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Original Article

Changed signals of blood adenosine and cytokines are associated with parameters of sleep and/or cognition in the patients with chronic insomnia disorder

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ABSTRACT

Objectives: This study aimed to investigate whether plasma levels of adenosine, adenosine deaminase (ADA), and certain cytokines change in patients with chronic insomnia disorder (CID), and if so, whether these alterations are associated with poor sleep quality and cognitive dysfunction.
Methods: Fifty-five CID patients were selected for the study, along with fifty-five healthy controls (HC) matched to the patients according to their basic data. All subjects completed sleep, emotion, and cognition assessments, with some CID patients also completing an overnight polysomnography. The plasma level of adenosine was measured using liquid chromatography–tandem mass spectrometry, while ADA level was quantified using a quantitative sandwich enzyme-linked immunosorbent assay. Levels of cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ, were measured using Luminex liquid chip technology.
Results: CID patients had a lower adenosine level, and higher levels of ADA and some of the cytokines (IL-1β, IL-2, IL-6, IL-10 and TNF-α) compared with controls. In the CID group, plasma concentrations of adenosine were negatively correlated with Pittsburgh Sleep Quality Index scores, while concentrations of IL-1β, IL-6 and TNF-α were positively correlated with these scores. Concentrations of IL-1β and TNF-α were negatively correlated with scores on the Chinese-Beijing Version of the Montreal Cognitive Assessment. Moreover, levels of IL-1β, TNF-α, IL-6, and IL-2 were positively correlated with memory test errors by CID patients after controlling for confounding factors.
Conclusions: The reduced adenosine and elevated cytokine levels of CID patients were associated with the severity of insomnia and/or cognitive dysfunction.

1. Introduction

Insomnia is a sleep disorder characterized by dissatisfaction with sleep time and quality, accompanied by dysfunction during the daytime, despite adequate opportunities and conditions for sleep [1]. Long-term insomnia may lead to a series of internal diseases, including stroke, hypertension, coronary heart disease, diabetes [2–4], and neuroendocrine and immune dysfunction [5,6]. Unfortunately, the mechanisms underlying insomnia are still unclear, leading to difficulties in treating this condition. Moreover, insomniacs commonly complain of cognitive dysfunction, with a meta-analysis revealing that insomnia is associated with poorer cognitive performance in multiple specific cognitive domains [7]. In particular, insomnia is associated with poorer retention/capacity and manipulation of working memory, problem solving, and episodic memory, suggesting that it is a potential risk factor for dementia [8,9]. However, the mechanisms by which insomnia causes cognitive impairment are unclear. To date, studies on the topic have mainly focused on cerebral structural atrophy, abnormal functional connectivity, inflammatory stimulation, and other

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aspects [10]. Therefore, it is important to explore the pathogenesis of insomnia and cognitive impairments related to it.

Adenosine is an important neuromodulator in the central nervous system (CNS) [11], which is well known to promote sleep and regulate the human immune response [12,13]. For example, adenosine has been shown to promote sleep by derepressing GABAergic neurons located in the ventrolateral preoptic area, whose excitation can trigger sleep [14,15]. Numerous studies to date have focused on adenosine signal changes in brain areas regulating sleep in animals [16–18]. However, only a few have focused on the correlation between the metabolism of adenosine in the blood and sleep stages in humans [19], and none have yet reported whether blood adenosine level changes in patients with CID.

ADA, an adenosine hydrolase, plays a significant role in the adenosine signaling system [20]. Animal study has shown that both genetic and pharmacological manipulation can change the level of ADA and affect sleep status [21]. In genetic research at the population level, differences in sleep homeostasis have been linked to polymorphism of the ADA gene. For instance, individuals carrying the G/A allele of the ADA gene show greater slow wave power and higher electroencephalogram power during NREM than G/G allele carriers, with a greater number of the former feeling sleepy [22–24]. However, at present, data regarding the change in ADA level in patients with insomnia are scarce.

In addition to being a sleep-promoting neuromodulator, adenosine is also an important immune regulator, exerting immune-related effects on the CNS and via the peripheral circulation. Specifically, adenosine plays a crucial role in regulating immunosuppression [25], for example by inhibiting IL-1, TNF-α, and other cytokines [26]. In addition, in the CNS, adenosine and inflammatory factors are thought to interact and are strongly involved in the process of sleep-wake regulation [27]. In light of these findings, we aimed to explore the relationship between adenosine signaling in the peripheral blood and inflammatory factors in patients with CID.

It has been reported that injection of IL-1 into the lateral ventricle and cortex can increase slow wave sleep [28], while acute enhancement or inhibition of endogenous brain TNF-α respectively promotes or inhibits sleep [29]. In terms of its pathology, insomnia can affect cytokine secretion, such as by enhancing the production of IL-6 and TNF-α [30,31]. In addition, studies have shown that IL-2 can enhance NREM sleep, while IL-4 and IL-10 have the opposite effect [32–34]. However thus far, levels of IL-4 and IL-10 have not been studied in patients with CID.

In this study, we explored whether blood levels of adenosine signals (adenosine and ADA) and cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ) change in patients with CID. Furthermore, we examined correlations of parameters of sleep quality and cognition with blood indicators, and adenosine signals with immune measures.

2. Material and methods

2.1. Subjects

Fifty-five patients with CID were recruited from the Clinic of Sleep Disorders at Chaohu Hospital, Anhui Medical University. CID diagnostic criteria followed the International Classification of Sleep Disorders, 3rd Edition (ICSD-3) [35]. Patients also had to meet the following conditions: (1) aged between 18 and 65 years old; (2) received education for more than 6 years; (3) possessing normal comprehension, reading and motor function, and able to complete the assessment scales and the memory test. Furthermore, patients with physical diseases, including those affecting their heart, lung, liver, kidney, or gastrointestinal system, or with neurological diseases, autoimmune diseases, pathogenic microbial infections, tumors, or any other psychiatric disorders were excluded [36]. In addition, patients who had taken sedatives, antidepressants, antipsychotics, or any other drugs within four weeks, as well as pregnant and menstrual women, were not eligible for the study.

