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Original Article

Association of vitamin D and N-acetylcysteine supplementation with anxiety, cognition, and biomarkers in generalized anxiety disorder: a retrospective cohort study

Yulong Zhang[1], Xue Han[2], Fei Jiao[3], Guodong Huang[1], Yanhui Liang[1]

[1]Department of Psychiatry and Psychology (Ward 6), Beidahuang Group Neuropsychiatric Hospital (The Second Hospital of Beidahuang Group), No. 472 Anqing Road, Qianjin District, Jiamusi 154001, Heilongjiang, China; [2]Department of Psychiatry (Ward 2), Beidahuang Group Neuropsychiatric Hospital (The Second Hospital of Beidahuang Group), No. 472 Anqing Road, Qianjin District, Jiamusi 154001, Heilongjiang, China; [3]Department of Medical Insurance, Beidahuang Group Neuropsychiatric Hospital (The Second Hospital of Beidahuang Group), No. 472 Anqing Road, Qianjin District, Jiamusi 154001, Heilongjiang, China

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Abstract: Objective: To investigate the association between combined vitamin D and N-acetylcysteine (NAC) supplementation and clinical outcomes in patients with generalized anxiety disorder (GAD). Methods: This retrospective cohort study included 88 propensity-score-matched patients with GAD from Beidahuang Group Neuropsychiatric Hospital. Based on clinical records, patients were classified into an observation group (vitamin D3 + NAC + usual care) and a control group (usual care only). Anxiety symptoms and cognitive function were assessed using the Beck Anxiety Inventory (BAI), Automatic Thought Questionnaire (ATQ), and Dysfunctional Attitudes Scale (DAS). Serum levels of 25-hydroxyvitamin D [25(OH)D], inflammatory markers [high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6)], oxidative stress parameters [glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD)], and neurochemical markers [brain-derived neurotrophic factor (BDNF), dopamine (DA), Serotonin (5-HT), norepinephrine (NE)] were measured at baseline and week 8. Results: After 8 weeks, both groups showed significant improvements in BAI, ATQ, and DAS scores, with greater reductions in the observation group (all $P < 0.05$). The observation group also exhibited more favorable changes in biomarkers: greater increases in 25(OH)D, GSH, SOD, BDNF, DA, 5-HT, and NE, and greater decreases in MDA, IL-6, and hs-CRP compared to the control group (all $P < 0.05$). Adverse event incidence was low and similar between groups. Conclusion: In this retrospective cohort, combined vitamin D and NAC supplementation was associated with significantly greater improvements in anxiety symptoms, cognitive patterns, and relevant metabolic biomarkers in patients with GAD compared to usual care alone, supporting its potential as an adjunctive therapy.

Keywords: Vitamin D, N-acetylcysteine, generalized anxiety disorder, oxidative stress, neuroinflammation

Introduction

Generalized anxiety disorder (GAD) is a common anxiety condition characterized by persistent worry and impaired social functioning. Common physical symptoms include fatigue, restlessness, and difficulty sleeping [1]. The global prevalence of GAD ranges from 3% to 6%. Women and individuals experiencing chronic stress are at higher risk, and GAD can have detrimental effects on their long-term health [2]. First-line treatments for GAD include benzodiazepines and selective 5-HT reuptake inhibitors. Nevertheless, these drugs do not fully alleviate symptoms and can cause side effects such sexual dysfunction, weight gain, gastrointestinal problems, sleepiness, and cognitive impairment [3]. There is a clear need for adjuvant treatments with fewer side effects to enhance clinical results.

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GAD is caused by a combination of oxidative stress, inflammatory reactions, and neuronal dysregulation, which damages nerve cells and interferes with neurotransmitter networks [4]. Many multi-target medications with anti-inflammatory and antioxidant properties have been developed based on the previously outlined mechanisms. Due to their neuroprotective effects and safety profiles, vitamin D and N-acetylcysteine (NAC) are well-known candidate medications [5, 6].

Vitamin D, a steroid hormone, is necessary for maintaining proper neural function, promoting brain development, and regulating calcium levels in the body [7, 8]. It also plays a crucial role in the synthesis of 5-HT (5-hydroxytryptamine), inflammatory cytokines, and neurotrophic factors, all of which are critical for regulating emotions [9, 10]. Mao et al. discovered a substantial correlation between low serum levels of 25-hydroxyvitamin D [25(OH)D)] and a higher risk of anxiety and depression [11]. As a precursor to glutathione (GSH), NAC has shown notable efficacy in treating a range of mental health conditions. Studies have shown that it may alleviate anxiety symptoms by reducing oxidative damage and improving neuronal plasticity [12, 13].

