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ORIGINAL RESEARCH

# Mediation Effect of Relaxin in Cerebrospinal Fluid on the Association Between Smoking and Sleep

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**Objective:** This study investigates the influence of CSF relaxin (RLN) on the association between smoking and sleep quality, considering previous findings linking smoking and RLN with psychiatric conditions.

**Methods:** In a case-control study of 168 Chinese adult males (70 smokers, 98 non-smokers), levels of relaxin in cerebrospinal fluid (CSF) were measured. Sleep quality was assessed using the Pittsburgh Sleep Quality Index (PSQI), comprising seven scales. Logistic regression and mediation models analyzed the relationships between nicotine dependence, PSQI scores, and CSF relaxin. Logistic regression examined the interaction of nicotine dependence and relaxin gene on PSQI subdimension scores.

**Results:** Smokers exhibited more severe sleep problems in PSQI total score and four PSQI subdimension scores ($p < 0.05$). CSF relaxin levels were significantly higher in smokers ($20.7 \pm 7.0$ vs $16.3 \pm 6.5$, $p < 0.001$) and correlated closely with PSQI total score ($r = 0.275$, $p < 0.001$). Logistic regression found that CSF relaxin associated with PSQI subdimension scores, particularly in sleep disturbance (OR = 3.07 (1.61–5.99), adjusted $p < 0.01$). Mediation analysis indicated relationship between nicotine dependence and PSQI total score, with CSF relaxin as a mediator, and the indirect effect accounted for 25% of the total effect (Indirect effect = 0.124 (0.021–0.223), Total effect = 0.494 (0.193–0.807)). Additionally, polymorphisms in gene of relaxin and its receptors were closely tied to smoking behaviors and sleep quality ($p < 0.05$).

**Conclusion:** CSF relaxin levels were significantly elevated in smokers and closely associated with PSQI subdimension scores, particularly with the sleep disturbance subdimension score. Moreover, CSF relaxin mediated the relationship between nicotine dependence and sleep quality. Polymorphisms (RLN3 rs12327666, rs1982632, and rs7249702, RLN3R1 rs35399, and RLN3R2 rs11264422) also played a role in smoking behaviors or sleep quality.

**Keywords:** smoking, PSQI score, relaxin, psychiatric disorders, mediation effect

## Introduction

Cigarette smoking remains a pressing public health issue, impacting around 34 million individuals in the US alone, with approximately 14% of adults classified as smokers.[1] It stands as a leading preventable cause of chronic diseases in developed nations. Among its myriad consequences, sleep disorders emerge as a significant concern.[2] Chronic smoking disrupts sleep architecture and is implicated in conditions such as depression, obesity, diabetes, and cardiovascular diseases.[3] Smokers are predisposed to various sleep disturbances, encompassing sleep-disordered breathing, sleep apnea, insomnia, and compromised sleep quality marked by diminished duration, prolonged latency, and daytime dysfunction.[4,5] Such disturbances are linked to adverse psychosocial functioning and physical health outcomes, including delinquency,

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self-harm, and suicide.[6] Notably, the intricate interplay between smoking and sleep disruption is exacerbated by the stress response, with smokers often resorting to cigarettes as a coping mechanism, thereby exacerbating sleep problems.[7,8] However, the precise underlying risk factors and pathophysiology of sleep disorders associated with smoking remain incompletely understood.

Relaxin (RLN) belongs to the insulin superfamily, comprising seven peptides exhibiting high structural similarity but low sequence homology.[9] While relaxin 2 exerts anti-inflammatory, anti-fibrotic, and blood pressure regulatory effects peripherally,[10,11] relaxin 3 predominantly operates within the central nervous system, particularly in the nucleus incertus (NI) located near the fourth ventricle midline tegmentum.[12] Previous research has predominantly focused on peripheral relaxins (RLN2) and their regulatory role in vascular smooth muscle and blood pressure,[13] leaving the relationship between RLN3 and sleep underexplored. RLN3 demonstrates considerable resemblance to established ascending arousal systems.[14] The NI/relaxin-3 system, receiving inputs from various brain regions including the prefrontal cortex, lateral habenula, and interpeduncular and median raphe nuclei, projects to arousal-related pathways, potentially modulating sleep.[15] Behavioral studies involving RLN3 knockout mice indicate reduced activity and prolonged sleep duration.[16] Additionally, RLN3 gene polymorphisms, located on chromosome 19q13, have been linked to sleep quality.[17,18]

Smoking and sleep disorders are intricately intertwined, with smoking serving as a major catalyst for sleep disruptions. The neuropeptide NI/relaxin3 system emerges as a pivotal player in arousal regulation.[19] However, the nuanced interplay among smoking, sleep, and these neurohormones remains inadequately understood. This study seeks to elucidate the role of relaxin in mediating the relationship between nicotine dependence and sleep quality. Additionally, we hypothesize that genetic variations within RLN3 and its receptors, RXFP3 and RXFP4, may influence sleep quality and smoking dependence.