Based on similar background information to that of the experimental group, Fifty-five HC subjects were selected from the Health Examination Center of our hospital. None of these subjects had any of the aforementioned physical diseases, nor did they suffer from other conditions such as insomnia, depression, anxiety, or schizophrenia. They all had Pittsburgh Sleep Quality Index (PSQI) scores < 7 [37] and scores ≥ 26 on the Chinese-Beijing Version of Montreal Cognitive Assessment (MoCA-C) [38], indicating good sleep quality and normal cognition. The study was discussed and approved by the Clinical Trial Ethics Committee at Chaohu Hospital, Anhui Medical University (approval number: 201805-kyxm-01). All subjects signed a written informed consent form.

2.2. Baseline data collection

2.2.1. Background information

Background information on the subjects, including their age, gender, educational information, history of illness, individual history, family history, daily life habits, and so on, were collected.

2.2.2. Sleep assessment

2.2.2.1. Subjective sleep parameters. Subjective sleep quality was evaluated using the PSQI, a sleep evaluation scale that is widely used to assess sleep quality during the past month in patients with sleep disorders or mental disorders, and in the general population [39]. The scale includes 18 self-assessment questions, while the remaining five questions are answered by the bedmate of the subject and are not counted in the total score. The scale is scored up to a maximum of 21 points, with a higher score indicating a worse quality of sleep. In China, a PSQI score higher than seven is considered to indicate clinical insomnia [40].

2.2.2.2. Objective sleep parameters. A polysomnogram (PSG) was recorded to collect objective sleep parameters, including total sleep time (TST), sleep efficiency (SE), sleep onset latency (SOL), time of wake after sleep onset (WASO). In addition, measures of sleep structure consisted of the percentages of time spent in non-rapid eye movement sleep stages 1–3 (N1%, N2%, and N3%) and the percentage of time spent in REM sleep. The PSG was obtained using a Condi Grael v2 (Australia) sleep monitor ($n = 29$). Variables monitored included electroencephalogram, electrocardiogram (lead II), electrooculogram, electromyogram, respiratory airflow, and chest and abdomen movement.

2.2.3. Assessment of depression

The severity of depressive symptoms was evaluated using the 17-item Hamilton Depression Rating Scale (HAMD-17) [41], with each item either rated using five grades (0–4) or three grades (0–2). The HAMD-17 is composed of five factors: (1) anxiety and somatization, consisting of psychic anxiety, somatic anxiety, gastrointestinal symptoms, hypochondriasis, insight, and general symptoms; (2) weight loss; (3) cognitive disorder, consisting of feelings of guilt, suicide, and agitation; (4) symptoms of retardation, consisting of depressive mood, loss of interest in work and other activities, retardation, and sexual symptoms; (5) sleep disorders, consisting of difficulties in falling asleep, poor sleep quality, and early awakening. Total scores range from 0 to 52, with higher scores indicating more severe depression. Specifically, >24 points indicates severe depression, >17 points indicates moderate depression, >7 points indicates mild depression, and <7 is considered to indicate a lack of depressive symptoms [42].

2.2.4. Cognitive assessment

2.2.4.1. General cognition. The MoCA scale is widely used to assess mildly impaired cognitive function. The MoCA-C scale is widely used in China and has been shown to have high reliability and validity for Chinese subjects [43]. It assesses eight aspects of cognition: attention, visual-spatial and executive function (VSEF), naming, language, abstraction, short-term memory, delayed memory, and orientation [44]. In Chinese subjects, a total score ≥26 is considered to indicate normal cognitive function [38]. It is worth noting that one point is added to the score of patients with <12 years of education to balance the cognitive bias caused by this lower level of education.

2.2.4.2. Multi-dimensional memory. The nine-box maze task has been improved to simultaneously measure multidimensional aspects of memory function, namely spatial reference/working memory (SRM/SWM), object reference/working memory (ORM/OWM), and object recognition memory (ORcM) [45,46]. As required, the experiment was carried out in a spacious, bright room with visible cues on one wall. A round table was placed in the center of the room and nine identical commercial cups made from plain, opaque pasteboard were placed equidistantly along the edge of the table. Ten common objects, such as an eraser, key, coin, and button, were used in the experiment. Moreover, five photographs containing ten objects (five of the ten common objects along with another five corresponding similar objects) were prepared for the subjects to observe, with the objects arranged in different sequences in each photograph. During the training phase (not included in the statistical analysis), the examiner introduced the experiment to each subject and instructed them to name the ten objects until they could correctly name them all. Then, the examiner placed two of the objects in two cups and asked the subject to remember the objects and their corresponding locations. The subject was asked to close their eyes and guided around the table twice clockwise and twice counterclockwise. Then, the previously prepared photograph containing the ten objects was displayed, and the subject was required to recognize the two objects and the corresponding cups on the table. If their answer was correct, the test proceeded to the next phase, the testing phase. In this phase, two objects were put into two cups respectively and the subject was asked to remember the two objects and their positions (to test ORM and SRM), with neither the objects or positions changed during this phase of the experiment. Then two different objects were placed randomly into two of the other cups. The subject was informed that in each trial, the two objects would be placed in different cups and in different positions (to test OWM and SWM). In total, each subject completed four trials, during which the experimenter made sure to instruct them to change direction and kept them safe. Finally, after the subject had completed all four trials, they were shown the photographs and asked to identify the objects presented during the testing phase (to test ORcM). The number of errors made in each memory test were recorded as the performance score for each subject.

