# Clinical Analysis of Children with Mixed Macrolide-Resistant Mycoplasma pneumoniae Infection

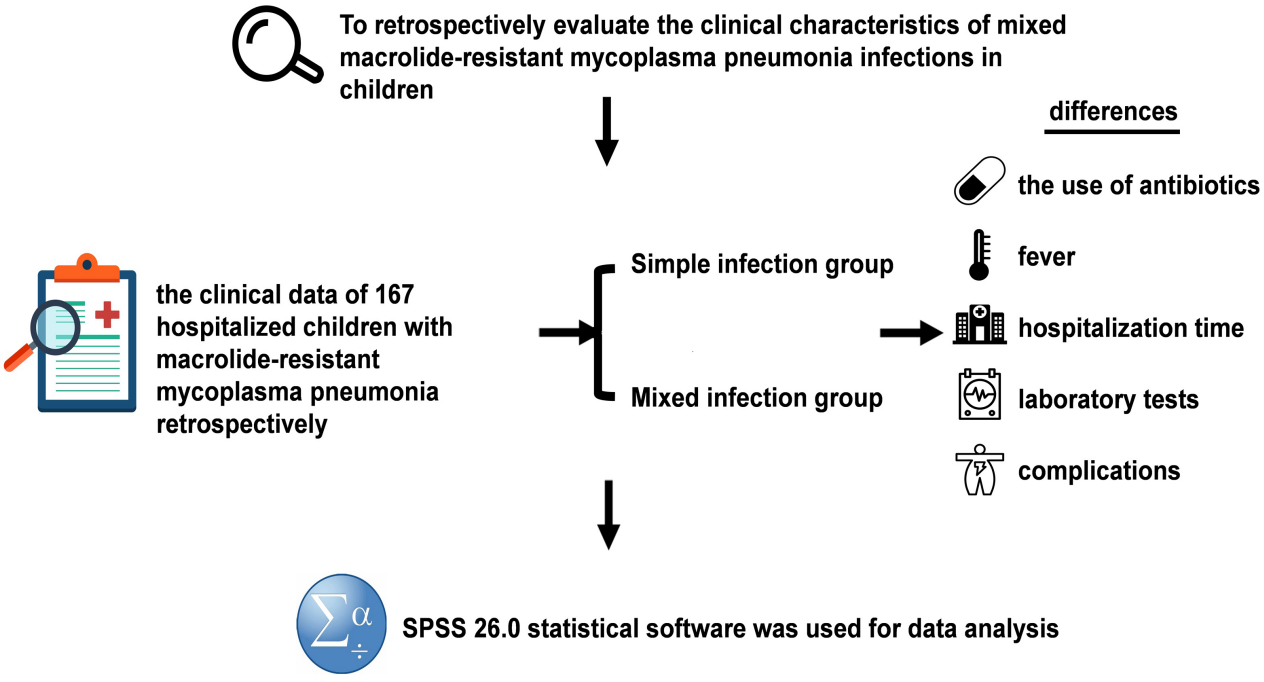
## Authors

**Zhengyuan Wang,** Yuanhong Xu

## Correspondence

xyhong1964@163.com (Y. Xu)

## Graphical Abstract

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# Clinical Analysis of Children with Mixed Macrolide-Resistant Mycoplasma pneumoniae Infection

**Zhengyuan Wang** [1] , Yuanhong Xu [1*]

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## Abstract

**Purpose:** Epidemiological data from Asia have documented the rapid and widespread emergence of macrolide resistance in Mycoplasma pneumoniae (MP). This paper aims to retrospectively evaluate the clinical characteristics of mixed macrolide-resistant Mycoplasma pneumoniae (MRMP) infections in children.

**Methods:** The study included 167 hospitalized children with MRMP. We analyzed the children's clinical data retrospectively, and compared the differences in the use of antibiotics, fever, hospitalization time, laboratory tests and complications between the mixed infection group and the separately infected group before admission.

**Results:** Compared with the separately infected group, hospitalized children with mixed infection had a longer hospital stay, and there was a significant difference between the two ($P < 0.05$); Alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), and platelet count (PLT) of children in the mixed infection group were significantly higher than those in the separately infected group ($P < 0.05$); and the incidence of hepatic function impairment, cardiac enzyme profile abnormality, and pleural effusion in the children in the mixed infection group was higher than that in the separately infected group ($P < 0.01$).

**Conclusion:** Patients with MRMP combined with mixed infections had prolonged hospital stays, increased systemic inflammatory response, and increased incidence of extrapulmonary complications compared with those with simple infections.

**Keywords:** Mycoplasma pneumoniae; Macrolide resistance; Mixed infection; Hospitalized children

## Introduction

MP is one of the major pathogens causing community-acquired pneumonia (CAP) in children and adolescents, accounting for up to about 40% of cases [1]. While pneumonia caused by MP is typically a self-limiting disease [2], some patients may develop refractory Mycoplasma pneumoniae pneumonia (RMPP) [3] or extrapulmonary complications [4,5]. Macrolides are currently the first-line agents for treating Mycoplasma pneumoniae pneumonia (MPP) in pediatric populations. However, the widespread use of macrolides has resulted in a sustained increase in antibiotic resistance prevalence, with significant regional variations. Mutations at nucleotide sites A2063, A2064, A2067, and C2617 within the 23S rRNA domain V of MP are recognized as markers of MRMP [6]. Among these, the A2063G mutation is the most frequently identified in resistant isolates [7]. Mixed infections have been reported to be more common in children with MPP, in which disease severity is higher than in single pathogen infections [8,9]. Relevant studies have shown that co-infection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) with MP during the Coronavirus Disease 2019 (COVID-19) pandemic may lead to prolonged Intensive Care Unit (ICU) hospitalization or exacerbation of clinical symptoms in patients [10,11].

In this study, we analyzed the clinical characteristics and laboratory test results of children with MRMP and mixed infections in Hefei, aiming to identify children with MRMP with mixed infections at an early stage, and to guide clinicians to intervene in the rational use of antibiotics and therapeutic measures as a means of reducing complications, shortening the hospitalization time of the children, and improving the prognosis.

## Methods

### Study Design and Population

The clinical data of 167 children diagnosed with MPP in the First Affiliated Hospital of Anhui Medical University were collected. All children were diagnosed with MPP according to WHO's CAP clinical diagnostic criteria [12], including symptoms and chest X-rays assessed by clinicians. Inclusion criteria: (1) age from 5 months to 15 years; (2) positive MP PCR or positive serologic tests; (3) macrolide antibiotic resistance; (4) complete clinical data. Exclusion criteria: (1) immunocompromised; (2) chronic lung disease (except asthma), chronic heart disease; (3) diseases of the blood

1 Department of Clinical Laboratory, The First Affiliated Hospital of Anhui Medical University, Hefei, China

* Corresponding Author.

system; (4) incomplete clinical data.

**Data Collection and Variables**

The clinical data of the children were collected, including gender, age, previous antibiotic use during the disease and clinical symptoms such as fever, cough, sputum, rash, and duration of symptoms before hospitalization. In addition white blood cell count (WBC), percentage of neutrophils (NEU), percentage of monocytes (MON), PLT, fibrinogen (FIB), thrombin time (TT), D-dimer (DD), fibrinogen degradation product (FDP), CK, urea (UREA), blood creatinine (CRE ), uric acid (UA), estimated glomerular filtration rate (eGFR), C-reactive protein (CRP), procalcitonin (PCT), activated partial thromboplastin time (APTT), prothrombin time (PT), ALT, AST, LDH, and total protein (TP), chest X-ray, and complications: Hepatic impairment, pleural effusion, ketonuria, pulmonary atelectasis, coagulation disorders, micronutrient deficiencies and abnormalities in cardiac enzyme profiles.

**Detection of MP drug resistance genes**

The presence of mutations at the A2063 and A2064 loci was determined by pyrophosphate sequencing of the two portions of the 23S rRNA gene structural domain V. The extracted nucleic acids were reverse transcribed into the nucleic acids of the A2063 and A2064 loci. The extracted nucleic acids were reverse transcribed into cDNA for amplification (11121ES, Yeason Biotech Co., Shanghai, China). Positions 2063 and 2064 in the structural domain of the 23SrRNA gene were amplified using PCR as described below. PCR system: ddH20 16 μl, 2×Taq Master Mix 25 μl, Forward primer1 (10 μM) 2 μl, Reverse primer2 (10 μM) 2 μl. cDNA 5μl, the total system was 50μl. program: 95°C 3min; 95°C 15S, 60°C 15S, 72°C 60S cycle 35 times; 72°C 5min. the PCR products were compared with the MP standard gene (23SrRNA gene, GenBank no. X 68422) after bidirectional sequencing by Anhui General Biological Company [13].

**Statistical analysis**

SPSS 26.0 statistical software was used for data analysis. According to data types, categorical variables were expressed as frequencies and percentages, and continuous variables were expressed as medians and quartiles. Normal distribution was expressed as mean ± standard deviation, and non-normal distribution was expressed as interquartile spacing. When comparisons were made between two groups, normal distribution was tested by the t test; non-normal tests were tested by the rank sum test. Count data were compared using $\chi^2$or corrected $\chi^2$. $p<0.05$ was considered statistically significant.

**Ethics Approval**

The current investigation was duly approved by the human ethics council of Anhui Medical University's First Affiliated Hospital (Approval No: Quick-PJ 2023-08-41). The written informed consent was obtained from patients in accordance with the Declaration of Helsinki.

## Results

**Study of drug-resistant gene mutations of MP**

In this study, 167 MP nucleic acid positive patients were amplified and sequenced at the above loci. The results showed that the A2063G mutation in the V region of the 23SrRNA gene was found in all 167 (167/167,100%) patients, and no drug-resistant mutation was found in the A2064G/A2064C locus.

Comparison of clinical symptoms on admission between the two groups

According to the results in Table 1, most of the patients were on antibiotics before admission. The length of hospitalization in the mixed infection group was significantly longer than that in the simple infection group, and the difference between the two groups was statistically significant (Z=-6.607, p < 0.001); however, the clinical symptoms exhibited by the patients, such as fever, cough, sputum, vomiting, rash, and dizziness, were not statistically significant between the two groups.

**Table 1** Comparison between clinical symptoms in simple and mixed infection groups

| | Simple infection group (n=117) | Mixed infection group (n=50) | $\chi^2$/t/Z value | p value |
|---|---|---|---|---|
| Use of antibiotics before admission | 116(99.1) | 49(98.0) | 0.000# | >0.999 |
| Length of hospitalization (d) | 4.0(4.0,6.0) | 7.0(5.8,8.0) | -6.607 | <0.001 |
| ICU | 14(11.9) | 10(20.0) | 1.837 | 0.175 |
| Fever | 114(97.4 ) | 48(96.0) | 0.249 | 0.618 |
| Cough | 114(97.4) | 47(94.0) | 1.194 | 0.275 |
| phlegm | 72(61.5) | 24(48.0) | 2.627 | 0.105 |
| Vomiting | 14(28.0) | 10(20.0) | 1.837 | 0.175 |
| Rash | 4(3.4) | 0(0) | 0.594# | 0.441 |
| Dizziness | 2(1.7) | 0(0) | 0.024# | 0.878 |

#chi-square test continuity correction

**Comparison of relevant laboratory test indexes between the two groups**

The PLT, ALT, AST, LDH and CK of the children in the mixed infection group were elevated and higher than those in the simple infection group, and the difference was statistically significant (P < 0.05), especially for ALT and LDH (P < 0.001). While other blood routine related indexes, coagulation function indexes and renal function indexes were compared, the

difference was not statistically significant (P > 0.05). See Table 2.

**Comparison of complications between the two groups**

The incidence of complications of pleural effusion, liver function impairment and cardiac enzyme spectrum abnormality was higher in the mixed infection group than in the simple infection group, and the difference was statistically significant (P < 0.05). There was no significant difference in the incidence of coagulation dysfunction, ketonuria, pulmonary atelectasis and micronutrient deficiency between the two groups (P > 0.05).