# Materials and Methods

## Study Population

Regarding the population, the studied subjects were selected from a northern Chinese Han population. The studied sample was a subset of patients who were scheduled for anterior cruciate ligament reconstruction surgery between September 2014 and January 2016. These patients were screened according to the following exclusion criteria. Patients with 1) a family history of psychosis or neurological disorders and 2) systemic or central nervous system (CNS) diseases diagnosed via the Mini International Neuropsychiatric Interview; 3) reported smoking less than 10 cigarettes per day were not invited to this study. Patients who were eligible for this study were given a concise briefing of the study and asked to voluntarily provide informed consent, informed consent also obtained from the legal guardians of participants aged under 18. Accordingly, all 191 eligible patients (age range 17–64 years) provided informed consent. After excluding individuals lacking relevant data, 168 participants were included, comprising 70 smokers and 98 non-smokers. Sociodemographic information, including age, years of education, and body mass index (BMI), was recorded. Clinical data, encompassing smoking history (age of smoking initiation, smoking duration), and Fagerström Test for Nicotine Dependence (FTND) scores,[20] were collected via self-report, corroborated by close relatives.

Participants with no history of smoking or substance abuse were categorized as non-smokers. Participants consuming at least half a pack of cigarettes (ie, 10 cigarettes) daily for over a year were categorized as smokers. Smokers who consumed fewer than 10 cigarettes per day were excluded. The Institutional Review Board of Inner Mongolian Medical University approved the study, conducted in accordance with the Declaration of Helsinki.

## Biosample Collection and Laboratory Tests

Cerebrospinal fluid (CSF) samples were collected following established protocols[21] and promptly frozen at −80°C. The levels of relaxin in CSF were quantified using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the manufacturer's instructions.[22] The entire detection process adhered to double-blind principles.

## Assessment of PSQI

The Pittsburgh Sleep Quality Index (PSQI) assessed sleep quality over the preceding month. Following guidelines for the Chinese version of PSQI.[23] The measure consists of seven component scores, each of which has a range of 0–3 points (0 = "no difficulty" to 3 = "severe difficulty"). Responses formed seven subscales: subjective sleep quality, latency, duration, efficiency, disturbances, use of sleep medication, and daytime dysfunction. Scores ranged from 0 to 21, with higher scores indicating poorer sleep quality. Each questionnaire was administered in Chinese.

## Single Nucleotide Polymorphism Selection

Single nucleotide polymorphisms (SNPs) in the RLN3, RXFP3, and RXFP4 genes were selected based on prior technical articles. These SNPs were chosen from regions potentially regulating gene expression and included the following tag SNPs: rs12327666, rs1982632, and rs7249702 in RLN3; rs35399 in RXFP3; and rs11264422 in RXFP4.

## Statistical Analysis

Categorical variables were expressed as number (percentage) and compared using the Chi-square test. Continuous variables were presented as mean ± standard deviation. Group differences were evaluated using the independent *t*-test and Wilcoxon rank-sum test respectively. Spearman's rank correlation was employed to examine the relationship between nicotine dependence and PSQI scores (both total and subdimension scores) in smokers. Correlation between CSF relaxin levels and PSQI scores (both total and subdimension scores) was assessed using Spearman's rank correlation coefficients in all population.

To explore the mediation effect of relaxin on the association between nicotine dependence and PSQI total score, traditional linear regression models were employed. Linear relationship and homogeneity of variance were acceptable for all models (see Supplementary Figures 1 and 2). Model 1 included nicotine dependence as the independent variable and PSQI total score as the dependent variable. Model 2 included nicotine dependence as the independent variable and the relaxin level in CSF as the dependent variable. In model 3, both nicotine dependence and CSF relaxin level were included as the independent variables and PSQI total score as the dependent variable. Subsequently, multivariable logistic regression model was conducted with relaxin levels in CSF as the independent variable and the five PSQI subdimension scores (sleep quality, sleep latency, sleep duration, sleep disturbance and daytime dysfunction scores) as the dependent variables, with Bonferroni correction applied to adjust for multiple comparisons. Specifically, CSF relaxin was categorized into low level (≤median) and high level (> median), and five PSQI subdimension scores were categorized into two groups (0 score and ≥1score). All linear and logistic regression models were adjusted for age (continuous), BMI (continuous), marital status (married/unmarried), and living arrangements (with family/with others).

Additionally, mediation analysis was conducted using the "Bruce R" package. The model was based on a mediation method with 10,000 bootstrap bias-corrected 95% confidence intervals (95% CI). The direct effect refers to the impact of an independent variable on a dependent variable, after accounting for the mediating variable. Indirect effects arise from the influence of independent variables on mediators and mediators on dependent variables. The total effect combines the direct and indirect effects, representing the impact of the independent variable on the dependent variable without considering the mediating variable.

Chi-square comparisons assessed genotype distribution for each polymorphism, with differences between different genotypes compared using the Wilcoxon rank-sum test. Regression was used to explore the specific effects of nicotine dependence and relaxin-related genes on the PSQI subdimension scores. Logistic regression models were employed to investigate the interaction between nicotine dependence and relaxin gene polymorphisms on PSQI subdimension scores. The models were conducted with nicotine dependence x relaxin gene polymorphism as the independent variable and the five PSQI subdimension scores (sleep quality, sleep latency, sleep duration, sleep disturbance and daytime dysfunction scores) as the dependent variables, with Bonferroni correction applied to adjust for multiple comparisons. Specifically, smoking status was categorized into two levels (Yes/No), and gene polymorphism was classified into two levels based on genotype. The five PSQI subdimension scores were grouped into two categories: a score of 0 and a score of ≥1.