2.3. Blood sample collection and storage

Two milliliters of blood were collected from each subject between 9 a.m. and 10 a.m. while they were sitting quietly after 30 min of rest. Plasma was centrifuged (3000 r/min at room temperature for 5 min) and quickly placed in a −80 °C refrigerator for subsequent analyses.

2.4. Measurement of adenosine, ADA, and cytokine levels

Adenosine level was measured using liquid chromatography–tandem mass spectrometry. The standard used was adenosine with a Chemical Abstracts Service (CAS) number of 58-61-7 and a molecular formula of $C_{10}H_{13}N_5O_4$ (Sigma, USA). The level of ADA was quantified using a quantitative sandwich enzyme-linked immunosorbent assay, according to the manufacturer's instructions (CK-E10995H and E20180201A; YuanYe Biotechnology Corporation, Shanghai, China). Levels of cytokines were measured using Luminex liquid chip technology (EPX180-12165-901; Thermo-Fisher).

2.5. Statistical analysis

Normally distributed variables were represented by the mean ± standard deviation (SD). For non-parametric data, variables were expressed as the 25th, 50th, and 75th percentiles [P50 (P25, P75)]. The chi-square test was used to analyze differences between groups of categorical variables. Student's *t* test was used for inter-group comparison of normally distributed data, while the Mann–Whitney *U* test was used to analyze non-normally distributed variables. Partial correlation analysis was employed to test correlations of the related blood indicators with the severity of insomnia and with the number of errors in the nine-box maze task. Multiple linear regression was used to explore the correlation between adenosine and cytokine levels and to identify the contribution of each cytokine to changes in the level of adenosine. To examine relationships among measures in the CID group, multivariate data reduction was performed using principal component analysis. The threshold for statistical significance was set at a two-tailed *P*-value of 0.05. All data obtained were processed using SPSS 16.0 statistical software.

3. Results

3.1. Baseline data and depressive mood

There were no significant differences between the CID and HC groups in terms of gender, age, or educational level (*P*s > 0.05; Table 1). Compared with the HC group, the CID group had significantly higher HAMD-17 scores [7.0 (6.0, 9.0)], which indicated mild depression in the CID group, which was expected in this study.

3.2. Sleep parameters

Subjective and objective sleep parameters are detailed in Table 1. Compared with the HC group, the CID group had significantly higher PSQI scores (13.9 ± 2.2), indicating moderate to severe insomnia. According to the PSG parameters obtained from the patients with CID, they had a shorter TST, longer SOL, decreased SE, and increased WASO and N1% compared to the norm. Overall, these results indicate that the patients selected for this study were insomniacs with objectively short sleep durations.

3.3. Cognitive functions

Compared with the controls, the patients with CID had significantly lower total MoCA-C scores ($P < 0.001$), and lower factor scores in several domains, including attention, VSEF, and delayed memory (*P*s < 0.05; Table 1). They also made significantly more errors in the SWM, OWM, and ORcM tests compared with the HC group (*P*s < 0.05; Table 2).

Table 1
General data, MoCA-C performance and sleep quality.

Terms	Variables	Insomniacs (n = 55)	Controls (n = 55)	Statistic	*p*-Values
General data	Male/Female	18/37	18/37	0.000	1.000
	Age (yr)	46.0 ± 12.5	46.2 ± 10.9	−0.057	0.955
	Education (yr)	9.0 (6.0, 12.0)	9.0 (6.0, 12.0)	−0.018	0.985
	HAMD-17 (score)	7.0 (6.0, 9.0)	3.0 (3.0, 4.0)	−7.581	<0.001
	Disease duration (yr)	3.0 (1.0, 9.0)			
Montreal Cognitive Assessment	Total score	24.7 ± 2.4	26.6 ± 0.8	−5.233	<0.001
	Factor scores				
	Visuo-spatial and executive functions	3.0 (3.0, 4.0)	4.0 (3.0, 4.0)	−3.131	0.002
	Naming	3.0 (2.0, 3.0)	3.0 (2.0, 3.0)	−0.930	0.352
	Attention	3.0 (2.0, 3.0)	3.0 (3.0, 3.0)	−2.847	0.004
	Calculation	3.0 (3.0, 3.0)	3.0 (3.0, 3.0)	−1.470	0.142
	Language	3.0 (2.0, 3.0)	3.0 (3.0, 3.0)	−1.861	0.063
	Abstraction	2.0 (1.0, 2.0)	2.0 (1.0, 2.0)	−0.458	0.647
	Delayed memory	2.0 (1.0, 3.0)	3.0 (3.0, 4.0)	−3.872	<0.001
	Orientation	6.0 (6.0, 6.0)	6.0 (6.0, 6.0)	−1.000	0.317
Sleep	Pittsburgh Sleep Quality Index (score)	13.9 ± 2.2	3.8 ± 0.8	31.355	<0.001
	Polysomnogram (N = 29)				
	Total sleep time (min)	357.6 ± 63.6			
	Sleep onset latency (min)	32.5 (16.5,54.8)			
	Sleep efficiency (%)	72.8 ± 12.1			
	Time in wake after sleep onset (min)	129.0 ± 63.0			
	NREM sleep stage 1 (%)	15.4 ± 10.9			
	NREM sleep stage 2 (%)	58.3 ± 15.0			
	NREM sleep stage 3 (%)	11.5 (2.1,21.1)			
	REM sleep (%)	11.4 ± 6.5			
	Apnea Hyponea Index (/hr)	1.1 ± 0.4			
	Periodic Limb Movement (/hr)	0.0 (0.0, 1.0)			

Abbreviations: HAMD-17: Hamilton Depression Rating Scale 17 items; NREM, non-rapid eye movement sleep; REM, rapid eye movement.
Expressions: the normally distributed variables were represented by the mean ± standard deviation. The non-normally distributed variables were expressed as the 25th, 50th and 75th percentiles.