## Discussion

MP is microorganisms intermediate between cells and viruses that cause respiratory infections [14] and is one of the major pathogens causing community-acquired pneumonia in children, prevalent in children aged 4-6 years [15].MP infections exhibit a range of signs and symptoms from asymptomatic infections to severe and potentially fatal

**Table 2** Comparison of laboratory test results between simple infection group and mixed infection group

| | Simple infection group | Mixed infection group | t/Z value | P value |
|---|---|---|---|---|
| WBC | 6.66(5.61,8.89) | 7.12(5.82,9.23) | -1.213 | 0.225 |
| NEU | 59.98±12.46 | 61.04±13.70 | -0.490 | 0.624 |
| LYM | 31.00±12.05 | 30.68±13.34 | 0.150 | 0.881 |
| MON | 6.50(5.35,8.30) | 5.95(4.78,7.75) | -1.384 | 0.166 |
| PLT | 301.00(251.00,362.50) | 344.00(266.00,414.25) | -1.966 | 0.049 |
| CRP | 9.24(3.11,20.56) | 10.33(2.21,20.21) | -0.687 | 0.492 |
| PCT | 0.08(0.05,0.10) | 0.08(0.05,0.14) | -0.055 | 0.956 |
| PT | 12.60(12.10,13.00) | 12.58(12.10,13.00) | -0.005 | 0.996 |
| APTT | 38.78±5.76 | 38.91±4.73 | -0.160 | 0.873 |
| FIB | 4.59(4.06,5.04) | 4.50(3.94,5.03) | -0.650 | 0.516 |
| TT | 16.80(16.20,17.20) | 16.57(16.05,17.20) | -1.269 | 0.204 |
| DD | 0.68(0.42,1.06) | 0.55(0.36,0.89) | -1.595 | 0.111 |
| FDP | 2.71(2.05,3.86) | 2.33(1.52,3.69) | -1.319 | 0.187 |
| TP | 68.4(64.9,72.7) | 67.4(64.1,72.18) | -0.606 | 0.544 |
| ALT | 16.00(13.00,20.00) | 23.00(15.00,33.25) | -4.073 | <0.001 |
| AST | 29.00(24.50,34.00) | 31.50(25.00,49.25) | -2.223 | 0.026 |
| LDH | 289.00(259.00,346.00) | 342.50(304.25,450.25) | -4.108 | <0.001 |
| CK | 71.00(45.5,106.00) | 92.00(60.25,230.20) | -2.473 | 0.013 |
| UREA | 3.19(2.47,3.83) | 3.23(2.64,3.89) | -1.148 | 0.251 |
| CRE | 32.60±7.79 | 31.03±7.61 | 1.198 | 0.860 |
| UA | 221.00(185.50,289.00) | 214.5(185.00,260.00) | -0.620 | 0.535 |
| eGFR | 185.00(178.00,209.00) | 195.00(179.25,223.50) | -0.699 | 0.485 |

**Table 3** Comparison of complications between the simple infection group and the mixed infection group (cases (x/%))

| | Simple infection group | Mixed infection group | $\chi^2$/t/Z value | P value |
|---|---|---|---|---|
| Pleural effusion | 5(4.3) | 13(26.0) | 17.195 | <0.001 |
| Lung atelectasis | 17(14.5) | 9(18.0) | 0.321 | 0.571 |
| Hepatic impairment | 5(4.3) | 10(20.0) | 10.597 | 0.001 |
| Ketonuria | 7(6.0) | 7(14.0) | 1.981 | 0.159 |
| Coagulation abnormalities | 36(30.8) | 10(20.0) | 2.124 | 0.145 |
| Abnormal cardiac enzyme profile | 1(0.8) | 7(14.0) | 13.727 | <0.001 |
| Micronutrient deficiencies | 44(37.6) | 17(34.0) | 0.197 | 0.658 |

intra- and extra-pulmonary complications.

It has been documented that the co-infection rate of MP with other respiratory infection pathogens is 88.49% [16]. However, in some cases, these co-infections can lead to severe disease. In this study, when MRMP was co-infected with other pathogens, the length of hospitalization was prolonged, and in addition, the serum levels of ALT, AST, LDH, and CK were increased in patients, which may be related to the extrapulmonary complications of MP infection and the activation of the immune response in children's organisms [17].

In recent years for the treatment of MPP in children the first choice is macrolides, which to some extent can relieve the symptoms of patients. However, overuse of macrolides has resulted in the development of macrolide-resistant antibiotic resistance. Since the first isolation of drug-resistant strains from patients in Japan in 2001, countries have begun to emphasize and monitor the occurrence of resistance rates. In China, there is some variability in the resistance rate in each region. For example, the resistance rate was 60.33% in Guangyuan [18] and 93.88% in Yunnan [19]. The results of this study found that the resistance mutation rate in Hefei region during the MP epidemic was as high as 100% and was a mutation at the A2063G locus, which is the mutation locus with the highest incidence in China at present [20].

Research has found that during follow-up of some pediatric patients treated with azithromycin according to standard protocols, some patients developed antibiotic-resistant gene mutations within 2 to 4 weeks[18], indicating that antibiotics can induce resistant mutations or select for resistant strains in the short term. This highlights the importance of avoiding unnecessary antibiotic use. Additionally, studies have shown that macrolide antibiotics have some efficacy against MRMP infections, but this is not because MRMP is sensitive to macrolide antibiotics, but rather due to the self-limiting nature of MP infections and the anti-inflammatory effects of macrolide drugs [21]. Other studies have also found that the use of macrolide antibiotics does not provide significant benefits for mild MP infections [22]. Therefore, the standardized use of macrolide drugs is essential. It is crucial to continue strictly adhering to the standards for the use of macrolide antibiotics to reduce the emergence and spread of drug-resistant strains. Clinicians should promptly monitor the drug resistance of MRMP patients and rationally select antimicrobial drugs to minimize the emergence of drug-resistant strains, thereby providing a scientific basis for MP treatment. Avoiding unnecessary antibiotic use and curbing the emergence of microbial drug resistance is of critical importance to children, families, and the healthcare system.

Children with MPP primarily present with fever and cough. Early pulmonary signs may not be prominent, but as the condition progresses, decreased breath sounds, dry rales, and wet rales may appear. Severe Mycoplasma pneumoniae pneumonia (SMPP) typically occurs around one week into the illness, often accompanied by pulmonary and extrapulmonary complications. A small number of MPP cases may progress to critical illness, often presenting with prominent symptoms of dyspnea and respiratory failure. The Guidelines for the Diagnosis and Treatment of Mycoplasma Pneumonia in Children (2023 Edition) emphasize that the key to managing mycoplasma pneumonia lies in the early identification and treatment of SMPP and fulminant Mycoplasma pneumoniae pneumonia (FMPP). The optimal treatment window is within 5–10 days after the onset of fever. Early diagnosis and intervention for children with MP are crucial in preventing the occurrence of complications.

This study indicates that the incidence of liver function impairment and complications is higher in the mixed infection group than in the single infection group. Previous studies have shown that there are differences in LDH elevation between the resistant group and the sensitive group, while CRP changes are similar [23]. This not only confirms that MRMP exhibits a stronger immune response but also suggests that LDH is a more sensitive immunological indicator than CRP. Imaging findings are one of the primary bases for clinically assessing the severity of MPP in children and evaluating prognosis. Early imaging examinations aid in the early identification and

treatment of SMPP and FMPP, enabling the development of individualized treatment plans based on classification. For mild cases, systemic corticosteroids should not be routinely used in addition to anti-MP therapy. However, corticosteroids are frequently used in cases of MP infection complicated by excessive inflammatory responses. Studies have shown that corticosteroids can rapidly improve clinical and imaging findings in severe MPP [24]. The Chinese Expert Consensus on the Diagnosis and Treatment of MPP in Children also states that systemic corticosteroids may be considered for acute-onset, rapidly progressing, and severe MPP [25].

Domestic studies have found that among MP-associated viral infections, respiratory syncytial virus accounts for the highest proportion, While among MP-associated bacterial infections, Streptococcus pneumoniae and Klebsiella pneumoniae are more common [26]. Children with Epstein-Barr virus(EBV)-associated MP infections experience longer fever durations and are more prone to respiratory distress or pleural effusion, suggesting that during clinical treatment, when prolonged fever, respiratory distress, or pleural effusion occurs, the possibility of co-infection should be considered [27]. In the Taipei study, 64.7% of patients requiring ICU admission presented with pleural effusion, indicating a high incidence of pleural effusion as a comorbidity [28], which is similar to our results. WBC, PLT, Hb levels, fever duration, dyspnea, and pleural effusion are useful in diagnosing MPP in children with EBV infection. Multi-organ damage is exacerbated by mixed infections, which may involve complex immune response mechanisms. Some scholars have suggested that most extrapulmonary manifestations may be caused by disturbances in the adaptive immune response. Cellular and humoral immunity, including tissue-specific T cells and autoantibodies, may be involved in the pathogenesis of extrapulmonary manifestations of MP [29]. The results of this study showed that the occurrence of liver damage and cardiac enzyme profile abnormalities and pleural effusion was significantly higher in the mixed infection group than in the simple infection group in Hefei area. Therefore, the possibility of mixed infections is alerted to the occurrence of some complications in combination with the occurrence of some comorbidities in the children attending the clinic in Hefei area.

Our study has several limitations, first, the sample size was too small and from a single center, and also the condition of the children admitted to the hospital during the Mycoplasma pneumoniae outbreak may have been inherently more severe. These two points suggest some bias in our results. We acknowledge that our study may be subject to the inherent limitations of retrospective data collection and have taken steps to mitigate their impact. Selection bias was addressed by applying strict inclusion and exclusion criteria during participant/study selection. While retrospective designs have inherent limitations, we believe our rigorous methodology provides meaningful clinical insights.

## Conclusion

In summary, this study found that during the outbreak of MP, the drug-resistant mutation rate of hospitalized children in Hefei was 100%, and that mixed infection of MRMP with other pathogens led to prolonged hospitalization and higher laboratory-related indicators in patients. In addition, there was an increased incidence of pleural effusion, hepatic impairment, and complications of abnormal cardiac enzyme profiles. This requires clinicians to pay close attention to the changes in the patient's disease during the course of treatment in order to prevent complications from affecting the quality of life of the child.

## Abbreviations

ALT: Alanine aminotransferase, APTT: activated partial thromboplastin time, AST: aspartate aminotransferase, CAP: community-acquired pneumonia, CK: creatine kinase, COVID-19: Coronavirus Disease 2019, CRE: creatinine, CRP: C-reactive protein, DD: D-dimer, EBV: Epstein-Barr virus, eGFR: estimated glomerular filtration rate, FDP: fibrinogen degradation product, FIB: fibrinogen, FMPP: fulminant Mycoplasma pneumoniae pneumonia, ICU: Intensive Care Unit, LDH: lactate dehydrogenase, LYM: lymphocyte, MON: monocytes, MP: Mycoplasma pneumoniae, MPP: Mycoplasma pneumoniae pneumonia, MRMP: macrolide-resistant Mycoplasma pneumoniae, NEU: neutrophils, PCT: procalcitonin, PLT: platelet count, PT: prothrombin time, RMPP: refractory Mycoplasma pneumoniae pneumonia, SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2, SMPP: severe Mycoplasma pneumoniae pneumonia, TP: total protein, TT: thrombin time, UA: uric acid, UREA: urea, WBC: white blood cell count.

## Author Contributions

Zhengyuan Wang conceived the project and collect clinical data. Zhengyuan Wang performed data analysis and wrote the initial manuscript. Yuanhong Xu provided useful advice regarding the manuscript. All authors read and approved the final manuscript.