# Results

## Basic Characteristics of Study Population

Table 1 presents the basic characteristics of the 168 participants, comprising 70 smokers and 98 non-smokers. Smokers exhibited older age compared to non-smokers ($P < 0.05$). Significantly elevated levels of relaxin in CSF were observed among smokers (16.3 ± 6.5 pg/mL vs 20.7 ± 7.0 pg/mL, $P < 0.001$). Moreover, smokers demonstrated higher PSQI total score compared to non-smokers (2.7 ± 2.5 in non-smokers vs 4.2 ± 2.3 in smokers, $P < 0.001$), particularly in these PSQI subdimension scores, such as sleep disturbance, sleep latency, subjective sleep quality, and sleep efficiency scores. No significant differences were detected in blood pressure, BMI and other dimensions of sleep between the two groups ($P > 0.05$).

## Correlation Between FTND Score, CSF Relaxin Level and PSQI Scores (Both Total Score and Subdimension Scores)

Spearman correlation analysis revealed associations between FTND score and PSQI scores (both total score and subdimension scores). Higher FTND score correlated with poorer PSQI subdimension sleep quality scores, and age of smoking initiation negatively correlated with FTND score in smokers (all $P < 0.05$) (Figure 1).

Spearman correlation analyses explored the relationship between cerebrospinal fluid relaxin levels and PSQI scores. A positive correlation was observed between CSF relaxin and PSQI total score in all participants ($P < 0.001$) (Figure 2). Notably, the correlation pattern varied across groups, with a positive correlation observed in non-smokers ($r = 0.28$, $P < 0.01$)

**Table 1** Comparisons of Baseline Characteristics Between Non-Smokers and Smokers

| Variable | Non-Smokers (Mean±SD) (n=98) | Smokers (Mean±SD) (n=70) | P value |
|---|---|---|---|
| **Age, y** | 29.7 (9.4) | 33.7 (10.1) | 0.009 |
| **BMI** | 24.9 (4.1) | 25.6 (3.5) | 0.054 |
| **SBP** | 129.5 ± 12.7 | 127.4 ± 13.7 | 0.301 |
| **DBP** | 74.9 ± 8.8 | 75.9 ± 11.3 | 0.518 |
| **Relaxin (pg/mL)** | 16.3 ± 6.5 | 20.7±7.0 | <0.001 |
| **PSQI total score** | 2.7 ± 2.5 | 4.2 ± 2.3 | <0.001 |
| **PSQI Components** | Mean±Sd (no/with symptom) | Mean±Sd (no/with symptom) | |
| 1. Sleep Quality | 0.5±0.6 (54/44) | 0.7±0.6 (24/46) | 0.008 |
| 2. Sleep Latency | 0.3±0.6 (70/28) | 0.6±0.6 (30/40) | <0.001 |
| 3. Sleep Duration | 0.7±0.7 (46/52) | 0.8±0.8 (31/39) | 0.733 |
| 4. Sleep Efficiency | 0.1±0.3 (90/8) | 0.3±0.8 (57/13) | 0.044 |
| 5. Sleep Disturbance | 0.4±0.6 (60/38) | 0.8 ±0.5 (18/52) | <0.001 |
| 6. Sleep Medication | 0.05±0.27 (94/4) | 0.01±0.118 (69/1) | 0.403 |
| 7. Daytime Dysfunction | 0.6±0.8 (52/46) | 0.9±0.9 (29/41) | 0.137 |
| **Marriage, n (%)** | | | 0.010 |
| Married | 49 (50%) | 21 (30%) | |
| Unmarried | 49 (47%) | 49 (70%) | |
| **Living, n (%)** | | | 0.001 |
| Living in family | 73 (74.49%) | 66 (94.29%) | |
| Living with others | 25 (25.51%) | 4 (5.71%) | |
| **FTND** | – | 3.2±2.2 | – |
| Age of smoking onset (years) | – | 20.0±3.8 | – |
| Smoking period (years) | – | 13.3±9.3 | – |

**Notes**: Marriage and living status are presented as number (%), and other data are presented as mean ± standard deviation. P-values between non-smokers and smokers were calculated using the Chi-square test for categorical variables and either the independent *t*-test or Wilcoxon rank-sum test for continuous variables.

**Abbreviations**: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; relaxin, relaxin-3; PSQI, Pittsburgh Sleep Quality Index.