Although vitamin D and NAC have demonstrated efficacy as supplementary treatments for mental disorders, few clinical trials have investigated their combined use in GAD [14]. Vitamin D and NAC act through distinct but complementary pathways. Specifically, vitamin D regulates inflammation and monoamine metabolism, while NAC mainly restores redox balance and provides neuroprotection. The combined use of the two may result in a stronger therapeutic effect. However, this potential synergistic effect requires validation in clinical trials.

This retrospective cohort study aimed to assess the safety and effectiveness of combined vitamin D and NAC supplementation as an auxiliary therapy for GAD in a real-world clinical setting, and to explore its use as a viable supplemental treatment strategy.

Patients and methods

Study design and setting

This retrospective cohort study was conducted utilizing clinical data from patients with GAD at the Beidahuang Group Neuropsychiatric Hospital between December 2024 and August 2025. The study protocol was reviewed and approved by the hospital's Ethics Committee (Approval No.: 2023001) and complied with the ethical principles of the Declaration of Helsinki. Given the retrospective nature of the analysis, the requirement for individual informed consent was waived. All patient data were anonymized and handled with strict confidentiality.

Participants

Patients aged 18-60 years with a diagnosis of GAD were retrospectively identified from the clinical database of Beidahuang Group Neuropsychiatric Hospital. The diagnosis was confirmed by licensed psychiatrists in patient medical records according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria [15]. These criteria require the presence of excessive anxiety and worry occurring more days than not for at least six months, accompanied by at least three of the following symptoms: restlessness, fatigue, difficulty concentrating, irritability, muscle tension, or sleep disturbance.

Eligible patients were initially identified based on the following criteria: (1) a documented baseline Beck Anxiety Inventory (BAI) score ≥ 45; (2) availability of complete clinical follow-up data over an 8-week period; and (3) stable doses of psychotropic drugs for at least 2 weeks before the index date (baseline).

Exclusion criteria were: (1) a concurrent diagnosis of other major psychiatric disorders (e.g., schizophrenia, bipolar disorder); (2) active substance abuse; (3) severe hepatic, renal, or endocrine dysfunction; (4) recorded use of vitamin D, NAC, or antioxidant supplements during the observation period; and (5) pregnancy or lactation.

Propensity score matching (PSM)

Given the retrospective nature of this cohort study, PSM was employed to enhance comparability between the observation group (which received combined vitamin D and NAC supplementation) and the control group (which did not). Eligible patients from the preliminary cohort (n = 118) were matched in a 1:1 ratio without replacement using the nearest-neighbor method, with a caliper width set to 0.2 of

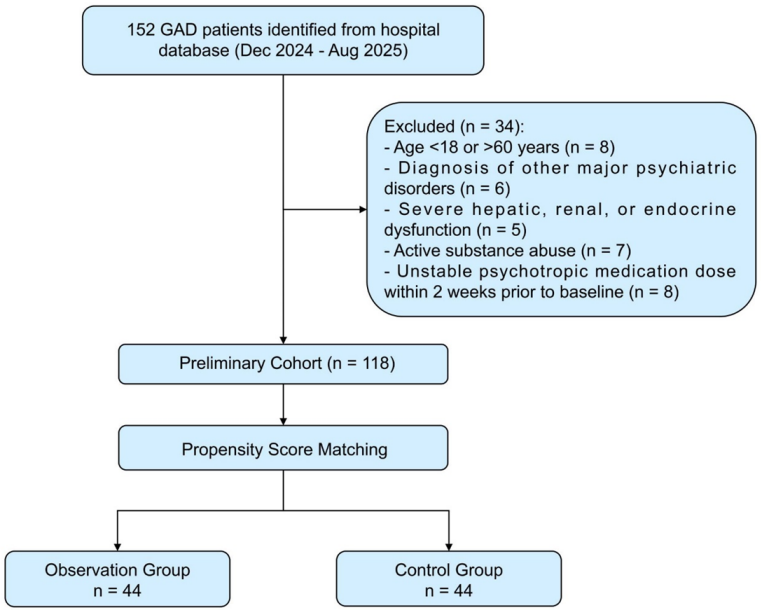

Figure 1. Flowchart of patient selection and cohort formation. GAD = generalized anxiety disorder.

the standard deviation of the logit propensity score. The propensity score was estimated by logistic regression, with exposure status (supplementation) as the dependent variable and the following pre-specified baseline covariates: age, sex, baseline BAI total score, first-episode status of GAD, and the presence of sleep disturbance. Following PSM, 44 matched pairs (n = 88) were successfully formed, comprising the final analytical cohort for the study (**Figure 1**).