## Acknowledgments

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## Ethics Approval and Consent to Participate

The current investigation was duly approved by the human ethics council of Anhui Medical University's First Affiliated Hospital (Approval No: Quick-PJ 2023-08-41). The written informed consent was obtained from patients in accordance

with the Declaration of Helsinki.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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# Transthoracic pulmonary artery radiofrequency denervation (TPARFD) concomitantly with cardiac operations in adult atrial septal defect with pulmonary arterial hypertension: case report

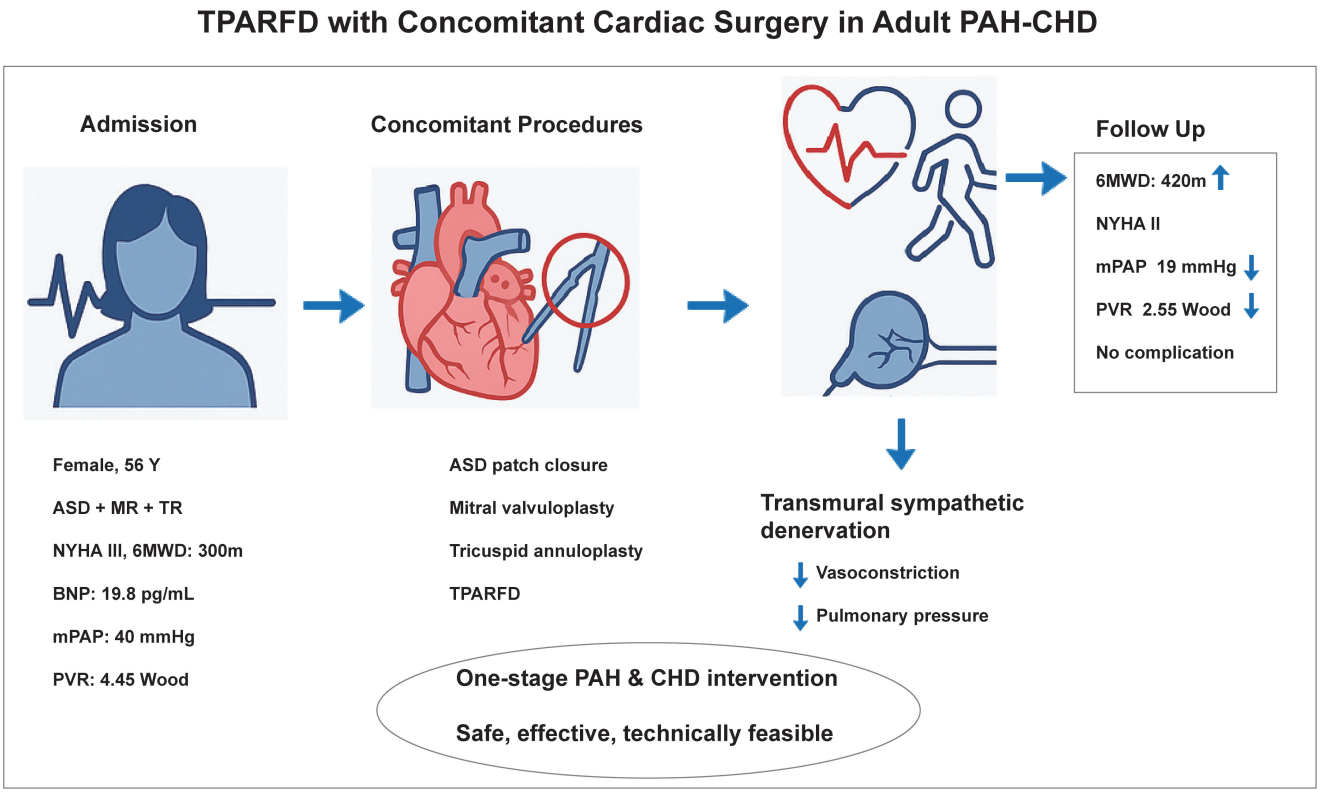

## Authors

**Wenpeng Dong,** Shenglin Ge, Min Lin, Weida Zhang

## Correspondence

517027671@qq.com (W. Dong)


## Graphical Abstract

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# Transthoracic pulmonary artery radiofrequency denervation (TPARFD) concomitantly with cardiac operations in adult atrial septal defect with pulmonary arterial hypertension: case report

**Wenpeng Dong** [1*], Shenglin Ge [1], Min Lin [1], Weida Zhang [2]

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## Abstract

Pulmonary arterial hypertension (PAH) is a life-threatening progressive disorder caused by increased resistance in the pulmonary vasculature. The condition may lead to right-sided heart failure and death. A 56-year-old woman was referred for pulmonary arterial hypertension (PAH) with atrial septal defect, mitral regurgitation, and tricuspid regurgitation. Hemodynamic parameters were obtained by right heart catheterization. She underwent transthoracic pulmonary artery radiofrequency denervation (TPARFD), to relieve the pulmonary artery pressure, while concomitantly undergoing mitral valvuloplasty with 28 # Sorin ring, patch closure of atrial septal defect, and tricuspid annuloplasty with 28 # Edwards MC3 ring. Her hemodynamic parameters and functional capacity showed marked improvement after TPARFD.

**Keywords:** Pulmonary artery radiofrequency denervation; Pulmonary arterial hypertension; Congenital heart disease.

## Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening progressive disorder. The high prevalence of pulmonary arterial hypertension in adults is commonly associated with congenital heart disease [1] . A majority of the cardiac defects are successfully corrected by surgery; however, a considerable proportion of patients experience aggravation of PAH and remain at a high risk [2] . Several clinical studies have demonstrated the safety and effectiveness of percutaneous pulmonary artery denervation (PADN). However, pre-operative patients with congenital heart disease were not included in the clinical studies [3-5] . In addition, PAND alone, without repairing the simple pre- or post-tricuspid shunts, might insult the pulmonary pulmonary circulation in the PAH-CHD patients. Herein, we designed a new surgical operation method, transthoracic pulmonary artery radiofrequency denervation (TPARFD), to concomitantly relieve the pulmonary artery pressure during repair of the pre- or post-tricuspid shunts in PAH-ACHD (Figure 1).

## Case Description

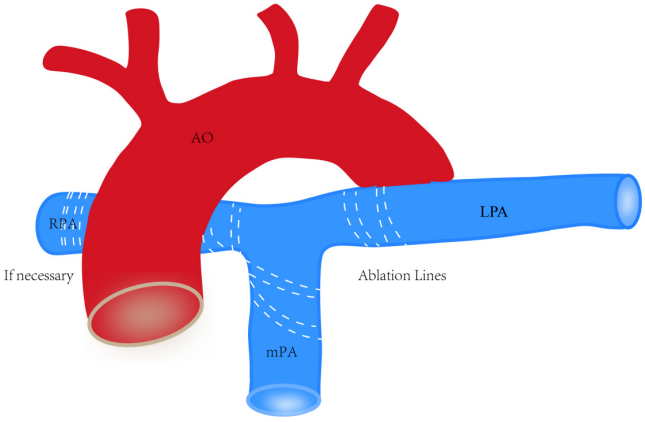
A 56-year-old woman was diagnosed with atrial septal defect seven months ago. The patient was asymptomatic and did not receive any treatment. On February 22, 2021, the patient came to our hospital for further treatment. At admission, her blood pressure was 125/68 mmHg and her pulse rate was 70 bpm. The New York Heart Association functional classification was class III. Six-minute walk distance was 300 m. The B-type natriuretic peptide (BNP) level was 19.8 pg/mL. Transthoracic echocardiography revealed an atrial septal defect (size: 35.9×35.6 mm) with left to right shunt, marked enlargement of the right atrium (size: 53×65.5 mm), left ventricle end-diastolic dimension of 40 mm, ejection fraction of 66%, tricuspid regurgitation, severe PH with a tricuspid systolic pressure gradient of 82 mmHg. Tricuspid Annular Plane Systolic Excursion (TAPSE) was 23.5 mm. Right ventricular fractional area of change (RV-FAC) was 47%. The diameter of the central pulmonary artery was 43.5 mm. Twelve-lead electrocardiogram showed sinus rhythm, complete right bundle branch block, and QRS wave width of 114 ms. Right heart catheterization (RHC) showed elevated mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance (PVR) of 40 mmHg and 4.45 Wood, respectively. Cardiac output was 8.31 L/min estimated by the Fick method. The Qp/Qs ratio was 1.85. We started supplemental oxygen therapy and medical therapy with ambrisentan (10mg/day) and sildenafil (75mg/day).
She underwent transthoracic pulmonary artery radiofrequency

1 Department of Cardiovascular Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, China.
2 Department of Cardiovascular Surgery, General Hospital of Southern Theatre Command of PLA, Guangzhou, China.
* Corresponding Author.

denervation (TPARFD) to relieve the pulmonary artery pressure, while concomitantly undergoing mitral valvuloplasty with 28 # sorin ring, patch closure of atrial septal defect, and tricuspid annuloplasty with 28 # Edwards MC3 ring.

The combination medical therapy was continued after procedure. Six-minute walk distance was increased to 350 m at 1 month and 420 m at 3 months. The New York Heart Association functional classification was class II without oxygen administration, and the chest X-ray and echocardiography demonstrated the improvement of right ventricular overload. Her BNP level was 98.8 pg/mL. RHC performed 3 months after operation showed marked improvement in cardiac hemodynamics 3 days after stopping taking medications, mPAP was 19 mmHg and PVR was 2.55 Wood units. There was no airway injury, hemoptysis, PA dissection, vessel defect, pulmonary embolism, or hemorrhagic pleural effusion.

**Figure 1.** Schematic illustration of pulmonary artery radiofrequency denervation.

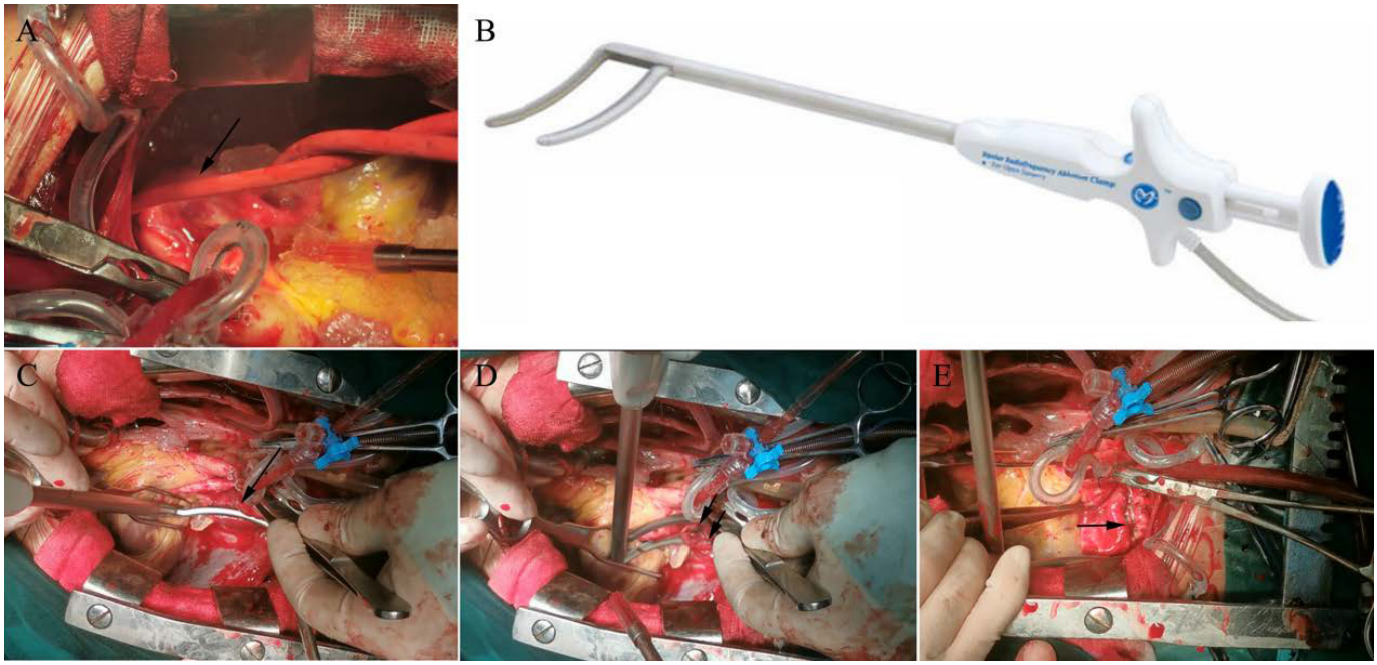



## Transthoracic Pulmonary Artery Radiofreqency Denervation

A straight vertical midline skin incision was made through a median sternotomy while placing the patient on cardiopulmonary bypass using bicaval cannulation. Initially, the space between the aorta and the dilated main pulmonary artery and the left pulmonary artery was bluntly dissected. Occasionally, a sharp division was necessary. After cardiopulmonary bypass, the posterior wall of the dilated main pulmonary artery, and the anterior and posterior walls of the right pulmonary artery were dissected. The bipolar clamp was placed from the right side, after carefully developing the space between the right pulmonary artery and the ascending aorta using blunt dissection to avoid any injury (Figure 1). On the left side, the ligament of Marshall was divided with cautery, while the left pulmonary artery was bluntly dissected and surrounded by a red catheter (Figure 2A).

PARFD was performed with linear ablation using bipolar radiofrequency (RF) energy simultaneously with cardiopulmonary bypass rewarming. The ablation device (Med-Zenith Ltd., Beijing, China) (Figure 2B) delivers RF energy through two closely approximated 6-cm electrodes embedded in the jaws of the clamp. The RF device was placed around the left pulmonary artery bifurcation (Figure 2C) for delivering the RF energy until the algorithm confirmed transmurality for two times (Figure 2D). Following the initial ablation, the device was unclamped, moved proximally by several millimeters on the left pulmonary artery, and re-clamped for a second ablation to ensure electrical isolation.