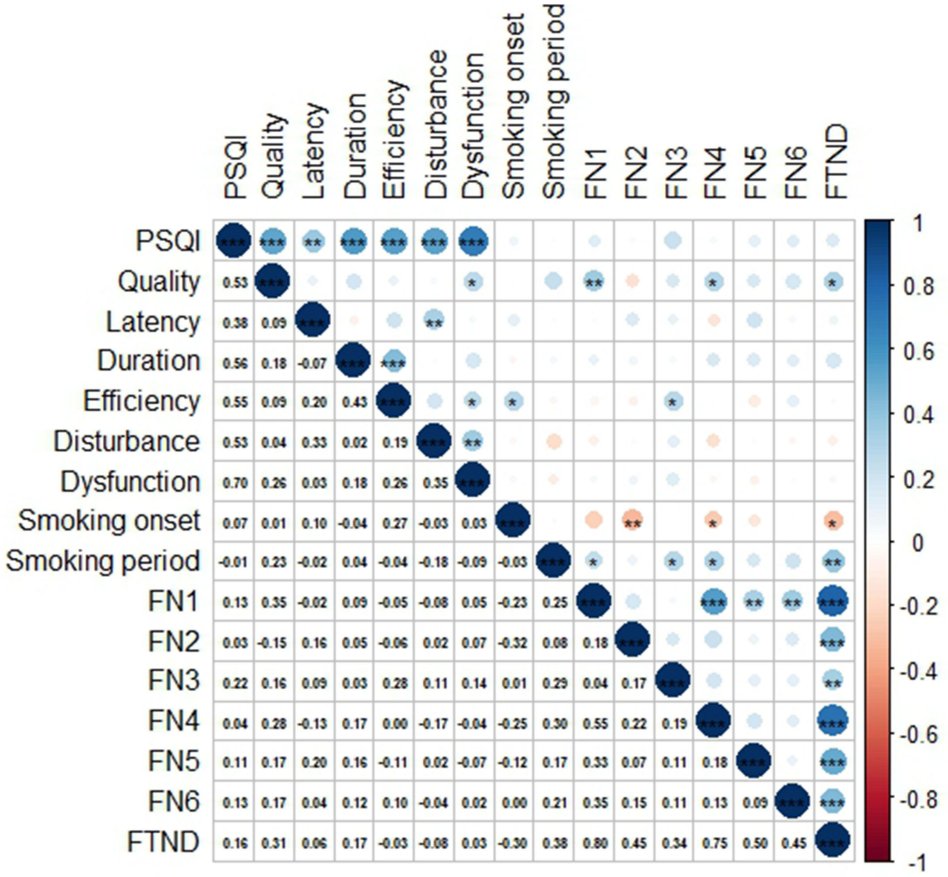


**Figure 1** Correlation analysis between smoking behaviors, FTND, and PSQI scores (total and subdimension scores) in smokers.
**Notes**: The numbers on the left indicate the correlation coefficients between variables. Blue circles indicate positive correlations, and red circles indicate negative correlations. The darker the color, the greater the absolute value of the correlation coefficient. FN1:6: the scores of the six questions on the FTND scale. *P < 0.05, **P < 0.01, ***P < 0.001.

but not in smokers (r = 0.02, P = 0.72) (Figure 2). Logistic regression analysis revealed significant associations between CSF relaxin levels (divided by median) and different PSQI subdimension scores, with sleep disturbance showing significant contrasts after adjustment with Bonferroni correction (adjusted p < 0.05) (Figure 3).

## Mediation Effect of Relaxin

Mediation models were conducted based on regression analyses (see Table 2). A mediation effect was observed in the PSQI total score. Initially, we assessed the impact of nicotine dependence on PSQI total score after adjusting for age, living situation, and BMI. The linear regression results indicated a positive effect of nicotine dependence on PSQI total score in model 1 (β = 0.494, t = 3.161, p < 0.01). Subsequently, we investigated the influence of nicotine dependence on CSF relaxin levels after the same adjustments in model 2. Linear regression results revealed a positive effect of nicotine dependence on CSF relaxin levels (β = 0.586, t = 3.752, p < 0.01). In model 3, nicotine dependence and CSF relaxin levels were treated as independent variables, while the PSQI total score was the dependent variable. The results demonstrated that nicotine dependence independently contributed to higher PSQI total score (β = 0.370, t = 2.317, p < 0.05). Similarly, CSF relaxin levels independently predicted increased PSQI total score (β = 0.211, t = 2.744, p < 0.01).

The Bootstrap sampling method was employed to dissect the effects in the mediation models. As illustrated in Table 3 and Figure 4, nicotine dependence exerted a direct effect on PSQI total score (effect value = 0.370, bootstrap 95% CI = 0.074–0.690, p = 0.020), an indirect effect (effect value = 0.124, bootstrap 95% CI = 0.021–0.223, p = 0.016), and a total