Treatment exposure and data collection

Based on retrospective review of medical records, patients were assigned to the observation group if they had documented combined oral supplementation with vitamin D3 and NAC for at least eight weeks alongside their regular psychiatric care. The documented supplementation regimen consisted of vitamin D3 at 2,000 IU once daily and NAC at 600 mg twice daily. Patients in the control group received only regular psychiatric care without documented supplementation of vitamin D or NAC during the same observation period. All patients continued their predetermined psychiatric treatment plans. Adherence to the supplementation regimen in the observation group was assessed based on pharmacy dispensing records and documented pill counts from outpatient notes during the 8-week follow-up.

Outcome measures

Anxiety symptoms were assessed using the BAI [16]. This 21-item self-report scale measures the frequency and severity of anxiety symptoms over the past week. Each item is rated on a 4-point Likert scale from 0 (“not at all”) to 3 (“severely”). The total score ranges from 0 to 63, with higher scores indicating greater anxiety severity. The BAI demonstrates strong internal consistency across diverse populations, with a Cronbach’s α typically exceeding 0.90 [17].

Secondary outcome measures included psychological evaluations and biochemical tests. The psychological evaluations comprised the following: (1) Automatic Thoughts Questionnaire (ATQ) [18]: A 30-item scale measuring the frequency of negative automatic thoughts on a 5-point Likert scale (total score range: 30-150); and (2) Dysfunctional Attitude Scale (DAS) [19]: A 40-item scale assessing maladaptive cognitive tendencies on a 7-point Likert scale (total score range: 40-280). Biochemical analyses included serum 25(OH)D, inflammatory markers [interleukin-6 (IL-6) and high-sensitivity C-reactive protein (hs-CRP)], oxidative stress markers [GSH, malondialdehyde (MDA), and superoxide dismutase (SOD)], and neurochemical markers [brain-derived neurotrophic factor (BDNF), dopamine (DA), serotonin (5-HT), and norepinephrine (NE)]. Fasting venous blood samples (5 mL) were collected at baseline and the 8-week follow-up using standard venipuncture techniques as part of routine clinical care. After clotting at room temperature, samples were centrifuged at 3000 ×g for 10 minutes at 4°C to separate serum. The serum was aliquoted and kept at -80°C until analysis. Serum biomarkers were quantified using the following methods: chemiluminescence immunoassay for 25(OH)D; immunoturbidimetry for hs-CRP; enzyme-linked immunosorbent assay for IL-6 and BDNF; spectrophotometry for GSH, MDA, and SOD; and high-performance liquid chromatography with electrochemical detec-

tion for DA, 5-HT, and NE. All assays were performed by a certified central laboratory.

All reported adverse events (AEs) were retrospectively extracted and reviewed from the patients' medical records. The severity of AEs was categorized as mild (easily tolerated), moderate (interfering with function but not requiring intervention), or severe. Investigators assessed a potential association with supplementation based on clinical documentation and categorized it as unrelated, possibly related, or probably related. Adherence to the supplementation regimen in the observation group was assessed retrospectively using the Grymonpre method [20]. This method was applied to pharmacy dispensing records and documented pill counts from outpatient clinical notes during the 8-week period. Adherence was calculated as: (Number of pills dispensed - Number of pills returned)/(Number of pills prescribed) × 100%.

The psychological scale data analyzed in this study were obtained from the hospital's clinical records. These routine clinical assessments had been administered using standardized, validated Chinese versions of the BAI, ATQ, and DAS by licensed clinical psychologists in a uniform setting with consistent instructions, as documented by the clinical notes. As these evaluations were part of standard care, the assessors were inherently blinded to any future research grouping, thereby mitigating assessment bias for this retrospective analysis.