**Figure 2.** The left pulmonary artery is bluntly dissected and surrounded with a red catheter (**A**). The radiofrequency ablation device (**B**). The bipolar radiofrequency device is placed around the left pulmonary artery bifurcation (**C**). The black arrows indicate the two ablation lines at the ostial left pulmonary artery (**D**). The black arrow indicates the two ablation lines at the bifurcation of the main PA (**E**).



Following completion of the left pulmonary artery isolation, the dilated main pulmonary artery and the right pulmonary artery bifurcation were isolated in a similar manner with the RF device (Figure 2E).

## Discussion

At first, we were planning to perform transthoracic denervation with the cut-and-sew method, or by stripping the adipose and connective tissues around the main pulmonary artery trunk and its bifurcation and the proximal regions of the left and right pulmonary artery, similar to the transthoracic PADN procedure in monocrotaline PH rat models [8] . However, these surgical procedures are time-consuming and it is challenging to achieve hemostasis. Considering the safety and efficacy of RF ablation in the treatment of atrial fibrillation, the bipolar RF ablation device may prove more effective and safer for transthoracic denervation.

Unlike the other techniques available for the treatment of PAH-CHD, such as sympathetic ganglion block, catheter-based renal denervation, and PADN, TPARFD is a novel therapeutic option, although an invasive and high-risk procedure. Therefore, it is better to perform PARFD only in adult PAH-CHD cases, concomitantly with cardiac operations.

In contrast to the percutaneous PADN, TPARFD is performed with two ablation lines at the ostial left pulmonary artery and the bifurcation of the main pulmonary artery and ostial right pulmonary artery, and not just the ten points embedded on the circular tip of the ablation catheter.

## Conclusion

TPARFD is a novel and effective therapeutic option for the treatment of PAH-ACHD which may be performed concomitantly with other cardiac surgeries. Further clinical study is required to confirm the efficacy of TPARFD in PAH-ACHD.

## Abbreviations

ASD: Atrial Septal Defect; PAH: Pulmonary Arterial Hypertension; TPARFD: Transthoracic Pulmonary Artery Radiofrequency Denervation; PADN: Percutaneous Pulmonary Artery Denervation; PAH-CHD: Pulmonary Arterial Hypertension associated with Congenital Heart Disease; PAH-ACHD: Pulmonary Arterial Hypertension in Adult Congenital Heart Disease; mPAP: mean Pulmonary Arterial Pressure; PVR: Pulmonary Vascular Resistance; BNP: B-type Natriuretic Peptide; TAPSE: Tricuspid Annular Plane Systolic Excursion; RV-FAC: Right Ventricular Fractional Area Change; RHC: Right Heart Catheterization; Qp/Qs: Pulmonary-to-Systemic blood flow ratio; RF: Radiofrequency; 6MWD: 6-Minute Walk Distance.

## Author Contributions

All authors read and approved the final manuscript. Wenpeng Dong: Conceptualization, Methodology, Data curation, Writing - Original Draft, Writing - Review & Editing. Shenglin Ge: Data collection, Formal analysis, Validation, Writing - Review & Editing. Min Lin: Supervision, Project administration, Funding acquisition, Writing - Review & Editing. Weida Zhang: Investigation, Resources, Visualization, Writing - Review & Editing. All authors read and approved the final manuscript.

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## Ethics Approval and Consent to Participate

Not Applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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# Mapping heterogeneous molecular subtypes of circadian misalignment underlying lung adenocarcinoma risk

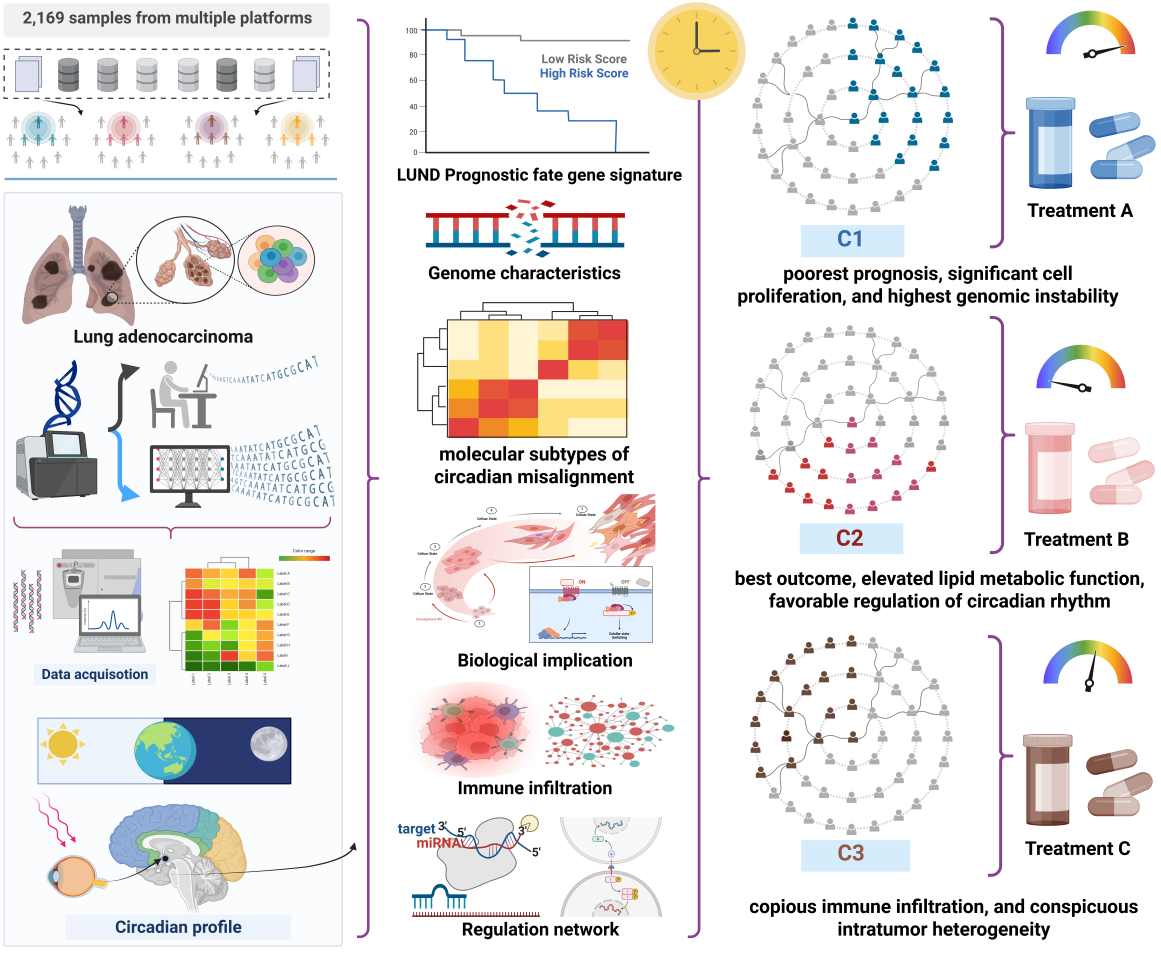

## Authors

**Ruhao Wu,** Teng Li, Kaisaierjiang Kadier, Pengyuan Xu, Peiyu Yuan, Tinglan Fu, Yu Yang, Xufeng Huang, Zhou Shujing, Zhengrui Li, Pengpeng Zhang, Song-Bin Guo, Haonan Zhang, Shiqian Zhang , Chaoyang Yu, Ge Zhang

## Correspondence

zg381492242@163.com (G. Zhang)


## Graphical Abstract

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Research Article Life Conflux

# Mapping heterogeneous molecular subtypes of circadian misalignment underlying lung adenocarcinoma risk

**Ruhao Wu**[1,2], Teng Li [3], Kaisaierjiang Kadier [4], Pengyuan Xu [5], Peiyu Yuan [2], Tinglan Fu [2], Yu Yang [2], Xufeng Huang [6], Zhou Shujing [6], Zhengrui Li [7], Pengpeng Zhang [8], Song-Bin Guo [9], Haonan Zhang [10], Shiqian Zhang [11], Chaoyang Yu [12], Ge Zhang [2*]

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## Abstract

**Background:** The circadian rhythm coordinates multiple physiological and behavioral processes. Substantial evidence illustrates that circadian rhythm disruption (CRD) dramatically influences tumor initiation, progression, and the tumor immune microenvironment remodeling. However, there is a dearth of exploration for CRD heterogeneity's underlying clinical significance in lung adenocarcinoma (LUAD).

**Methods:** 2090 LUAD patients and 79 immunotherapy patients were enrolled from nine public independent datasets. The nonnegative matrix factorization (NMF) was applied to develop molecular classification after collecting CRD-related genes. Subsequently, the reliability and robustness of classification were evaluated through the nearest template prediction (NTP) method. Furthermore, clinical outcomes, functional characteristics, genomic alterations, and immune landscape were explored. The efficacy of clinical common treatment was detected for the specific classification.

**Results:** Three heterogeneous LUAD subtypes were identified based on the expression profile of CRD-related genes. Different expression characteristics and clinical outcomes of distinct subtypes were revealed. Relative similar clinical outcomes and proportion of each subtype were verified in multiple independent cohorts, which indicated the reliability of classification. Distinguish features of three subtypes were further explored: (i) C1, the poorest prognosis, significant cell proliferation, and highest genomic instability. (ii) C2, the best outcome, elevated lipid metabolic function, favorable regulation of circadian rhythm, and (iii) C3, copious immune infiltration, immunosuppressive microenvironment, and conspicuous intratumor heterogeneity. The evaluation of treatment strategies suggested that C1 patients might benefit from chemotherapeutics agents, including docetaxel and paclitaxel, patients in C2 were suitable for glucocorticoids, whereas C3 patients were recommended to accept immunotherapy.

**Conclusions:** We identified three CRD subtypes with distinct characteristics, including clinical outcomes, biological function, genomic alterations, and immune landscape. For individualized subtypes, befitting therapy approaches were proposed. Our study could provide more efficient and precise management to LUAD patients.

**Keywords:** Circadian Rhythm Disruption, Lung Adenocarcinoma, Molecular Subtype, Immunotherapy, Tumor Immune Microenvironment.

## Introduction

Lung adenocarcinoma (LUAD) is the most predominant type of lung cancer, with a high invasion and mortality rate. [1] Presently, with a thorough understanding of LUAD development, earlier diagnosis, earlier detection, and diverse treatments were conducted for patients. However, dismal median overall survival (OS) and 5-year survival rates of LUAD patients persist. [2] Moreover, substantial

1 Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

2 Department of Cardiology, The First Affiliated Hospital of Zhengzhou University; Key Laboratory of Cardiac Injury and Repair of Henan Province; Henan Province Clinical Research Center for Cardiovascular Diseases, Zhengzhou, China

3 Department of Ophthalmology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

4 Department of Cardiology, First Affiliated Hospital of Xinjiang Medical University, Ürümqi, China

5 Cardiac and Osteochondral Tissue Engineering (COTE) group, School of Medicine, The Chinese University of Hong Kong, Shenzhen, China

6 Faculty of Medicine, University of Debrecen, Debrecen, Hungary

7 Department of Oral and Cranio-Maxillofacial Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; College of Stomatology, Shanghai Jiao Tong University; National Center for Stomatology; National Clinical Research Center for Oral Diseases; Shanghai Key Laboratory of Stomatology; Shanghai Research Institute of Stomatology, Shanghai, China

8 Department of Lung Cancer, Tianjin Lung Cancer Center, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China

9 Department of Medical Oncology, Sun Yat-Sen University Cancer Center; State Key Laboratory of Oncology in South China, Collaborative Innovation Center of Cancer Medicine,Sun Yat-Sen University Cancer Center, Guangzhou, China

10 Department of Thyroid Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

11 Department of Colorectal Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

12 Department of Vascular and Endovascular Surgery, First Affiliated Hospital of Zhengzhou University, China

* Corresponding Author.

prognostic differences exist in LUAD patients with similar clinical characteristics, which indicated unsatisfactory risk stratification ability of traditional clinical classification strategy based on clinicopathological characteristics. [3-5] It is inevitable for clinical workers to deduce the tumor heterogeneity and develop a novel stratification approach to improve prognosis and treatment efficacy[6].