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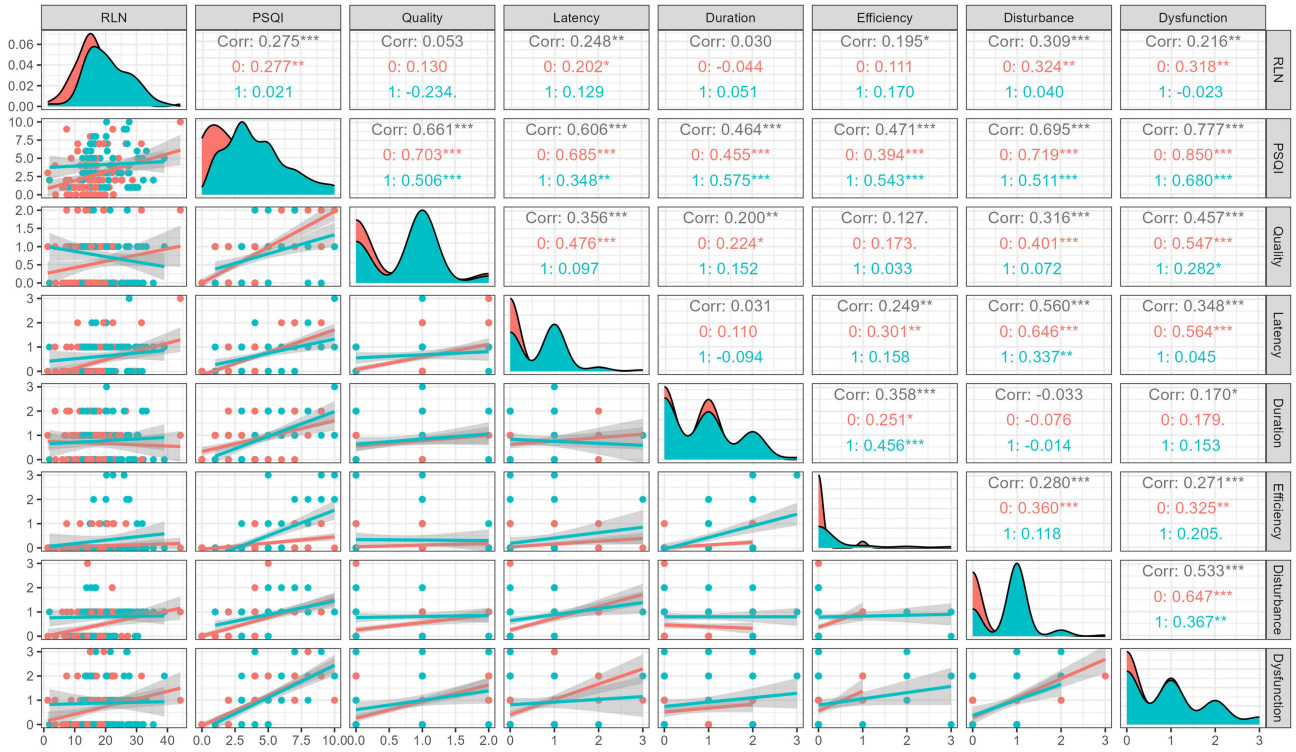


**Figure 2** Correlation analysis between CSF relaxin levels and PSQI scores (total and subdimension scores) in non-smokers and smokers.
**Notes**: Black, pink, and blue colors represent data for all participants, non-smokers, and smokers, respectively. The figure shows population distribution (lower left corner) and correlation coefficients for the entire population, non-smokers, and smokers. *P < 0.05, **P < 0.01, ***P < 0.001.

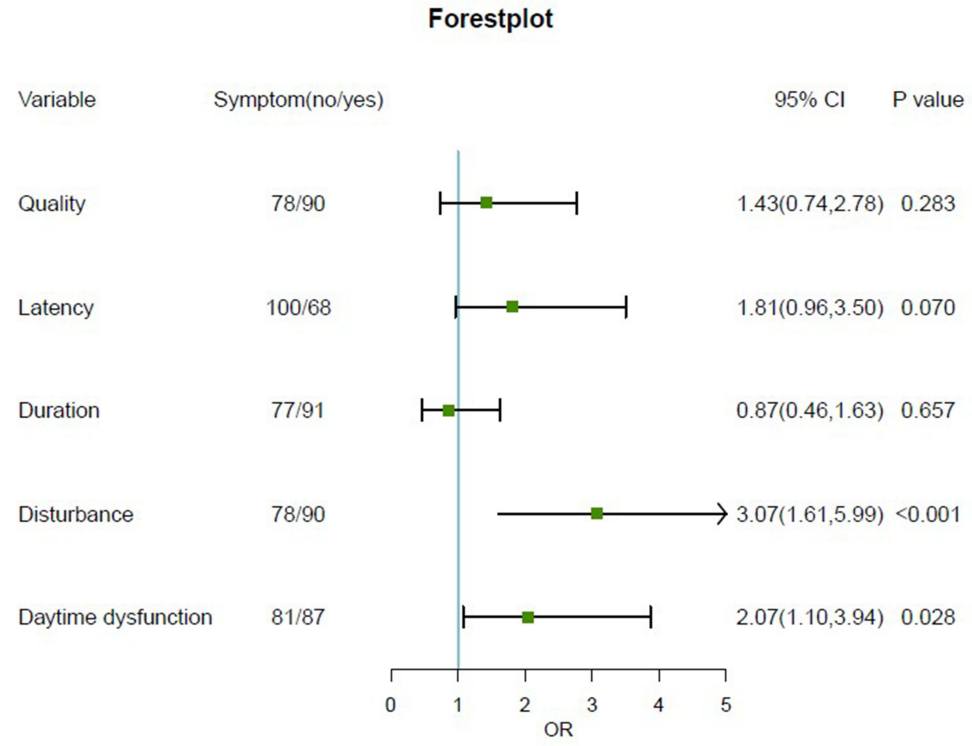


**Figure 3** Logistic regression analysis of CSF relaxin levels and PSQI subdimension scores.


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**Table 2** Mediation Analysis Examining the Association Between Nicotine Dependence and PSQI Total Score, with CSF Relaxin Levels as a Mediator