Statistical analysis

GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA) was used to construct the charts, and SPSS 27.0 (IBM Corporation, Armonk, NY) was used for all statistical analyses. Continuous variables were presented as mean ± standard deviation or median (interquartile range) based on their distribution, which was assessed using the Shapiro-Wilk test. Categorical variables were expressed as frequencies (percentages). For between-group comparisons of continuous variables, independent samples t-tests were used for normally distributed data, and Mann-Whitney U tests for non-normally distributed data. For within-group changes from baseline to week 8, paired samples t-tests were used for normally distributed data, and Wilcoxon signed-rank tests for non-normally distributed data. Categorical variables were compared using the chi-square (χ^2) test or Fisher's exact test, as appropriate. To account for multiple comparisons across the pre-specified secondary outcomes, the False Discovery Rate correction was applied to all corresponding *P*-values, and the corrected values were reported. A two-sided *P*-value of less than 0.05 was deemed significant.

Results

Baseline characteristics of the matched cohort

After PSM based on age, sex, baseline BAI score, first-episode status, and sleep disturbance, the final matched cohort comprised 88 patients with GAD (44 matched pairs). Excellent balance was achieved between the observation and control groups for all five matched covariates, with standardized mean differences ranging from 0.000 to 0.074 (all below the 0.1 threshold). There were no significant differences in these or other baseline characteristics between groups (all $P > 0.05$; **Table 1**).

Integrated results of psychological outcomes

BAI, ATQ, and DAS scores decreased significantly from baseline after the 8-week period in the total cohort (all $P < 0.05$; **Figure 2**). At the endpoint, these scores were significantly lower in the observation group than in the control group (all $P < 0.05$; **Figure 2**).

Serum 25(OH)D and inflammatory markers

Serum 25(OH)D concentrations increased significantly from baseline after the 8-week period, while hs-CRP and IL-6 levels decreased substantially (all $P < 0.05$; **Figure 3**). At the endpoint, the observation group exhibited significantly higher serum 25(OH)D levels and lower hs-CRP and IL-6 concentrations compared to the control group (all $P < 0.05$; **Figure 3**).

Oxidative stress biomarkers

Following the 8-week observation period, GSH and SOD levels increased significantly, while MDA levels decreased substantially from baseline (all $P < 0.05$; **Figure 4**). At the end of follow-up, compared to the control group, the observation group exhibited significantly lower MDA levels along with notably higher GSH and SOD levels (all $P < 0.05$; **Figure 4**).

Table 1. Baseline demographic and clinical characteristics of participants

Variable	Observation group (n = 44)	Control Group (n = 44)	t/χ^2	*P*
Age (years, $\bar{x} \pm s$)	36.21 ± 9.44	35.92 ± 8.79	0.149	0.882
Sex (n, %)			0.000	1.000
Male	18 (40.91)	18 (40.91)		
Female	26 (59.09)	26 (59.09)		
Baseline BAI total score, ($\bar{x} \pm s$)	48.45 ± 3.34	48.21 ± 3.18	0.345	0.731
Duration of GAD (months, $\bar{x} \pm s$)	17.43 ± 6.6	17.85 ± 7.44	0.275	0.784
Education (years, $\bar{x} \pm s$)	10.81 ± 2.26	11.12 ± 2.09	0.668	0.506
Marital status (n, %)			0.183	0.669
Married	23 (52.27)	25 (56.82)		
Unmarried	21 (47.73)	19 (43.18)		
First-episode GAD (n, %)			0.000	1.000
Yes	31 (70.45)	31 (70.45)		
No	13 (29.55)	13 (29.55)		
Sleep disturbance (n, %)			0.000	1.000
Yes	23 (52.27)	23 (52.27)		
No	21 (47.73)	21 (47.73)		

Note: BAI, Beck Anxiety Inventory; GAD, generalized anxiety disorder.