The circadian rhythm is a vital biological mechanism in almost all organisms, which coordinates multiple physiological and behavioral processes through the construction of the circadian clock. [7, 8] In recent years, mounting research demonstrate circadian rhythm disruption (CRD) and altered hub circadian genes expression are linked to abnormalities in cell metabolism, cell proliferation, tumor microenvironment (TME), and intratumoral heterogeneity, which contribute to cancer development and progression. [9-11] For instance, enhanced stemness of tumor cells and an immunosuppressive TME were detected in breast cancer mice with chronic circadian disruption. [12]A previous study revealed the existence of intratumoral heterogeneity and related resistance to anti-cancer treatments in LUAD patients with CRD based on single-cell RNA-seq analysis. Besides, Ruan et al. indicated that CRD was a potential target to facilitate the anti-tumor therapeutic efficacy. [13] With the continuous advancements in circadian rhythm research, several CRD-related genes have been detected, such as BMAL1, CLOCK, PER, and CRY.[14, 15] Nevertheless, the relationship among CRD status, molecular characteristics, and clinical outcome in LUAD remains to be elucidated.

Besides, with the advancement of tumor research, plentiful effective therapies have been developed (e.g., chemotherapy, radiation therapy, targeted therapy, and immunotherapy) [5]. Due to the provision of diverse treatment options, the need for individualized treatment and precision medicine ensues. [5, 16] Obviously, a traditional therapy strategy with an insufficient understanding of molecular characteristics was powerless for this requirement. [17-19] Thus, individualized comprehensive treatment which included novel approaches, such as immunotherapy and targeted therapy, was barged to the forefront. [20, 21] However, an effective and rational classification is the essential prerequisite to determining the appropriate treatments. Therefore, it is warranted to identify CRD status heterogeneity and propose new insights for molecular classification, which could offer proper clinical management and precision medicine to LUAD patients.

In our present study, we aimed to address a significant clinical gap in the stratification of LUAD patients by identifying three heterogeneous subtypes based on the expression of CRD-related genes. By utilizing six independent databases, we validated the robustness of the CRD-related subtypes, demonstrating consistent relative fractions, gene expression profiles, and prognostic outcomes across cohorts. Furthermore, we explored the distinct differences among the three subtypes from multiple perspectives, including biological functions, genomic variations, and tumor microenvironment (TME) characteristics. This comprehensive approach allowed us to illustrate how CRD-based classification uniquely adds clinical value compared to existing biomarkers. We also assessed patients' sensitivity to common clinical therapies, which is essential for advancing personalized medicine strategies. Overall, our findings suggest that LUAD patients may benefit from more efficient and precise management if our promising CRD-based stratification platform is implemented in clinical practice.

## Method

**Data acqui sition and procession**

In this study, LUAD cohorts were collected from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) dataset according to the following inclusion criteria: 1. Patients were primary lung adenocarcinoma. 2. The number of patients is more than 100. 3. Patients in the dataset have complete gene expression profiles and corresponding survival information. 4. The probe and gene ID were clearly labeled, with more than 20000 genes. Finally, 2090 patients from TCGA-LUAD (n = 497), GSE72094 (n = 442), GSE68465 (n = 462), GSE50081 (n = 127), GSE42127 (n = 133), GSE41271 (n = 183) and GSE31210 (n = 246) were included. The expression profile and clinical information of the TCGA-LUAD cohort were from UCSC Xena portal. The expression data were converted from fragments per kilobase of million mapped reads (FPKM) transcripts to trans per million (TPM) format and log2 transformed. The remaining cohorts were retrieved from GEO, normalized, and processed using Affy and Lumi packages based on different platforms subsequently[22]. Furthermore, TCGA-LUAD somatic mutation and segmented copy number variation data were received from the TCGA portal. Across all cohorts, the expression of each gene was converted into a Z-score value before model construction.

**Development and validation of CRD-related subtypes**

With the help of previous studies, we retrieved a total of 2091 circadian rhythm disruption-related genes from CircaDB and MSigDB for the development of CRD-related subtypes (Table S1). [23] Nonnegative matrix factorization (NMF) algorithm was applied to identify the optimal number of consensus clusters from the TCGA-LUAD cohort through the NMF package. After decomposing the nonnegative matrix of CRD-related genes and iterating, the cophenetic coefficient was executed to determine the optimal factorization rank. The identification criteria were as follows: possible factorization ranks = 2 – 7, number of iterations = 100, and method = "lee". In general, the rank before the most obvious decrease of the cophenetic coefficient value was considered to be the optimal rank. [24] Then, Partial Least Squares Discriminant Analysis (PLS-DA) was applied to evaluate the separation of three subtypes. PLS-DA was A linear classification method that uses partial least squares regression to identify latent variables that maximize class separation. It is particularly effective in high-dimensional data settings. The limma package was used to decipher the differences among distinct CRD-related subtypes and obtain signature genes for each subtype (log2 fold change (log2 FC) > 1 and adjust P value < 0.05 ). A flexible technique, the nearest template prediction (NTP), was a helpful tool to assess class prediction confidence for single patient. [25, 26] Using signature genes, we implemented the NTP algorithm with the CMScaller package to evaluate the stability and robustness of clusters across multiple GEO validation cohorts from different platforms. An FDR threshold of less than 0.05 was applied to establish appropriate classification confidence

thresholds.

**Exploring specific biological characteristics of three subtypes**

To further explore the biological functional heterogeneity among the three subtypes, we conducted the gene set variation analysis (GSVA), which was widely applied to evaluate the activity of pathways.[5, 27] GSVA was a method used to assess variability in gene sets across samples or conditions. GSVA transforms gene expression data into enrichment scores for gene sets, helping to reveal changes in the activity of biological processes or signaling pathways. Differentially expressed genes (DEGs) were analyzed by limma package and the expression matrix was obtained by arranging all genes in descending order according to log2 FC. Based on this matrix and gene sets from Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and HALLMARK, the GSVA package was implemented to determine specific biological characteristics between each subtype and the others. Meanwhile, the gene set enrichment analysis (GSEA) algorithm was performed to exhibit CRD-related pathway activities through the clusterProfiler package.

**Somatic mutation and copy number variation analysis**

The maftools package was applied for processing and visualizing the genomic alteration data. Based on CNV data obtained from GISTIC 2.0 pipeline, the burden of copy number alteration, including amplification and deletion, was quantified at focal and arm levels. We also calculated the fraction of genome alteration (FGA), fraction of genomic gained (FGG), and fraction of genome lost (FGL) to evaluate genetic changes in three CRD-related clusters.

**Depicting distinct immune landscape and evaluating Immunotherapy**

For deciphering features of the tumor immune infiltration, single-sample gene set enrichment analysis (ssGSEA) was exploited for the quantification of 28 immune cell subsets[28]. Besides, GSVA package was conducted to evaluate the relative infiltration of 24 TIME cells. Six other algorithms including ESTIMATE, TIMER, quantTIseq, MCP counter, EPIC, and xCell were further implemented to verify the stability and reliability of the results. Meanwhile, the assessment of immunogenicity and immunosuppression status was accomplished by computing Immunophenoscore (IPS) and novel S score respectively (Table S2). [29] The IPS of TCGA-LUAD patients was acquired from the cancer-immune group atlas (TCIA, https://tcia.at/home). Human leukocyte antigen (HLA) molecule expression was compared to assess the antigen presentation ability of three subtypes [30]. A range of immune escape-related signatures was collected and estimated to reveal underlying distinct immune escape mechanisms among three subtypes. [31]

For distinct subtypes, immune checkpoint molecules (ICM) expression, T cell inflammatory signature (TIS), and subclass mapping (Submap) algorithm were employed to deduce immunotherapeutic efficacy. A total of 27 ICM were enrolled, including the B7-CD28 superfamily, TNF superfamily, and eight other molecules. [32] As a signature obtained based on ssGSEA algorithm for 18 inflammatory genes, TIS could play a predictor of the response to PD-1 inhibitors. GSEA was an extension of traditional gene set enrichment analysis that evaluates the enrichment of gene sets in individual samples. Unlike GSEA, ssGSEA provides specific scores for each sample, allowing for the analysis of subtle differences between samples [33-35]. A higher TIS score represents a better response to PD-1 inhibitors. Moreover, the Submap algorithm was utilized to estimate the similarity between the three phenotypes and the patients with different immunotherapy responses from two independent immunotherapy cohorts.

**Personalized management for distinct subtypes**

Among the considerable number of published LUAD signatures, we aimed to identify an optimal signature tailored for distinct subtypes to facilitate personalized management. In our research, we retrieved a total of 151 published signatures that were based on various biological processes [36]. To strengthen the rationale for their use, we employed a systematic approach that included univariate Cox regression analysis, followed by a comparison of the concordance index (C-index) across these candidates. This allowed us to identify and prioritize the superior prognostic signatures for each subtype.

**Statistical analysis**

All data processing, plotting, and statistical analysis were conducted in R 4.1.2. Cox regression and Kaplan–Meier analyses were performed via the survival package. The comparison of the survival of categorical variables was completed through the log-rank test. Kruska-Wallis test was applied to compare the difference among the three clusters. A two-sided $P < 0.05$ was considered a statistical significance *1 for all statistical tests.

## Results

**Identification of three CRD-related subtypes**

Based on expression profiles of 2091 CRD-related genes, the NMF approach was applied to decode heterogeneous phenotypes. We selected three subtypes as the optimal rank based on the cophenetic coefficient score and consensus matrix (Figure 1A, S1A). Meanwhile, PLS-DA exhibited the obvious separation of three distinct subtypes (Figure 1B). We further explored the prognostic value of CRD-related subtypes to boost their practice in the clinic. For three subtypes called C1, C2, and C3, C2 displayed better overall survival (OS), while C1 harbored a dismal prognosis (Figure 1C).

**Validation of CRD-related subtypes**

To further demonstrate the stability and reliability of CRD-related subtypes, NTP analysis was performed in five independent cohorts, including GSE72094, GSE68465, GSE41271, GSE50081, GSE42127, and GSE31210 (Figure 1D-F, S1B-D). Signature genes for NTP were defined as specific upregulated DEGs in the individual subtype. Corresponding with a previous study, patients with a false discovery rate (FDR) of more than 0.05 were eliminated in subsequential analysis. Then, a resemblant proportion of three clusters in divergent datasets was exhibited, which hinted stability of CRD-related subtypes (Figure 2A). Moreover, we evaluated the difference in clinical outcomes for distinct subtypes through Kaplan-Meier curves in validation cohorts (Figure 2B-F, S2A). Relatively

**Figure 1 Identification and validation of CRD-related subtypes by nonnegative matrix factorization (NMF) analysis.**
(**A**) Consensus map generated from NMF clustering analysis of The Cancer Genome Atlas-Lung Adenocarcinoma (TCGA-LUAD) cohort. (**B**) Two-dimensional principle component plot of three CRD-related subtypes in the TCGA-LUAD cohort. (**C**) Kaplan–Meier curves of overall survival according to three subtypes in the TCGA-LUAD cohort. (**D-F**) Heat maps depicting the expression levels of template features among three subtypes in the GSE72094, GSE68465, and GSE41271 cohorts.