Table 2
The nine-box maze test performance.

Terms	Insomniacs (n = 55)	Controls (n = 55)	Statistic	*p*-Values
Object reference	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	−1.034	0.301
Spatial reference	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	−1.762	0.078
Object working	0.0 (0.0, 3.0)	0.0 (0.0, 1.0)	−4.027	<0.001
Spatial working	1.0 (1.0, 2.0)	0.0 (0.0, 1.0)	−6.412	<0.001
Object recognition	3.0 (2.0, 4.0)	0.0 (0.0, 0.0)	−3.115	0.002

Expressions: the non-normally distributed variables were expressed as the 25th, 50th and 75th percentiles.

3.4. Levels of adenosine, ADA, and cytokines

Patients with CID had lower levels of adenosine ($P = 0.001$), and higher levels of ADA ($P = 0.31$) than the control subjects. In addition, they exhibited increased plasma levels of five cytokines (IL-1β, IL-2, IL-6, IL-10, and TNF-α; Ps < 0.05; Table 3), but there were no significant differences between groups in the concentrations of the other cytokines measured (IL-4, IL-12, and IFN-γ).

3.5. Comparison of indicators grouped by objective sleep duration

Compared to those with TST >360 min, the subgroup of patients with TST<360 min were younger ($P = 0.011$), and had a lower TST ($P < 0.001$), SE ($P < 0.001$), and adenosine level ($P = 0.016$), and a higher WASO ($P = 0.019$) and REM% ($P = 0.040$; Table 4). In order to remove the influence of age, we performed a covariance analysis and found that the adenosine level of the patients with TST <360 min was still significantly lower ($F = 5.678$, $P = 0.025$) than that of the patients with TST >360 min.

3.6. Partial correlation analysis of biomarker levels with sleep quality and cognition

The results of the partial correlation analysis (controlling for gender, age, education, and severity of depression) of plasma biomarker levels in the patients with subjective and objective sleep parameters are shown in Table 5. The concentration of adenosine correlated negatively with PSQI score ($r = -0.427$, $P = 0.002$), and positively with the TST ($r = 0.556$, $P = 0.004$) and SE ($r = 0.512$, $P = 0.009$) measured by the PSG; however, ADA level did not correlate with any of the sleep parameters. In terms of cytokine levels, significant correlations with sleep parameters were only observed for IL-1β, IL-6, and TNF-α. Specifically, plasma IL-1β and IL-6 levels correlated positively with PSQI score ($r = 0.485$, $P < 0.001$ and $r = 0.377$, $P = 0.006$, respectively), while IL-1β alone correlated negatively with N3% ($r = -0.429$, $P = 0.033$). Furthermore, TNF-α level correlated positively with PSQI score ($r = 0.280$, $P = 0.047$) and WASO ($r = 0.402$, $P = 0.047$), and negatively with TST ($r = -0.491$, $P = 0.013$), SE ($r = -0.439$, $P = 0.028$), and N1%

Table 3
The plasma levels of biomarkers.

Biomarkers	Insomniacs (n = 55)	Controls (n = 55)	Statistic	*p*-Values
Adenosine (μg/ml)	14.3 ± 4.1	17.5 ± 5.4	−3.547	0.001
ADA (U/ml)	47.0 ± 9.3	43.1 ± 9.2	2.188	0.031
IL-1β (pg/ml)	1.3 (1.0, 1.7)	1.0 (0.8, 1.4)	−4.120	<0.001
IL-2 (pg/ml)	3.0 ± 0.5	2.7 ± 0.4	2.939	0.004
IL-4 (pg/ml)	0.9 (0.5, 1.3)	0.7 (0.6, 1.1)	−0.575	0.565
IL-6 (pg/ml)	4.0 ± 1.0	3.5 ± 0.8	3.005	0.003
IL-10 (pg/ml)	0.8 ± 0.3	0.7 ± 0.3	2.127	0.036
IL-12 (pg/ml)	1.1 (0.9, 1.3)	1.0 (0.9, 1.4)	−0.359	0.720
TNF-α (pg/ml)	2.7 ± 0.7	2.4 ± 0.5	3.133	0.002
IFN-γ (pg/ml)	17.4 (12.1, 28.5)	13.9 (10.9, 25.5)	−0.356	0.722

Abbreviations: ADA, Adenosine deaminase; IL, Interleukin; TNF-α, Tumor necrosis factor Interferon-α; IFN-γ: Interferon-γ
Expressions: the normally distributed variables were represented by the mean ± standard deviation. The non-normally distributed variables were expressed as the 25th, 50th and 75th percentiles.

Table 4
Comparison of indicators grouped by objective sleep duration (list only items with statistically significant differences).

Terms	TST <360 min (n = 16)	TST > 360 min (n = 13)	Statistic	*p*-Values
Age (yr)	43.0 ± 11.8	53.2 ± 7.4	3.083	0.011
TST (min)	330.8 (278.1, 350.4)	411.5.7 ± 33.6	−4.561	<0.001
Sleep efficiency (%)	66.6 (63.3, 69.7)	81.2 ± 7.7	−3.508	<0.001
Time in wake after sleep onset (min)	153.0 ± 66.2	99.2 ± 45.2	0.912	0.019
REM sleep (%)	13.6 ± 7.1	8.7 ± 4.5	2.298	0.040
Adenosine (μg/ml)	14.3 ± 2.6	17.7 ± 4.5	4.763	0.016

Abbreviations: TST, total sleep time; REM, rapid eye movement.
Expressions: the normally distributed variables were represented by the mean ± standard deviation. The non-normally distributed variables were expressed as the 25th, 50th and 75th percentiles.

Table 5
The correlations between plasma biomarkers and sleep parameters.