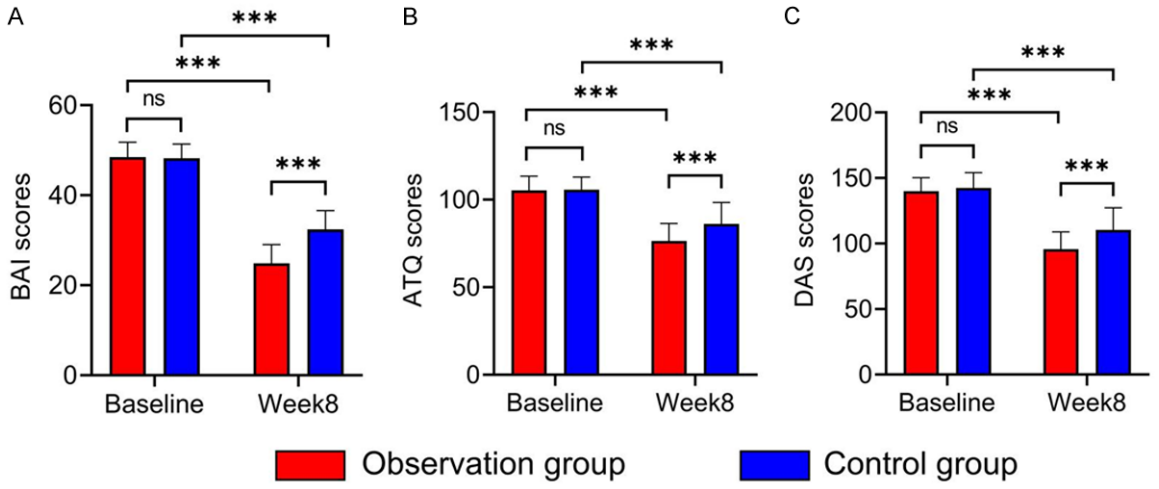

Figure 2. Changes in psychological outcomes from baseline to week 8 between the observation and control groups. A. BAI; B. ATQ; C. DAS. BAI = Beck Anxiety Inventory; ATQ = Automatic Thoughts Questionnaire; DAS = Dysfunctional Attitude Scale; ***$P < 0.001$, False Discovery Rate-corrected; ns = not significant.

Serum neurotransmitter biomarkers

Following the 8-week observation period, serum levels of BDNF, DA, 5-HT, and NE increased significantly from baseline (all $P < 0.05$; **Figure 5**). Moreover, at the end of follow-up, concentrations of all four biomarkers were substantially higher in the observation group compared to the control group (all $P < 0.05$; **Figure 5**).

AEs and adherence

The overall incidence of AEs did not differ significantly between the two groups ($P > 0.05$, **Table 2**). Treatment adherence, calculat-

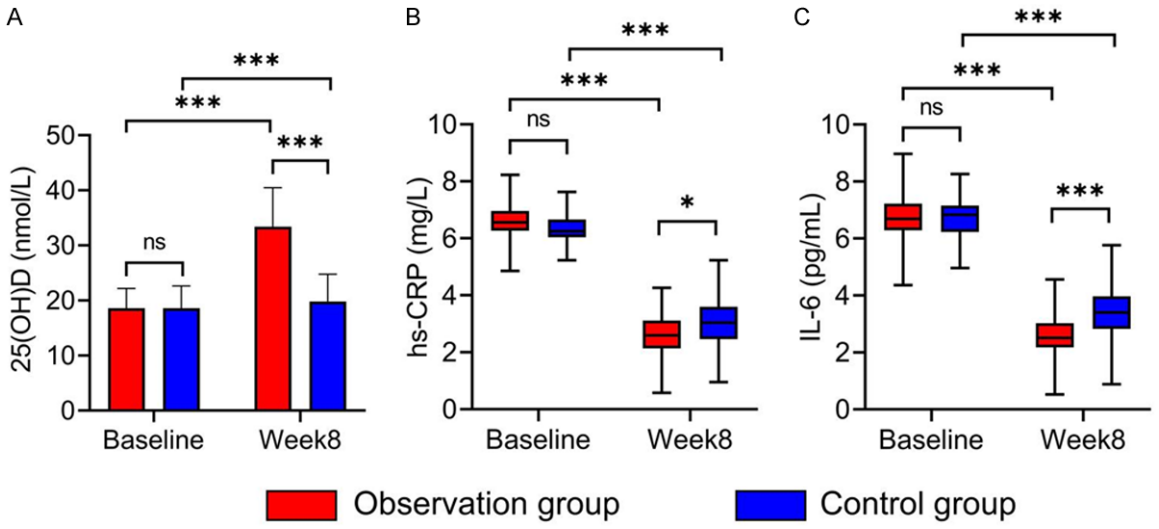

Figure 3. Changes in serum 25(OH)D and inflammatory markers levels before and after the 8-week period. A. 25(OH)D; B. hs-CRP; C. IL-6. [25(OH)D = 25-hydroxyvitamin D; hs-CRP = high-sensitivity C-reactive protein; IL-6 = interleukin-6; *P < 0.05, ***P < 0.001, False Discovery Rate-corrected; ns = not significant].