| | Model1 (PSQI) | | Model 2 (RLN) | | Model 3 (PSQI) | |
|---|---|---|---|---|---|---|
| | β | t | β | t | β | t |
| Age | 0.123 | 1.166 | –0.031 | −0.292 | 0.129 | 1.252 |
| BMI | 0.084 | 1.114 | –0.057 | −0.755 | 0.096 | 1.297 |
| Marriage | 0.014 | 0.063 | 0.161 | 0.750 | –0.020 | −0.097 |
| Living | –0.102 | −0.427 | –0.171 | −0.718 | –0.066 | −0.280 |
| Nicotine dependence | 0.494** | 3.161 | 0.586*** | 3.752 | 0.370* | 2.317 |
| RLN | | | | | 0.211** | 2.744 |
| *F* | 4.022 (5162) | | 4.006 (5162) | | 4.742 (6161) | |
| Adj. $R^2$ | 0.083 | | 0.083 | | 0.119 | |
| Num. obs. | 168 | | 168 | | 168 | |

**Notes**: Model descriptions: Model 1: Linear regression including nicotine dependence as the independent variable and PSQI total score as the dependent variable. Model 2: Linear regression including nicotine dependence as the independent variable and CSF relaxin as the dependent variable. Model 3: Linear regression including nicotine dependence and CSF relaxin as independent variables and PSQI total score as the dependent variable. All models were adjusted for age, BMI, marital status, and living situation. Data are reported as mediation analysis. *P < 0.05, **P < 0.01, ***P < 0.001.
**Abbreviations**: RLN, relaxin-3; PSQI, Pittsburgh Sleep Quality Index; 95% CI, lower and upper limits of the 95% confidence interval; BMI, body mass index.

**Table 3** Significance Test for Mediating Effect of CSF Relaxin on Nicotine Dependence and PSQI Total Score

| Effect Decomposition | Effect Value | Bootstrap 95% CI | | P value |
|---|---|---|---|---|
| | | Boot LLCI | Boot ULCI | |
| Indirect effect | 0.124 | 0.021 | 0.223 | 0.016* |
| Direct effect | 0.370 | 0.074 | 0.690 | 0.020* |
| Total effect | 0.494 | 0.193 | 0.807 | 0.002** |

**Notes**: *P < 0.05, **P < 0.01.
**Abbreviations**: Boot LLCI, lower 95% confidence interval; Boot ULCI, upper 95% confidence interval of the indirect effects estimated by the Bootstrap method.

effect (effect value = 0.494, bootstrap 95% CI = 0.193–0.807, p = 0.002). These results indicated that the mediating effect of CSF relaxin was incomplete, with only approximately 25% (indirect effect / total effect) of the association between nicotine dependence and PSQI total score mediated by CSF relaxin.

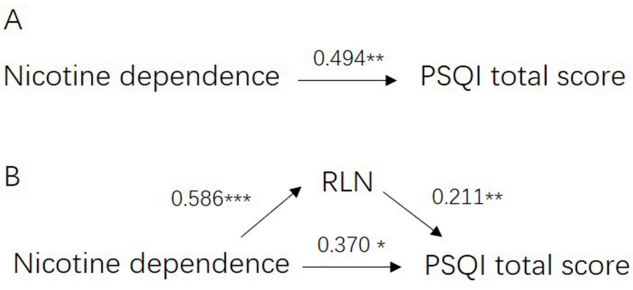


**Figure 4** Mediation effect of RLN on the relationship between nicotine dependence and PSQI total score.
**Notes**: (**A**) Effect of nicotine dependence on PSQI total score. (**B**) Association between nicotine dependence and PSQI total score, with CSF relaxin (RLN) as the mediator. *P < 0.05, **P < 0.01, ***P < 0.001.


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## The Role of Polymorphisms in Relaxin 3 and RXFP3/RXFP4 Receptors

All SNPs were found to be in Hardy-Weinberg equilibrium (Table 4). As depicted in Table 5, two polymorphisms within the RLN3 gene (rs12327666 and rs1982632), along with a relaxin receptor 2 (RXFP4) polymorphism (rs11264422), exhibited associations with nicotine dependence ($P < 0.001$), smoking duration ($P = 0.020$), and age of smoking onset ($P = 0.024$), respectively. Additionally, a polymorphism in the relaxin-3 receptor 1 (RXFP3) gene (rs35399) demonstrated significant associations with PSQI total score. Logistic regression showed that the interaction role of RLN3 gene (rs72497022) and PSQI subdimension sleep latency score (OR = 0.129, p = 0.014) (Table 6), while there was no significant association after Bonferroni adjustment (adjusted p = 0.070) (Table 6).