Biomarkers	PSQI (score)	TST (min)	SOL (min)	SE (%)	WASO (min)	N1 (%)	N2 (%)	N3 (%)	REM sleep (%)
Adenosine (μg/ml)	−0.427[b]	0.556[b]	−0.221	0.512[b]	−0.381	0.151	0.188	−0.224	−0.321
ADA (U/ml)	0.124	0.068	0.217	0.138	−0.243	−0.102	−0.117	0.296	−0.125
IL-1β (pg/ml)	0.485[b]	−0.193	−0.083	−0.187	0.164	0.096	0.219	−0.429[a]	0.101
IL-2 (pg/ml)	−0.067	−0.295	−0.047	−0.267	0.146	0.162	−0.220	0.067	0.108
IL-4 (pg/ml)	−0.199	0.081	−0.160	0.175	−0.117	0.074	−0.133	0.087	−0.081
IL-6 (pg/ml)	0.377[b]	0.026	0.357	0.107	−0.093	0.168	0.112	−0.128	−0.338
IL-10 (pg/ml)	−0.196	0.067	−0.244	0.158	−0.146	−0.264	−0.019	0.179	−0.094
IL-12 (pg/ml)	−0.062	−0.121	−0.178	−0.050	0.010	−0.008	0.308	−0.210	−0.374
TNF-α (pg/ml)	0.280[a]	−0.491[a]	0.139	−0.439[a]	0.402[a]	−0.435[a]	0.189	0.098	0.128
IFN-γ (pg/ml)	−0.144	−0.060	0.020	0.003	−0.009	0.113	−0.056	0.036	−0.020

Abbreviations: ADA, Adenosine deaminase; IL, Interleukin; TNF-α, Tumor necrosis factor Interferon-α; IFN-γ: Interferon-γ; PSQI, Pittsburgh Sleep Quality Index; TST, total sleep time; SOL, sleep onset latency; SE, sleep efficiency; WASO, time of wake after sleep onset; N1%, N2% and N3%, percentage of the NREM sleep stage 1,2,3; REM, rapid eye movement.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level (2-tailed).

($r = -0.435$, $P = 0.030$). After Bonferroni correction, adenosine level correlated negatively with PSQI score ($r = -0.427$, $P = 0.002$), and positively with TST ($r = 0.556$, $P = 0.004$), and plasma IL-1β level correlated positively with PSQI score ($r = 0.485$, $P < 0.001$).

The results of the correlation analysis (controlling for gender, age, education, sleep quality, and severity of depression) between plasma biomarker concentrations and levels of cognitive performance in the insomniacs are shown in Table 6. With respect to MoCA-C scores, only a few of the biomarkers yielded significant correlations. Specifically, adenosine positively correlated with the attention score ($r = 0.348$, $P = 0.012$), while IL-1β correlated negatively with the MoCA-C total score ($r = -0.365$, $P = 0.009$) and the factor scores for VSEF ($r = -0.327$, $P = 0.019$) and delayed recall ($r = -0.307$, $P = 0.029$). The level of TNF-α correlated negatively with the MoCA-C total score ($r = -0.314$, $P = 0.025$) and delayed recall score ($r = -0.319$, $P = 0.022$). In terms of the results from the nine-box maze task, adenosine level correlated negatively with the number of errors in SRM ($r = -0.449$, $P = 0.001$) and SWM ($r = -0.335$, $P = 0.016$). There were positive correlations of IL-1β level with OWM errors ($r = 0.334$, $P = 0.017$), IL-6 level with SRM errors ($r = 0.307$, $P = 0.029$), and TNF-α level with SRM errors ($r = 0.391$, $P = 0.005$) and with SWM errors ($r = 0.386$, $P = 0.005$). Moreover, IL-2 correlated negatively with SWM errors ($r = -0.287$, $P = 0.041$). After Bonferroni correction, adenosine level was still significantly negatively correlated with the number of errors in SRM ($r = -0.449$, $P = 0.001$).

3.7. Multiple linear regression between adenosine and cytokine levels

For the regression analysis, the level of adenosine was defined as the dependent variable and those of all of the cytokines were defined as independent variables. The results showed that a statistically significant linear regression ($r = 0.547$, $P = 0.026$), in

Table 6
The correlations between plasma biomarkers and cognitive performance.

Biomarkers	Factor scores in MoCA-C							
	VSEF	Naming	Attention	Calculation	Language	Abstraction	DR	Orientation
Adenosine (μg/ml)	0.141	−0.211	0.348[a]	−0.041	0.137	−0.065	0.051	−0.008
ADA (U/ml)	0.218	0.132	−0.173	0.107	−0.134	0.244	−0.014	0.196
IL-1β (pg/ml)	−0.327[a]	−0.156	0.215	−0.113	-0.032	−0.093	−0.307[a]	−0.083
IL-2 (pg/ml)	0.026	0.216	0.216	−0.042	0.197	0.069	0.124	−0.052
IL-4 (pg/ml)	−0.118	−0.185	0.088	0.038	0.222	−0.041	−0.089	−0.194
IL-6 (pg/ml)	−0.068	−0.129	−0.061	0.016	−0.115	0.000	−0.008	0.097
IL-10 (pg/ml)	−0.072	0.106	−0.118	0.043	−0.033	0.079	−0.196	−0.094
IL-12 (pg/ml)	−0.166	0.145	0.190	0.011	−0.107	−0.152	−0.248	0.098
TNF-α (pg/ml)	−0.202	0.117	−0.097	0.029	−0.242	−0.137	−0.319[a]	0.082
IFN-γ (pg/ml)	0.027	−0.044	0.008	0.244	0.118	0.066	0.173	−0.066
Biomarkers	MoCA-C	Numbers of errors in nine-box maze test						
	Total	ORM	SRM	OWM	SWM	ORcM		
Adenosine (μg/ml)	0.107	−0.056	−0.449[b]	0.030	−0.335[a]	0.212		
ADA (U/ml)	0.156	0.105	-0.231	−0.157	0.143	−0.086		
IL-1β (pg/ml)	−0.365[b]	0.123	0.146	0.334[a]	0.033	−0.151		
IL-2 (pg/ml)	0.218	0.182	0.092	−0.167	−0.287[a]	0.058		
IL-4 (pg/ml)	−0.109	0.040	−0.181	0.027	−0.267	0.078		
IL-6 (pg/ml)	−0.117	0.095	0.307[a]	−0.043	0.038	−0.091		
IL-10 (pg/ml)	−0.122	0.098	−0.254	0.028	−0.147	0.037		
IL-12 (pg/ml)	−0.176	0.129	0.142	−0.105	−0.046	−0.118		
TNF-α (pg/ml)	−0.314[a]	0.188	0.391[b]	0.068	0.386[b]	−0.139		
IFN-γ (pg/ml)	0.156	−0.106	−0.065	−0.023	−0.024	−0.083		