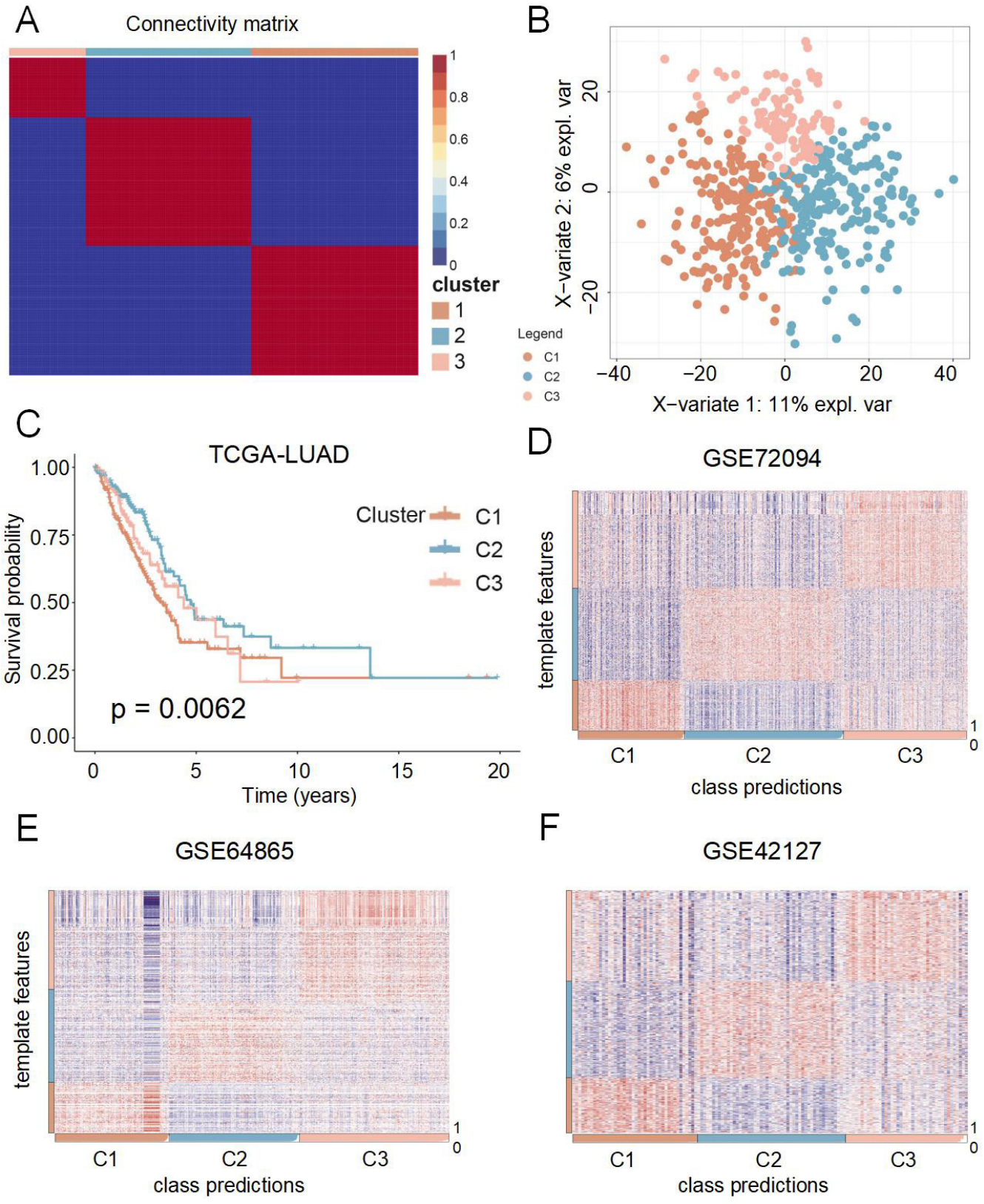

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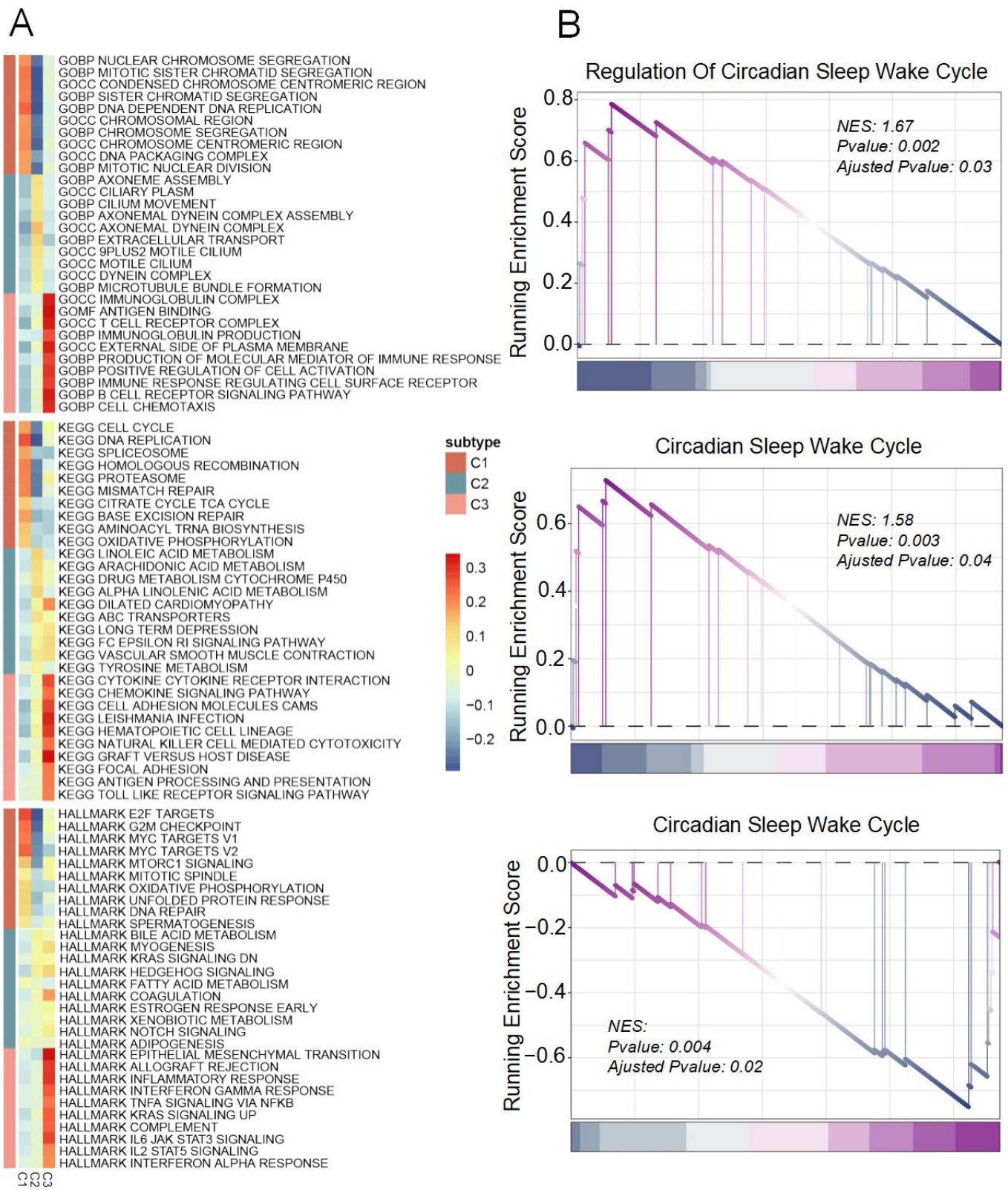
**Figure 2. Similar proportions and heterogeneous overall survival rates in the three subtypes.**
(**A**) Proportions of three subtypes among seven cohorts from distinct platforms. (**B-F**) Kaplan–Meier curves of overall survival rates for three subtypes in the GSE41271, GSE72094, GSE42127, GSE68465, and GSE31210 cohorts.



speaking, C2 subtypes possessed the most favorable OS ($P < 0.05$), whereas C1 exhibited the most frustrating OS, which was consistent with the above findings. Taken together, CRD-related subtypes were stable and robust in LUAD patients and revealed discrepant clinical prognoses.

**Underlying biological functional processes linked to three subtypes**

Based on GO, KEGG, and HALLMARK, enrichment analysis was performed to decode specific biological functions of LUAD patients in distinct subtypes. As illustrated in Figure 3A, significantly activated proliferation pathways were revealed in C1, including cell cycle, DNA replication, and E2F targets. C2 possessed a high correlation with lipid metabolisms such as bile acid metabolism and fatty acid metabolism. Notably, positive regulation of the circadian sleep-wake cycle was detected in C2 patients (Figure 3B), which demonstrated a healthy circadian rhythm. For tumor patients with CRD, clock ablation supports glycolysis and fatty acid synthesis, which was the signature of proliferative metabolism. Consistent with that, patients in C1 displayed negative regulation of the circadian sleep-wake cycle (Figure S2B), which signified its CRD status. However, elevated lipid metabolism activity impeded such a metabolism change, which coincided with depressed proliferation pathways in C2. Meanwhile, we observed C3 mainly harbored enhanced immune response and up-regulated signaling pathways of immune factors including cytokine, chemokine, and complement. Therefore, C3 was characterized as immune LUAD. Analogously, we defined C1 as proliferative LUAD and C2 as lipid-metabolic LUAD.

**Genomic alterations of three distinct subtypes**

By displaying and comparing the mutation frequency of the top 20 mutant genes in three subtypes, we detected C1 patients possessed the highest mutated frequency, especially TP53 and TTN mutations (Figure 4A). Meanwhile, an overview of single-nucleotide polymorphism (SNP), insertion and deletion (INDEL), and tumor mutation burden (TMB) were displayed to describe somatic variants comprehensively. C1 demonstrated the richest somatic mutations, which was consistent with the result of mutated frequency (Figure 4A). As a general mutation in LUAD, TP53 mutation conferred more vigorous malignant proliferation and poorer prognosis to patients. [37-39] In line with that, a significantly higher stemness index of C1 was indicated, which represented powerful malignant proliferation ability (Figure S2C). C2 had lower somatic mutation compared to other subtypes, implying better outcomes. A comparison of CNV in three CRD-related subtypes was also performed to further decode genomic alterations. Strikingly, C1 harbored obvious CNV at bases, fragments, and chromosome levels, which suggested a higher likelihood of cell proliferation and immune escape (Figure 4B). [40] Overall, prominent genomic alterations were revealed for patients in C1, suggesting a subtype with high genomic instability.

**The depiction of immune infiltration and immune escape landscape**

Immune infiltration and immune escape play an essential role in tumorigenesis and the development and prognosis of patients. Therefore, we depicted the landscape of immune infiltration and immune escape in three heterogeneous subtypes. Firstly, we quantified the relative abundance of 28 immune cells in three subtypes. Consistent with our results through multiple algorithms, C3 showed a more abundant immune-cell infiltration than the other two subtypes (Figure 5A, Figure S2D). However, IPS and the S score hinted lowest immunity and conspicuous immunosuppression of C3 patients, which might be associated with elevated infiltration of immunosuppressive cells, such as MDSC, Treg, and Th17. Conversely, C2 possessed vigorous immunity and the least immunosuppression (Figure 5B-C). Besides, we explored underlying mechanisms of immune escape for the three subtypes. Among the three subtypes, C3 exhibited the highest expression of HLA molecules, which represented superior power for antigen presentation, while C1 displayed the deficient capability to present antigen (Figure 5D). Furthermore, a spectrum of immunogenicity indicators was evaluated, including neoantigen load (single nucleotide variant (SNV) and indel neoantigens), cancer/testis-antigens score (CTA Score), and genomic instability-related indicators. As illustrated in Figure 5E, C1 owned a high level of immunogenicity but insufficient antigen processing and presentation capabilities, including TCR Richness and Shannon. Meanwhile, C2 and C3 showed lower immunogenicity, and C3 showed the highest intratumor heterogeneity (ITH) specifically. Taken together, inadequate immune cell infiltration and deficient antigen procession and presentation capacity were the main immune escape mechanism for the C1 subtype. The absence of immunogenicity might be responsible for immune escape in C2. For C3, copious infiltration of immunosuppressive cells, high level of ICI expression, and ITH contributed to immune escape.

**The assessment of response to immunotherapy**

Immunotherapy is recommended for clinical treatment because of its good efficacy and fewer side effects. Therefore, we evaluated the benefit of immunotherapy in CRD-related subtypes to further guide the clinical application of immunotherapy approaches. Notably, C3 harbored the highest level of immune checkpoints (Figure 6A), which suggested the benefit of immune checkpoint inhibitors (ICIs). We further enrolled TIS and Submap algorithm to evaluate the efficacy of immunotherapy. As expected, C3 had the highest TIS score, indicating that it was more likely to benefit from ICI treatment (Figure 6B). In line, in two independent immunotherapy cohorts, C3 showed an expression profile that was more similar to that of patients who responded to anti-PD-1 treatment (both Bonferroni corrected and Nominal P value < 0.05) (Figure 6C). In conclusion, immunotherapy was a recommended treatment modality for patients in C3.

**Potential drug development**

To facilitate the personalized treatment of each subtype, we used the pRRophetic package to evaluate the half-maximal inhibitory concentration (IC50) of potential sensitive drugs for C1 and C2 subtypes based on drug sensitivity data from CTRP and PRISM databases. For common clinical chemotherapeutic agents such as docetaxel and paclitaxel, C1 patients displayed better sensitivity, suggesting an effect of chemotherapy (Figure 6D, Figure S2E). Meanwhile, C2 patients may benefit more from corticosteroids, including dexamethasone and prednisone (Figure 6E, Figure S2F).

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**Figure 3. Biological function landscape of distinct CRD subtypes.**
(**A**) The activation states of GO, KEGG, Hallmark pathways of distinct CRD subtypes in the TCGA cohort. (**B**) Enrichment plots depicted by gene set enrichment analysis based on CRD-related gene sets from GO and KEGG.