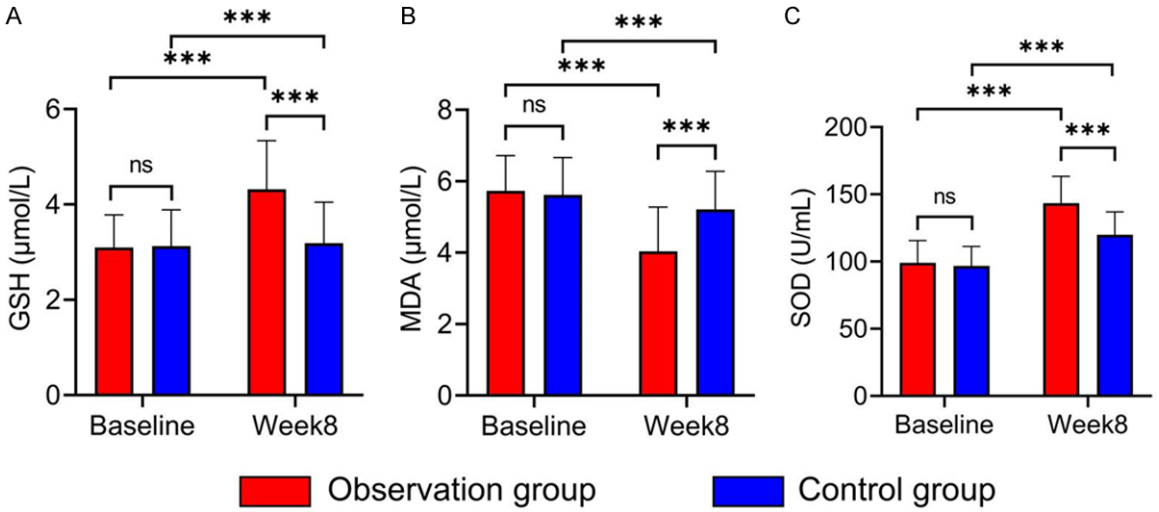

Figure 4. Changes in oxidative stress biomarkers before and after the 8-week period. A. GSH; B. MDA; C. SOD. GSH = glutathione; MDA = malondialdehyde; SOD = superoxide dismutase; ***P < 0.001, False Discovery Rate-corrected; ns = not significant.

ed using the Grymonpre method, was high (> 90%) and comparable between groups.

Discussion

Currently, pharmacological treatments for GAD are often limited by suboptimal efficacy and undesirable side effects, prompting increased research into antioxidant nutraceuticals such as vitamin D and NAC for their potential anxiolytic characteristics [21]. In this retrospective cohort study, we investigated the association of combined vitamin D and NAC supplementation with clinical outcomes in GAD patients receiving usual care. The observation group demonstrated significantly greater reductions in anxiety symptoms compared to the control group, indicating an association between this adjunctive antioxidant regimen and enhanced clinical improvement.

Although both NAC and vitamin D are recognized to have neuroregulatory properties, the effect of their combined supplementation on