Abbreviations: ADA, adenosine deaminase; IL, Interleukin; TNF-α, Tumor necrosis factor Interferon-α; IFN-γ: Interferon-γ; MoCA-C: Chinese-Beijing Version of Montreal Cognitive Assessment; VSEF: Visuo-spatial and executive functions; ORM, Object reference memory; SRM, Spatial reference memory; OWM, Object working memory; SWM, Spatial working memory; ORcM, Object recognition memory; DR: delayed recall.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level (2-tailed).

which TNF-α level ($\beta = -0.325$, $P = 0.022$) was independently negatively correlated with adenosine level (Table 7).

3.8. Principal component analysis of the CID group

Analyses of the CID group ($n = 55$, of which 29 completed the PSG evaluation) with the Kaiser–Meyer–Olkin (KMO) test yielded 0.519 (>0.500), while Bartlett's test of sphericity was significant at $P < 0.001$. These results suggested that the sample size was suitable and that the indexes selected were appropriate for principle component analysis. The variables adenosine, IL-1, TNF-α, PSQI, TST, SE, N1%, SRM, SWM, VSEF, attention, and delayed recall were included in the analysis based on their sufficient degree of correlation in the partial correlation analysis, and represented three aspects of the dataset, namely the blood biomarkers, sleep parameters, and cognitive parameters. The principal component analysis yielded five significant factors, with the component loading of variables for each of the rotated factors shown in Table 8. Plasma biomarker levels (adenosine and TNF-α), sleep parameters (TST and SE), and VSEF score were loaded heavily on factor 1. TST, SE, and VSEF and delayed recall scores were loaded on factor 2, while PSQI score and SRM errors were loaded on factor 3.

Table 7
Multiple linear regression of adenosine and cytokines.

Variable	Standardized β	*t*-Value	*p*-Value	B (95% CI)
adenosine (μg/ml)		4.706	0.000	14.608–36.445
IL-1β (pg/ml)	−0.187	−1.418	0.163	−3.457–0.599
IL-2 (pg/ml)	−0.117	−0.834	0.409	−3.288–1.362
IL-4 (pg/ml)	0.265	1.839	0.072	−0.168–3.716
IL-6 (pg/ml)	−0.225	−1.637	0.108	−1.966–0.202
IL-10 (pg/ml)	−0.156	−1.131	0.264	−5.559–1.56
IL-12 (pg/ml)	0.133	0.972	0.336	−1.751–5.024
TNF-α (pg/ml)	−0.325	−2.364	0.022[a]	−3.348–0.269
IFN-γ (pg/ml)	0.032	0.233	0.817	−0.086–0.108

Abbreviations: ADA, Adenosine deaminase; IL, Interleukin; TNF-α, Tumor necrosis factor Interferon-α; IFN-γ: Interferon-γ
[a] Correlation is significant at the 0.05 level.

4. Discussion

4.1. Subjects

The patients with CID in the current study had moderate to severe insomnia, as indicated by their PSQI score (13.9 ± 2.2; Table 1). The objective sleep parameters showed that the patients experienced decreased TST, increased SOL, and increased WASO. Furthermore, they also suffered from a mildly depressive mood, consistent with our current understanding of chronic insomnia. The criteria used to diagnose CID were derived from the ICSD-3 [35], as previously used diagnostic criteria, such as those of primary insomnia, have been abandoned.

4.2. Adenosine and ADA levels in patients with CID

The current study demonstrated that, relative to levels in the HC group who slept well, levels of adenosine were reduced and levels of ADA were elevated in the patients with CID in the morning. The partial correlation analysis showed that the level of adenosine correlated negatively with PSQI score, and positively with the objective sleep parameters TST and SE. Although the correlation between adenosine level and SE indicated by the partial correlation analysis was no longer significant after Bonferroni correction, the principal component analysis still showed that adenosine was negatively correlated with TST and SE, with a high loading on factor 1. These results suggest that the decreased level of adenosine in patients with CID was indeed associated with poor sleep quality.

With regard to the objectively measured short sleep duration, the subgroup of patients with CID with TST <360 min were younger and had a lower adenosine level than those with TST >360 min. In

Table 8
Principal component analysis for CID.

