p70; IFN-γ: Interferon-γ; GM-CSF: Granulocyte- macrophage Colony Stimulating Factor.

Table 3 Associations between inflammatory cytokines and measures of anxiety

Cytokines	GAD-7 or	SAI or	TAI or
CRP	0.25*	0.19	0.15
IL-1α	0.92**	0.75**	0.76**
IL-2	0.42	0.33**	0.35**
IL-5	0.07	-0.02	-0.03
IL-6	0.80**	0.59**	0.63**
IL-8	0.30**	0.32**	0.35**
IL-12p70	0.19	0.26*	0.29**
IFN-γ	0.30**	0.26*	0.24*
GM-CSF	0.28**	0.27**	0.24*

Note: or: odds ratio; CRP: C-reactive protein; IL-1α: Interleukin-1α; IL-2: Interleukin-2; IL-5: Interleukin-5; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-12p70: Interleukin-12p70; IFN-γ: Interferon-γ; GM-CSF: Granulocyte- macrophage Colony Stimulating Factor. *P<0.05, **P<0.01.

Highlights

- This is the first case-controlled investigation of peripheral proinflammatory cytokines in Chinese patients with first episode GAD.

- Serum levels of CRP, IL-1α, IL-2, IL-6, IL-8, IL-12, IFN-γ, and GM-CSF were significantly higher in the GAD group in comparison to the control group.
- The study demonstrates an elevated peripheral proinflammatory response in GAD.
- Growing understanding of cytokines in GAD may help identify an inflammatory signature for diagnosis and treatment response, and guide the search for new pharmacological interventions.