**Table 4** Distribution of Genotypes and Hardy-Weinberg Equilibrium (HWE) Test Results

| SNPs | Genotype Counts, n | | | $X^2$ | P value |
|---|---|---|---|---|---|
| | CC | GC | GG | | |
| **RLN3rs1982632** | 2 | 49 | 124 | 1.402 | 0.240 |
| | AA | GA | GG | | |
| **RLN3rs12327666** | 0 | 17 | 158 | 0.456 | 0.324 |
| | AA | GA | GG | | |
| **RLN3rs7249702** | 27 | 95 | 53 | 2.126 | 0.122 |
| | CC | TC | TT | | |
| **RLN3R1rs35399** | 4 | 51 | 120 | 0.275 | 0.583 |
| | AA | TA | TT | | |
| **RLN3R2rs11264422** | 3 | 41 | 131 | 0.010 | 1.000 |

**Note**: Hardy-Weinberg equilibrium was tested using the chi-square test.

**Table 5** Comparisons of PSQI Total Score and Smoking Behaviors Between Genotype Groups

| Independent Variables | Genotypic Groups | | T value | P value |
|---|---|---|---|---|
| | **RLN3R1rs35399** | | | |
| **PSQI total score** | TT(n=120) 3.7±2.7 | TC/CC(n=55) 2.8±2.1 | −2.43 | 0.016* |
| | **RLN3rs12327666** | | | |
| **FTND score** | GG(n=158) 3.3±2.3 | GA/AA(n=17) 5.0±0.6 | 4.66 | <0.001*** |
| | **RLN3rs1982632** | | | |
| **Smoking period (years)** | GG(n=124) 12.3±7.8 | GC/CC(n=51) 18.1±11.4 | 2.43 | 0.020* |
| | **RLN3R2rs11264422** | | | |
| **Age of smoking onset (years)** | TT(n=131) 20.5±4.1 | TA/AA(n=44) 18.8±2.5 | −2.31 | 0.024* |

**Notes**: *P < 0.05, ***P < 0.001.

**Table 6** Interaction Effects of rs7249702 and Genotypes on PSQI Subdimension Scores

| Predictors | OR | 95% CI | P Value |
|---|---|---|---|
| | **Sleep quality** | | |
| RLN3rs7249702 | 1.503 | −0.512 to 1.362 | 0.390 |
| Nicotine dependence | 4.571 | 0.386 to 2.742 | 0.011 |
| RLN3rs72497022*Nicotine dependence | 0.381 | −2.375 to 0.386 | 0.169 |
| | **Sleep latency** | | |
| RLN3rs7249702 | 3.485 | 0.052 to 2.765 | 0.063 |
| Nicotine dependence | 18.208 | 1.556 to 4.542 | <0.001 |
| RLN3rs72497022*Nicotine dependence | 0.129 | −3.825 to −0.496 | 0.014 * |
| | **Sleep disturbance** | | |
| RLN3rs7249702 | 1.350 | −0.653 to 1.315 | 0.546 |
| Nicotine dependence | 9.450 | 1.026 to 3.620 | 0.001 |
| RLN3rs72497022*Nicotine dependence | 0.463 | −2.328 to 0.684 | 0.312 |
| | **Sleep duration** | | |
| RLN3rs7249702 | 0.857 | −1.080 to 0.760 | 0.741 |
| Nicotine dependence | 1.457 | −0.719 to 1.491 | 0.501 |
| RLN3rs72497022*Nicotine dependence | 0.788 | −1.558 to 1.068 | 0.721 |
| | **Daytime dysfunction** | | |
| RLN3rs7249702 | 0.967 | −0.950 to 0.892 | 0.942 |
| Nicotine dependence | 1.256 | −0.852 to 1.320 | 0.678 |
| RLN3rs72497022*Nicotine dependence | 1.692 | −0.780 to 1.831 | 0.428 |

**Note**: *P < 0.05.

## Discussion

Previous research has consistently underscored the strong correlation between smoking and sleep quality. In our study, smokers indeed exhibited poorer sleep quality. Notably, we observed elevated levels of relaxin 3, a neurotransmitter intricately linked with the arousal system, in the cerebrospinal fluid of smokers. Statistical analyses further corroborated a positive correlation between CSF relaxin levels and sleep quality across all participants. We delved deeper into the relationship between RLN and sleep quality, uncovering the significant association between relaxin and one of PSQI subdimensions, namely sleep disturbance, in all participants. Mediation analysis shed light on the intricate interplay between nicotine dependence, relaxin, and sleep quality, revealing that the association between nicotine dependence and PSQI total score was mediated by relaxin in CSF. These findings suggest a pivotal role for relaxin-3 in modulating sleep quality, implicating the NI/relaxin-3 system in the association between nicotine dependence and sleep quality. Furthermore, specific genotypes in relaxin and its receptors were closely tied to smoking behaviors, correlating with longer smoking durations (RLN3rs1982632), earlier smoking initiation (RLN3R2rs11264422), and heightened tobacco dependence (RLN3rs12327666), while RLN3R1rs35399 TT genotype was linked to poorer sleep quality.