Terms	Measures	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Plasma biomarkers	Adenosine	0.828[a]	−0.070	0.118	−0.131	0.193
	IL-1β	−0.362	−0.445	0.439	0.046	0.138
	TNF-α	−0.625[a]	−0.004	0.084	0.428	0.388
Sleep parameters	PSQI	−0.474	−0.298	0.538[a]	0.161	−0.158
	TST	0.745[a]	−0.551[a]	−0.184	0.079	0.011
	Sleep efficiency	0.683[a]	−0.503[a]	−0.193	0.182	−0.0113
	N1%	0.156	0.339	0.484	−0.369	−0.615[a]
Cognition	SRM	−0.366	−0.083	−0.600[a]	−0.519[a]	0.129
	SWM	−0.142	0.209	−0.444	0.627[a]	−0.325
	VSEF	0.558[a]	0.584[a]	0.109	0.461	−0.023
	Attention	0.481	0.234	0.462	−0.022	0.482
	Delayed recall	0.021	0.730[a]	−0.174	−0.161	0.227
Eigenvalues		3.174	1.942	1.624	1.306	1.030
Variance contribution rate (%)		26.451	16.182	13.530	10.881	8.584

Abbreviations: IL-1β, interleukin-1β; TNF-α, tumor necrosis factor α; PSQI, Pittsburgh Sleep Quality Index; TST, total sleep time; N1%, percentage of the NREM sleep stage 1; SRM, Spatial reference memory; SWM, Spatial working memory; VSEF: Visuo-spatial and executive functions.
[a] Loadings higher than 0.5.

order to avoid the influence of age, a covariance analysis was performed and the adenosine level of patients with TST <360 min was still found to be lower. These results suggest that the reduced level of adenosine is mainly associated with an objective sleep deficiency in patients with CID. The current results are consistent with existing studies showing that subjects with insomnia characterized by an objectively short sleep duration are more likely to suffer from deficits in neuropsychological performance [47], and cardiovascular risk factors such as hypertension or diabetes [48]. However, more subject data will need to be collected in our future studies in order to classify different chronic insomnia phenotypes.

Adenosine is an inhibitory neurotransmitter and neuromodulator in the CNS, the dysfunctional signaling of which is closely linked to multiple CNS diseases. In the past decades, the relationship between adenosine and sleep regulation has been extensively studied [49]. The regulation of sleep by adenosine involves a number of targets in the brain, such as the cerebral cortex, brainstem (locus coeruleus and pons), basal forebrain, and hypothalamus (ventrolateral preoptic area and tuberomammillary nucleus) [50]. One study also suggested that adenosine provides a global feedback signal to neuronal networks, including those in subcortical and cortical structures [51]. The majority of animal studies exploring changes in adenosine level have primarily focused on examining changes in the brain after sleep deprivation. Little attention has been paid to the level of plasma adenosine in subjects with CID, which may be because of the difficulties of measuring adenosine level circulating in the peripheral blood [49]. Only a few studies to date have examined adenosine level in the peripheral blood, finding that it exhibits a circadian rhythm [52].

Why does the level of adenosine in peripheral blood decrease in patients with CID? First of all, this can be partially attributed to the enhancing activity of adenosine-metabolizing enzymes, such as ADA, which reduce adenosine level. ADA can accelerate the conversion of adenosine to inosine, via the main metabolic pathway for adenosine [53]. Interestingly, the current study showed an elevated level of ADA in patients with CID, supporting the suggestion that adenosine hypermetabolism accounts for the decreased blood concentration of adenosine. Secondly, insufficient release of adenosine triphosphate (ATP) into the extracellular space due to the malfunction of astroglial cells may result in a decreased adenosine level, since ATP secreted by astrocytes is the main source of extracellular adenosine [54]. Our group has demonstrated that astrocytes in patients with CID exhibit a number of structural and functional impairments [55,56], which may affect the secretion of ATP. Thirdly, the reduced level of adenosine may be partly explained by the chronic loss of sleep in the patients. One study has shown that, although the level of adenosine in the brain increases on the first day after acute sleep deprivation, it decreases after sleep restriction for several days [57].

4.3. Elevated levels of multiple cytokines

Our results showed increased concentrations of four proinflammatory factors (IL-1β, IL-2, IL-6, and TNF-α). Studies to date have not systematically investigated or reasonably explained the levels of proinflammatory cytokines measured in patients with CID. Explanations of the relationship between insomnia and cytokines have mainly focused on the proinflammatory factor IL-6 [30,31,58], suggesting an elevated level of IL-6 in subjects with insomnia. In terms of the other proinflammatory factors, the results of previous studies in humans are limited to reports that long-term sleep restriction increases the mRNA level of IL-1 and the concentration of TNF-α [59,60]. Moreover, there have been few studies of the association between IL-2 and insomnia/sleep restriction, with no change observed in IL-2 depending on the sleep stage [61].

Why did the patients with CID in this study have elevated concentrations of proinflammatory factors? Firstly, the increased levels may be attributed to the decreased concentration of adenosine in peripheral blood. Adenosine is a well-known immunoregulatory molecule that inhibits the function of immune cells and the release of inflammatory factors [25]. The results of the multiple linear regression analysis in our study showed that levels of the five upregulated cytokines correlated negatively with adenosine level, while TNF-α level had an independent effect, further supporting our explanation. Secondly, these results may also be related to the effect that the disruption of circadian rhythms during chronic insomnia has on the secretion of cytokines [30]. Specifically, patients with CID show a shift of the major peak of IL-6 secretion from 4 a.m. to 7 p.m. [30], while the secretion peak of good sleepers continues to occur during the night [30,62]. Therefore, concentrations of proinflammatory factors detected in the morning in patients with CID may be higher than those of normal sleepers. In addition, the higher concentration of proinflammatory factors may only be indicative of a physiological peak in the current study, which also partly supports this explanation. Thirdly, patients with CID suffer from an overactive Hypothalamus-Pituitary-Adrenal axis along with immune activation [63], possibly accounting for the increase in proinflammatory factors [64].

The current study showed for the first time that the level of the anti-inflammatory factor IL-10 was higher in patients with CID than

that in HC subjects with good sleep. The increase of IL-10 concentration in patients with CID can be explained in two ways. Firstly, based on the observed increase in the level of IL-10 following chronic sleep loss; for instance, in one study the concentration of IL-10 was found to increase after 192 h of continuous sleep deprivation [65]. Secondly, the elevation in the level of IL-10 may be attributed to the response of the body to the upregulation of proinflammatory factors. However, the increase in the concentration of IL-10 may promote insomnia, with a previous study showing that injection of IL-10 inhibited NREM sleep in animals [34]. IL-10 also inhibits the release of other sleep-promoting regulators, such as neurotrophic factor and nitric oxide [66,67].