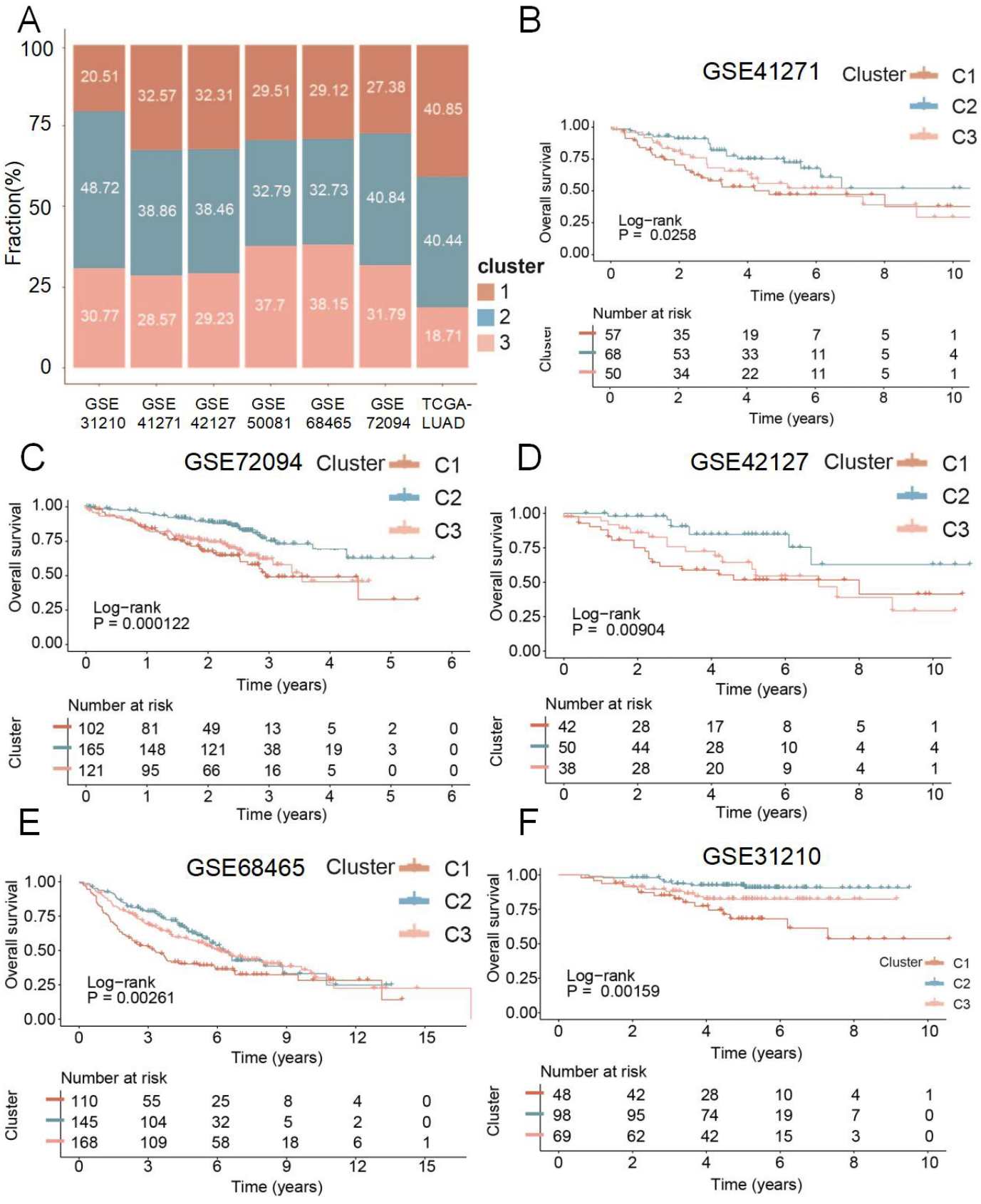

**Figure 4. Genomic alterations of the CRD subtypes.**
(**A**) The waterfall plot depicted the differences in frequently mutated genes (FMGs) of among three subtypes. (**B**) Distributions of fraction of genome alteration (FGA), fraction of genomic gained (FGG), fraction of genome lost (FGL), arm gain, arm loss, focal gain, and focal loss.

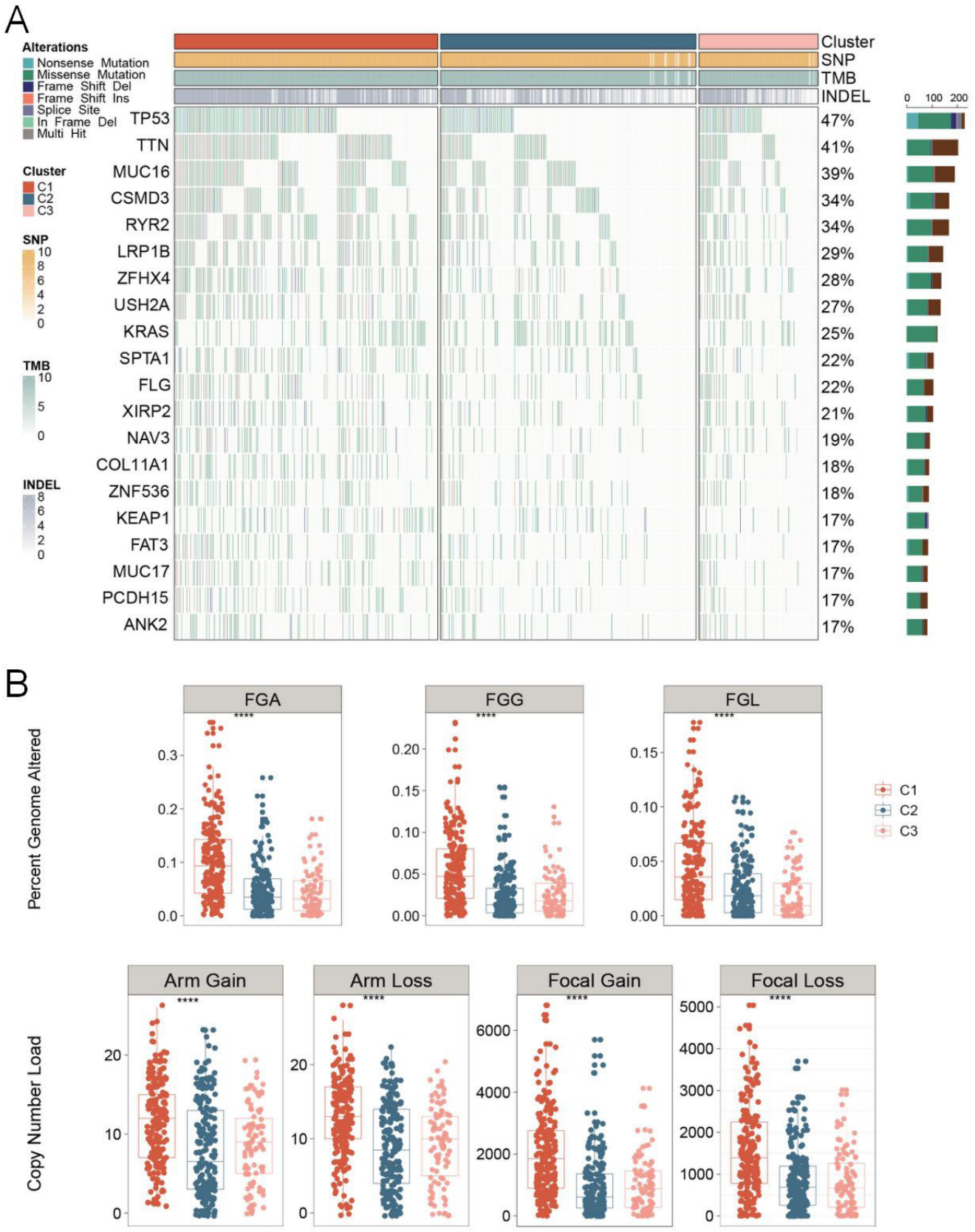

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**Figure 5. Immune landscape of distinct CRD subtypes.**

(**A**) Box plot of infiltration abundance for 28 immune cell subsets analyzed by a single-sample gene set enrichment algorithm. (**B**) Distribution difference of Immunophenoscore among three subtypes. (**C**) S score distribution across three subtypes. (**D**) Distribution of nine human leukocyte antigen molecular expressions among three subtypes. (**E**) Heat map of tumor underlying immune escape mechanisms among three subtypes.

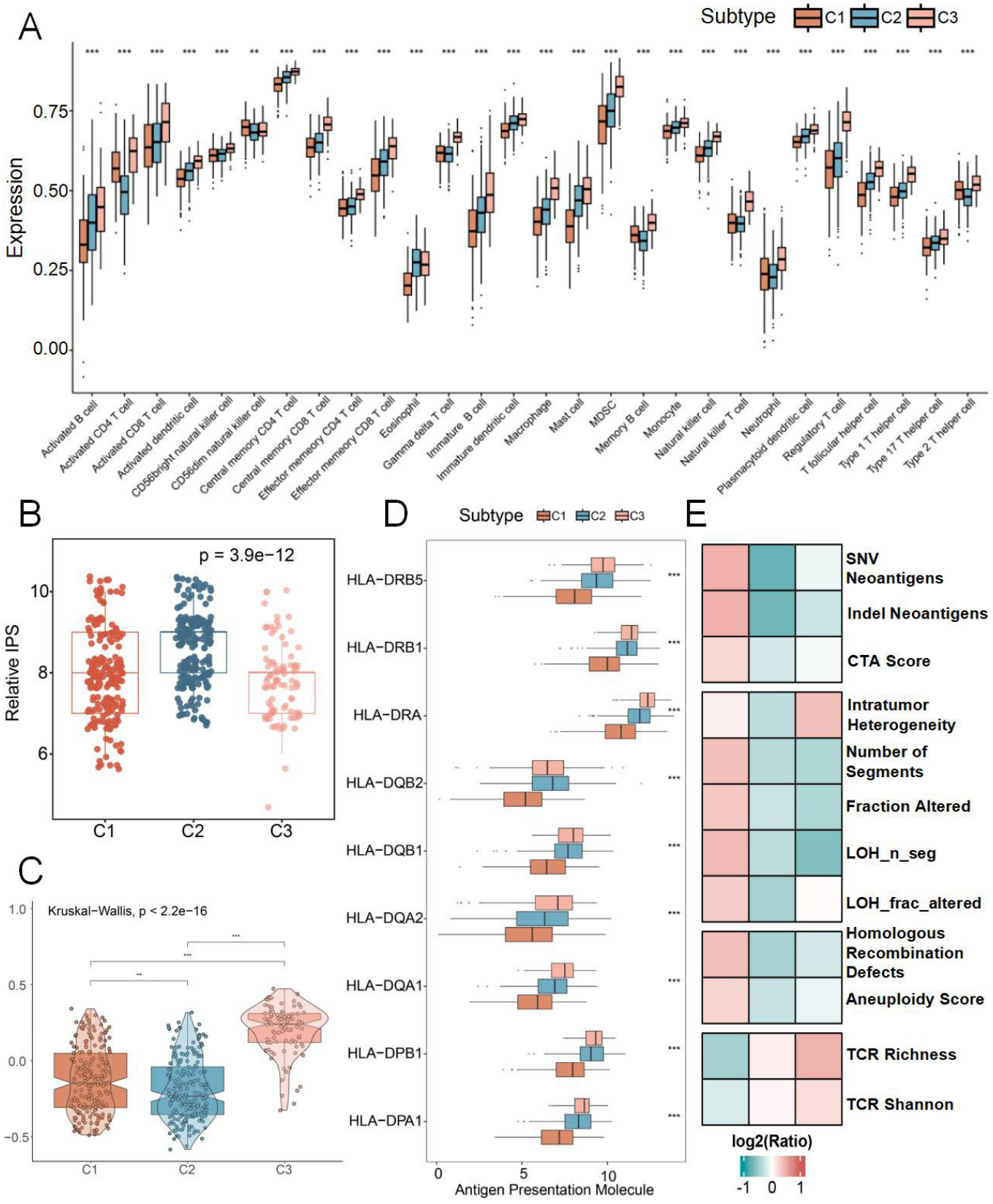
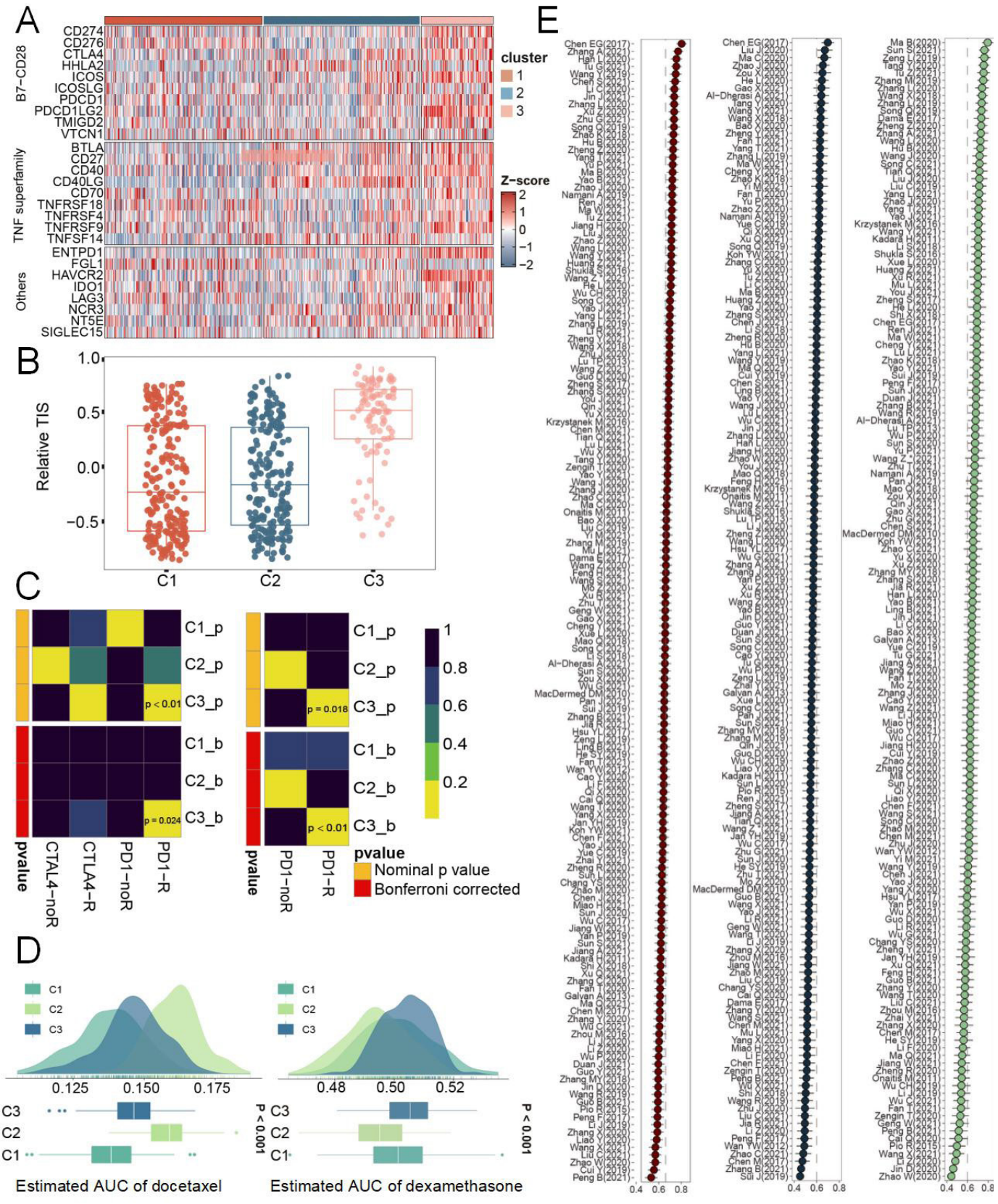