Cigarette smoking is intricately linked with stress.[24] Stress can trigger smoking behavior, leading smokers to increase cigarette consumption under stressful conditions.[25,26] Intriguingly, the relaxin-3/RXFP3 system plays a role in regulating stress responses, with evidence suggesting that stress induces rapid relaxin-3 expression.[27,28] However, direct literature elucidating the relationship between smoking and relaxin-3 is scarce.[29] Our results revealed higher levels of relaxin in CSF among smokers, potentially indicative of a stress response. Relaxin-3 plays a pivotal role in arousal regulation (sleep/wakefulness), and our findings regarding the association between CSF RLN levels and sleep align with previous studies.[12,16,19] Specifically, relaxin exhibited a positive association with PSQI total score in all participants. Further

exploration of the relationship between relaxin and sleep subdimensions unveiled heightened risks of sleep disturbance among participants with high CSF RLN levels. This could be attributed to the arousal effect of the NI/relaxin-3 system, particularly its projections to the lateral hypothalamus.[12] The elevation in relaxin associated disturbance of sleep after falling asleep.

Mediation analysis revealed an incomplete mediation effect of relaxin-3 between nicotine dependence and sleep quality. Linear regression models demonstrated significant positive associations between nicotine dependence and PSQI total score ($\beta = 0.494$, $t = 3.161$, $p < 0.01$) and between nicotine dependence and CSF relaxin levels ($\beta = 0.586$, $t = 3.752$, $p < 0.001$). CSF relaxin levels were further positively associated with PSQI total score ($\beta = 0.211$, $t = 2.744$, $p < 0.01$). Mediation analysis indicated relationship between nicotine dependence and PSQI total score, with CSF relaxin as a mediator, and the indirect effect accounted for 25% of the total effect (Indirect effect = 0.124 (0.021–0.223), Total effect = 0.494 (0.193–0.807)).

Furthermore, our findings underscored the close association between relaxin and arousal, particularly sleep disturbance in the entire population (Figure 3). This suggests a potential role for relaxin-3 as a mediator of the adverse effects of nicotine dependence on sleep quality, particularly regarding PSQI subdimension sleep disturbance.

Our study provides evidence that levels of relaxin-3 may mediate the association between nicotine dependence and sleep quality. The increase in cerebrospinal fluid may be related to smoking, while CSF relaxin may serve as a key neurotransmitter implicated in sleep quality. Under normal conditions, relaxin-3 within the central nervous system contributes to an ascending arousal system that regulates wakefulness. This mediation effect could be related to stress-induced up-regulation of relaxin expression, subsequently influencing sleep quality.[19]

Studies on relaxin-3 knockout (KO) mice and RXFP3 knockout (KO) mice have revealed distinct behavioral phenotypes, with relaxin-3 KO mice exhibiting decreased activity and prolonged sleep duration.[16] This underscores the significant influence of genes on phenotype.[30] Consequently, our study delved into gene-level associations to explore the relationships between RLN and sleep quality and nicotine dependence. Specifically, GG genotype in the RLN3 gene (rs12327666 and rs1982632) were associated with low FTND score, and short smoking duration, while RLN3R2rs11264422 TT genotype showed later age of smoking onset. Notably, RLN3R1rs35399 TT genotype associated with low sleep quality. These evidences showed that relaxin-related gene had tight association with smoking behaviors and sleep quality.

Additionally, nicotine dependence and RLN rs7249702 displayed a significant interaction effect on sleep latency before correction. These findings highlight the role of the NI/relaxin-3 system in modulating arousal levels,[14] further supporting the notion that RLN may play a crucial role in the association between nicotine dependence and sleep quality.

Despite the significant findings, several limitations warrant consideration. Firstly, the cross-sectional design of this study precludes the establishment of causality. Future longitudinal investigations are essential to unravel the temporal dynamics between relaxin-3, smoking, and sleep quality. Secondly, the exclusive inclusion of male participants limits the generalizability of the findings to female smokers. Subsequent research should explore whether the moderating effect of relaxin-3 on sleep quality extends to female smokers. Lastly, smoking behavior is multifaceted, influenced by various factors. This study did not explore potential confounders such as stress levels. Future research should incorporate these variables into their analyses for a more comprehensive understanding.

## Conclusion

In conclusion, this study unveils a positive association between nicotine dependence and PSQI total score, mediated by relaxin levels in cerebrospinal fluid among Chinese adult males. The impact of relaxin presents novel therapeutic avenues for addressing sleep disorders. Nonetheless, further validation and interpretation of these findings are warranted through in vitro and in vivo studies involving larger sample sizes. Different from the previous heterogeneity analysis, we mainly focused on exploring the mechanism of smoking and sleep.[31] In addition to focusing on nitric oxide synthase, we are also concerned with the effects of neuropeptides. In this paper, we have mainly studied the role of relaxin on the association between smoking and sleep. Additionally, we explored the relaxin-related genes and the association with smoking behaviors and sleep quality to better understand the mechanism of sleep problem associated with smoking.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

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