The partial correlation analysis showed poorer sleep correlates with higher levels of IL-1β and TNF-α. After Bonferroni correction, only the positive correlation between plasma IL-1β level and PSQI score remained. Nevertheless, principal component analysis further demonstrated that TNF-α level was loaded strongly on factor 1, with an inverse correlation for TST and SE. These results suggest that IL-1β and TNF-α have a disruptive effect on sleep in patients with CID and that TNF-α may be causally associated with insomnia.

4.4. Adenosine and proinflammatory factors were involved in cognitive impairment in patients with CID

One common complaint in subjects with insomnia is cognitive dysfunction, but the results from objective cognitive tests are often contradictory [68,69]. A recent meta-analysis of 48 studies showed that the cognitive impairment of subjects with insomnia mainly affects the cognitive domains of working memory, attention, executive function, and perceptual function [7]. Compared with healthy controls, patients with insomnia showed significantly poorer performance in working memory tasks [70]. Moreover, impairments in cognition due to insomnia also include effects on spatial memory, with a previous study finding that insomnia primarily affects SWM performance [71]. The short objective sleep time in subjects with insomnia has been linked to their poor performance in a short-term visual spatial memory task [72]. In two prior studies from our group, we observed poor level of performance for SWM and ORcM in the nine-box maze test, with or without OWM impairment [45,55]. The results of the current study showed that patients with CID had a lower total MoCA-C score and poorer performance in the cognitive domains of VSEF, attention, and delayed memory. Moreover, our results confirm the poorer performance of patients with CID on the SWM, OWM, and ORcM tests relative to the good sleepers from the HC group. Overall, the present study not only demonstrated impairments of working memory and attention in the patients with CID, but further showed that the poor cognitive performance mainly affected SWM, visual-spatial skills (reflected in scores on the cube and clock items of the MoCA-C), and delayed recall (reflected in performance on the ORcM of the nine-box maze test and on the delayed memory item of the MoCA-C).

Previous studies have explored the mechanisms underlying the cognitive dysfunction associated with insomnia. Insomnia leads to a number of pathophysiological changes that may have adverse effects on cognitive function, including the excessive release of cortisol [45], a reduction of BDNF level [72], an increase of amyloid-β in cerebrospinal fluid [73], and the expression of biomarkers of astroglial damage [55]. The results of the current study expand our understanding of the mechanisms underlying insomnia-related cognitive dysfunction. Firstly, our results showed for the first time that the decrease in the concentration of adenosine was linked to a decline in cognitive function. Specifically, partial correlation analysis showed that the level of adenosine was positively correlated with the attention domain score on the MoCA-C scale. Furthermore, it revealed that adenosine level was negatively correlated with the number of SRM errors (with the correlation remaining significant after Bonferroni correction) and SWM errors in the nine-box maze test. In the principal component analysis, the concentration of adenosine and VSEF score also had a higher load and positive correlation for factor 1 (in addition, attention had a load close to 0.5). Consistent with our results, relationships between adenosine and cognitive function have been reported in a number of studies [74–76]. Adenosine is effective at maintaining alertness [77] and is necessary for spatial memory [76]. In mice, the increase of adenosine induced by an adenosine nucleotide translocator inhibitor (which inhibits the transfer of adenosine to the extracellular space) led to an amelioration of spatial learning and memory impairments [75]. Secondly, after controlling for confounding factors, we found that the plasma concentrations of IL-1β and TNF-α in the patients with CID were negatively correlated with total MoCA-C score and the domain score for delayed memory. In addition, the level of IL-1β was negatively correlated with the domain score for VSEF. Furthermore, the higher levels of IL-1β, IL-2, IL-6, and TNF-α, either individually or in combination, correlated with the increased number of memory errors, such as on the SWM, SRM, and OWM tests. Although these correlations were no longer significant after Bonferroni correction, high loads and negative correlations were still observed between TNF-α and VSEF in factor 1 on the principal component analysis. These results suggest that the cognitive dysfunction observed may have resulted from an increase in levels of proinflammatory cytokines, consistent with the results of previous studies [78]. Thirdly, principal component analysis showed that classical sleep parameters (TST, SE) and cognitive performance scores (VSEF and delayed recall) were both positively loaded on factor 2. Similarly, PSQI score and the numbers of SRM errors were both loaded positively on factor 3. These results suggest that a short objective sleep time, low objective sleep efficiency, and poor subjective sleep quality were related to poor cognitive performance, supporting the view that cognitive dysfunction in subjects with insomnia can be attributed to sleep loss itself.

5. Conclusions

In summary, the patients with CID in this study had a lower level of adenosine and enhanced concentrations of a number of proinflammatory factors in their peripheral blood. The concentration of adenosine may be related to the levels of proinflammatory factors, in that the decreased adenosine level can partly explain the upregulation of proinflammatory factors. The altered plasma levels of adenosine and proinflammatory factors might also relate to the poor sleep quality and decline in cognitive performance in patients with CID.

The current study had several limitations. Firstly, PSG tests were consented to and conducted only in a subset of the patients with CID enrolled in the study, which may affected the results. Secondly, levels of adenosine and cytokines were only measured in peripheral blood, and not in cerebrospinal fluid. Thirdly, this experiment was a preliminary exploration and further investigation of the abnormal adenosine and cytokine signals in patients with CID are required. In future studies, appropriate measures to maintain the balance between adenosine and inflammatory factors may help to improve sleep quality and cognitive function in patients with CID.

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Conflict of interest

This was not an industry-supported study. The authors have indicated no financial conflicts of interest.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2021.02.005.

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