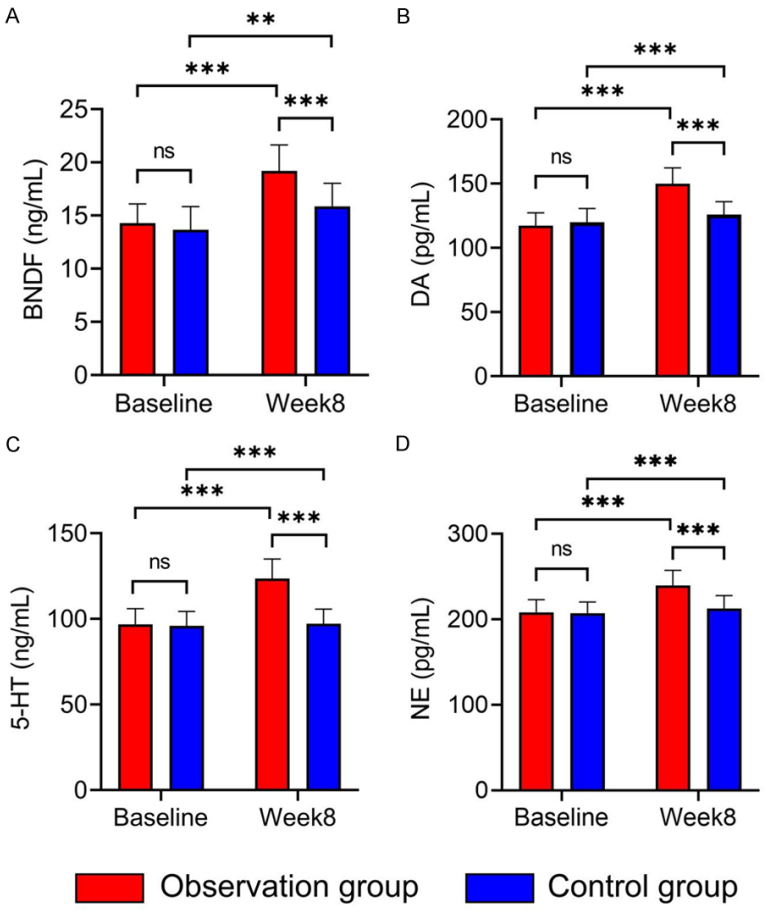

Figure 5. Changes in serum neurotransmitter biomarkers before and after the 8-week period. A. BDNF; B. DA; C. 5-HT; D. NE. BDNF = brain-derived neurotrophic factor; DA = dopamine; 5-HT = 5-hydroxytryptamine; NE = norepinephrine; $^{**}P < 0.01$, $^{***}P < 0.001$, False Discovery Rate-corrected; ns = not significant.

Table 2. Frequency of adverse events in observation and control groups [n (%)]

Adverse event	Observation group (n = 44)	Control Group (n = 44)	χ^2	*P*
Constipation	3 (6.82)	0 (0.00)		
Dry mouth	2 (4.55)	0 (0.00)		
Reduced appetite	2 (4.55)	0 (0.00)		
Insomnia	0 (0.00)	2 (4.55)		
Drowsiness	0 (0.00)	2 (4.55)		
Tremors	0 (0.00)	1 (2.27)		
Total	7 (15.91)	5 (11.36)	0.386	0.534

cognitive-emotional function remained unclear. In this analysis, the ATQ score at the 8-week endpoint was significantly lower in the observation group than in the control group ($P < 0.05$). This result is in line with previous research suggesting that these supplements could improve cognitive patterns [22]. Vitamin D's activities on neurotrophic factors and neurotransmitter pathways are the major mechanisms behind its neuroprotective qualities [5], whereas NAC is reported to enhance antioxidant defensed and modulate glutamatergic signaling [6]. Their complementary mechanisms - vitamin D in immunomodulation and neurotrophic support, and NAC in providing antioxidant precursors and regulating glutamate - suggest that their combination may have wider effects than either agent alone. Our findings indicate that combined supplementation was associated with reduced oxidative stress, enhanced cognitive flexibility, and diminished negative thought patterns related to failure and perfectionism [23, 24]. These results suggest that the combination of vitamin D and NAC may have promising therapeutic potential for ameliorating anxiety-related cognitive impairment and emotional symptoms. However, as this was not a factorial trial, we cannot determine whether the observed associations reflect a synergistic or merely additive interaction. Future studies, particularly those employing factorial designs, are needed to elucidate the precise nature of their interaction and confirm its therapeutic efficacy in GAD.

Serum 25(OH)D, hs-CRP, and IL-6 levels have been found to be important biomarkers for GAD, revealing its pathogenic mechanisms from the interconnected perspectives of neuroimmunology and inflammation. Consistent with prior reports linking low serum 25(OH)D to neuroimmune dys-

regulation and elevated hs-CRP and IL-6 to chronic inflammation and anxiety [2, 4, 11], the observation group in our analysis showed significantly higher 25(OH)D levels and lower hs-CRP and IL-6 levels compared to the control group (all $P < 0.05$). These findings align with the established roles of vitamin D and NAC: vitamin D is known to exert immunomodulatory and neurotrophic effects, and its deficiency has been strongly associated with anxiety symptoms, while NAC alleviates anxiety by mitigating oxidative damage and modulating glutamatergic transmission [25-29]. Mechanistically, vitamin D primarily reduces neuroinflammation, enhances neurotrophic support, and regulates 5-HT metabolism, whereas NAC mainly elevates GSH to mitigate oxidative stress in the nervous system. Our results thus support targeting these complementary immune-inflammatory and neurochemical pathways for novel therapeutic strategies in GAD. Future clinical studies should investigate the interplay between vitamin D and NAC in regulating mood and stress responses.

Oxidative stress, defined as an imbalance between the body's production of reactive oxygen species and antioxidant defenses, is a crucial component in the pathogenesis of GAD [4, 28]. In comparison to the control group, patients who were supplemented with the combination of vitamin D and NAC in this cohort had significantly lower MDA levels and significantly higher GSH and SOD levels (all $P < 0.05$). The different mechanisms of each supplement may be responsible for this improvement in redox balance markers: vitamin D is known to promote the production of antioxidant enzymes (like SOD) through the Nrf2 pathway [5, 27], while NAC provides the required precursor cysteine to facilitate the synthesis of GSH [25]. As a result, the combined regimen addresses oxidative stress in a number of ways - vitamin D by inducing antioxidant and anti-inflammatory enzymes, while NAC by directly bolstering GSH formation [12, 29]. Collectively, this broader restoration of redox balance may help to explain the observed improvements in cognitive function and reduction in anxiety symptoms associated with the combination.

It is advisable to interpret the observed changes in serum levels of BDNF, DA, 5-HT, and NE after supplementation as exploratory systemic biomarkers or peripheral correlates linked to clinical improvement in GAD. We specifically recognize a key limitation: circulating neurotransmitter levels do not accurately reflect central nervous system activity because they are altered by peripheral sources and do not consistently pass the blood-brain barrier [30]. As a result, our results should not be interpreted as concrete proof of changed central neurotransmission. However, studies indicate that these ancillary measurements may be useful as systemic correlates of mental states. As thoroughly examined, research on peripheral biomarkers (such as monoamines and BDNF) in anxiety disorders is still ongoing, aiming to identify accessible biological fingerprints and comprehending systemic pathogenesis [30]. For example, patients with GAD have been shown to have considerably lower serum BDNF levels than healthy controls, which supports its usefulness as a peripheral correlate of the condition [31]. Thus, the observed increase in BDNF in our cohort suggests a positive modulation within this systemic framework. Similarly, the established theoretical framework linking these pathways to anxiety can be used to interpret alterations in peripheral monoamines [32, 33]; their changes might reflect concurrent changes in peripheral biochemistry linked to symptom alleviation. In conclusion, our research contributes to the expanding literature on GAD's peripheral biological correlates [30]. These findings generate hypotheses regarding the systemic biochemical effects of the supplementation, which may be tangentially linked to central processes. Future research incorporating neuroimaging is crucial to establish clearer connections to brain function.

This study found that the combined vitamin D and NAC supplementation regimen showed good safety, with only minor, self-limiting adverse effects (such as gastrointestinal problems) recorded. None of these events required medical intervention, which is consistent with earlier reports [29]. Similarly, the dosage of vitamin D used in this study is well-established and associated with few adverse effects, most of which are non-specific (such as fatigue and dry mouth) [34]. No serious or long-lasting adverse effects were observed. These findings suggest that combined vitamin D and NAC supplementation is safe and feasible for clinical use. Both the observation and control groups

had high adherence to their respective regimens (> 90%), with no significant difference between them ($P > 0.05$). This high adherence rate is consistent with outcomes reported by many short-term clinical studies [35].

This retrospective cohort research has several significant strengths. The use of PSM strengthened the validity of the observed associations by improving comparability between the observation and control groups. Additionally, this study provides a multifaceted assessment that connects clinical symptom alleviation with likely biological processes by integrating a panel of serum biomarkers with established psychological measures, thereby enhancing the scientific value of the findings and informing future research.

However, this study has certain limitations. First, as a retrospective observational study, despite using PSM, residual confounding from unmeasured factors cannot be ruled out. Second, the small sample size may have limited the generalizability of the findings. Third, the 8-week observation period was insufficient to assess long-term outcomes. Fourth, the reliance on peripheral biomarkers, rather than direct measures of central nervous system activity (e.g., by neuroimaging), precludes definitive mechanistic conclusions. Finally, the requirement for stable psychotropic medication may restrict applicability to other patient populations. Future studies with larger samples, longer follow-up, and neuroimaging are warranted to verify and extend these findings.

Conclusion

In this retrospective cohort, combined vitamin D and NAC supplementation was associated with improvements in both emotional/cognitive assessments and relevant biomarker profiles among patients with GAD. Our results suggest that this supplementation regimen is associated with not only a reduction in clinical symptoms but also modulation of neurochemical pathways and cognitive processing patterns related to anxiety. These findings highlight the potential value of addressing oxidative stress and monoamine pathways within a comprehensive therapeutic strategy for anxiety disorders. Future research should therefore examine the underlying mechanisms and long-term effectiveness of this combined regimen in larger and more diverse patient populations.

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Disclosure of conflict of interest

None.

Address correspondence to: Yanhui Liang, Department of Psychiatry and Psychology (Ward 6), Beidahuang Group Neuropsychiatric Hospital (The Second Hospital of Beidahuang Group), No. 472 Anqing Road, Qianjin District, Jiamusi 154001, Heilongjiang, China. E-mail: 15946556042@163.com

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