**Figure 6. Potential treatment strategies and clinical delicacy management.**
(**A**) Heatmap of twenty-seven immune checkpoint profiles across three subtypes. (**B**) Variations in the distribution of T cell inflammatory signature prediction scores among three subtypes. (**C**) Immunotherapy responses of three subtypes from Submap analysis. (**D, E**) Potential sensitive drugs for C1(D) and C2(E). (**F**) The C-index of published prognostic models in the TCGA-LUAD cohort.



**Clinical delicacy management**

Technologies of next-generation sequencing and machine learning have flourished, which provided a powerful basement for the development of prognostic tools. Several prognostic signatures were constructed and validated to aid clinical decisions. After a systematic search of the published literature, a total of 151 LUAD prognostic signatures based on ample biological processes and robust machine-learning algorithms were collected. In the TCGA-LUAD cohort, the risk score of each model was recalculated for all patients and appraised their efficiency via the C-index (Figure 6F). Interestingly, Chen EG (2017) possessed the most accurate discrimination for both C1 (C-index = 0.741) and C2 (C-index = 0.698) patients. [41] Therefore, the model was suitable to predict their prognosis to optimize clinical management. Similarly, Ma B (2020) was the optimum signature for patients in C3 (C-index = 0.796). [42]

## Discussion

The heterogeneity of LUAD, which contributes to elusive prognosis and treatment sensitivity [43], continues to perplex clinicians and researchers. Considering the critical role of CRD in tumorigenesis and progression [44], it is essential for LUAD patients to accept proper stratification strategies based on CRD status, which helps with delicacy management and personalized treatment. As far as we know, deficiency has hitherto existed in CRD molecular heterogeneity research for LUAD. In this study, heterogeneous CRD subtypes were identified and systematically analyzed specific characteristics from multiple perspectives, including biological function, immune landscape, and genomic alteration. These results could improve our understanding of CRD and refine clinical management and personalized treatment.

We identified three CRD-related subtypes through the NMF algorithm. The reliability and stability of the three subtypes were validated in multiple ways. As an efficient tool, the NTP algorithm was applied to assess the stability of subtypes through the specific DEGs expression profile. Finally, similar specific gene expression profiles, proportions, and prognoses of each subtype were demonstrated in six independent GEO cohorts, suggesting the rationality of CRD-related subtypes. As displayed, C1 possessed the worst prognosis, C2 owned the best outcome, and the OS of C3 was between C1 and C2.

As described, there was distinct heterogeneity of biological functions in three subtypes. C1 was depicted by activated proliferation pathways, C2 was characterized by enrichment of lipid metabolic, whereas C3 was distinguished by prominent association to immune-related function. Besides, C1 displayed negative regulation of the circadian sleep-wake cycle. In contrast, C2 was enriched in the regulation of the circadian sleep-wake cycle. Notably, the CRD status of the three subtypes revealed similar trends with their prognosis. Besides, genomic characteristics of diverse genomic characteristics. C1 possessed the most profound genomic instability from analyses of both somatic mutation and CNV. Previous research revealed that TP53 mutations were associated with active DNA damage repair (DDR) and elevated cell proliferation levels [45, 46]. Besides, an increased risk of immune escape and dismal prognosis appeared with additional TP53 mutation [47]. Correspondingly, with the highest TP53 mutation, C1 presented enrichment in proliferation pathways and a poorer prognosis. Besides, we explored the immune landscape to further depicted the heterogeneous immune microenvironment of three subtypes. As illustrated, C3 possessed conspicuous immune cell infiltration and antigen presentation ability, which was consistent with the “immune-hot” phenotype. Moreover, the assessment of immune escape mechanisms demonstrated that C1 may achieve immune escape primarily due to insufficient TCR richness and TCR Shannon diversity; C2 appears to rely on deficient immunogenicity; and C3 likely utilizes a mechanism driven by high intratumor heterogeneity.

As described above, C1 displayed negative regulation of the circadian sleep-wake cycle, suggesting CRD status. For LUAD patients, systemic and somatic disruption of circadian rhythms contribute to enhanced proliferation and metabolic dysregulation, which resulting in cancer progression and related poor prognosis [48]. Besides, recent research provided evidence that CRD promoted genomic instability in LUAD patients[49], which was consistent with our findings in the C1 subtype. Nevertheless, the specific regulatory relationships among CRD, genomic instability and TP53 mutation still need to be further studied. Notably, CRD-related lipid metabolic dysregulation was a significant factor for tumor invasion and TME remodeling [50]. However, for C2 with relative healthy circadian rhythms, undisturbed lipid metabolism made a contribution for relatively good prognosis.

As is well acknowledged, it’s crucial and essential to provide individualized treatment. Development and clinical application of tumor immunotherapy, especially immune checkpoint inhibitor (ICI) therapy, has revolutionized treatment patterns in LUAD. As humanized monoclonal antibodies for blocking immune checkpoints (such as CTLA-4 and PD-1), ICI works by restoring effective immune cell function. [51, 52] C3, the “immune-hot” subtype, harbored abundant immune cell infiltration and higher expression of immune checkpoints, such as CD8+ T cell, CD4+ T cell, CD274 (PD-L1), PDCD1, and CTLA-4 molecular. Meanwhile, together with the highest TIS score and similar characteristics with patients who respond to ICIs, C3 was thought to derive potential benefits from ICIs treatment. Due to the heterogeneity of LUAD, ill-fitted chemotherapeutic caused additional side effects, which was severe challenges for LUAD patients. Our study suggested that C1 patients with significant TP53 mutation and increased proliferative activity may have better efficacy with two kinds of important chemotherapeutic drugs, docetaxel and paclitaxel. In addition, for C2 patients, glucocorticoids were recommended for their potential effect on adverse events for LUAD. Finally, we included 151 LUAD prognostic signatures to boost clinical management. The 21-gene model of Chen EG possessed the highest accuracy for both C1 and C2 and the 16-gene model of Ma B exhibited the best discrimination for C3 patients, implying their excellent ability to be applied for prognostic management to individual subtypes.

Several limitations need to be acknowledged in the present study. Firstly, the clinical information was incomplete for some patients in public datasets, which contribute to potential bias. Then, we focused on inter-tumor heterogeneity from the bulk RNA sequencing data, further intra-tumor heterogeneity studies haven’t been considered from the single cell level. Additional experimental and validation data for the biological

pathway characterizations associated with each subtype were meaningful. At last, further clinical validation for sensitivity to immunotherapy and specific drugs predicted by machine learning algorithms was still needed.
In conclusion, we revealed three heterogeneous CRD subtypes in LUAD. Among the three subtypes, different survival times, biological features, genomic alterations, immune landscape, and treatment responses were spotted. Our work provided a promising classification platform and individualized treatment strategies, which would be helpful for clinical management. Based on RNA sequencing results, clinicians can gain insights into the characteristics of different subtypes, enabling accurate classification and targeted treatment.

## Abbreviations

CI: Confidence Interva; CNV: Copy Number Variation; CRD: Circadian Rhythm Disruption; FGA: Fraction of Genome Alteration; FGG: Fraction of Genomic Gained; FGL: Fraction of Genome Lost; FPKM: Fragments Per Kilobase of Million Mapped Reads; GEO: Gene Expression Omnibus; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; HR: Hazard Ratio; KEGG: Kyoto Encyclopedia of Genes and Genomes; LUAD: Lung Adenocarcinoma; NMF: Non-negative Matrix Factorization; TCGA: The Cancer Genome Atlas; TCR: T Cell Receptor; TP53: Tumor Protein p53;
TPM: Transcripts Per Million

## Author Contributions

Ruhao Wu contributed study design and paper revisiting. Kaisaierjiang Kadier and Teng Li contributed project oversight. Pengyuan Xu, Peiyu Yuan, and Tinglan Fu contributed data analysis and visualization. Yu Yang, Xufeng Huang, and Zhou Shujing contributed paper writing. Zhengrui Li, Pengpeng Zhang, Song-Bin Guo, Haonan Zhang, Shiqian Zhang, Chaoyang Yu and Ge Zhang contributed paper revisiting. All authors approved this manuscript.

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## Funding Information

Not applicable.

## Ethics Approval and Consent to Participate

Not applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. Publicly available datasets analyzed during the current study are available in the TCGA database and GEO database under accession codes. TCGA (https://www.cancer.gov/tcga), GSE72094 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE72094) (54), GSE68465 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE68465)(55), GSE50081 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE50081) (56), GSE42127 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE42127)(57), GSE41271 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE41271)(58), GSE31210 (https://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE31210)(59), GSE93157 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93157)(60), and a comprehensive immunotherapy annotations cohorts(61). Additional data related to this paper may be requested from the authors.

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Case Report Life Conflux

# Dynamic Evolution of Hyperplastic Polyps in the Setting of Autoimmune Gastritis: An In-Depth Interpretation of Gastrin-Driven Patho-mechanisms in a Case

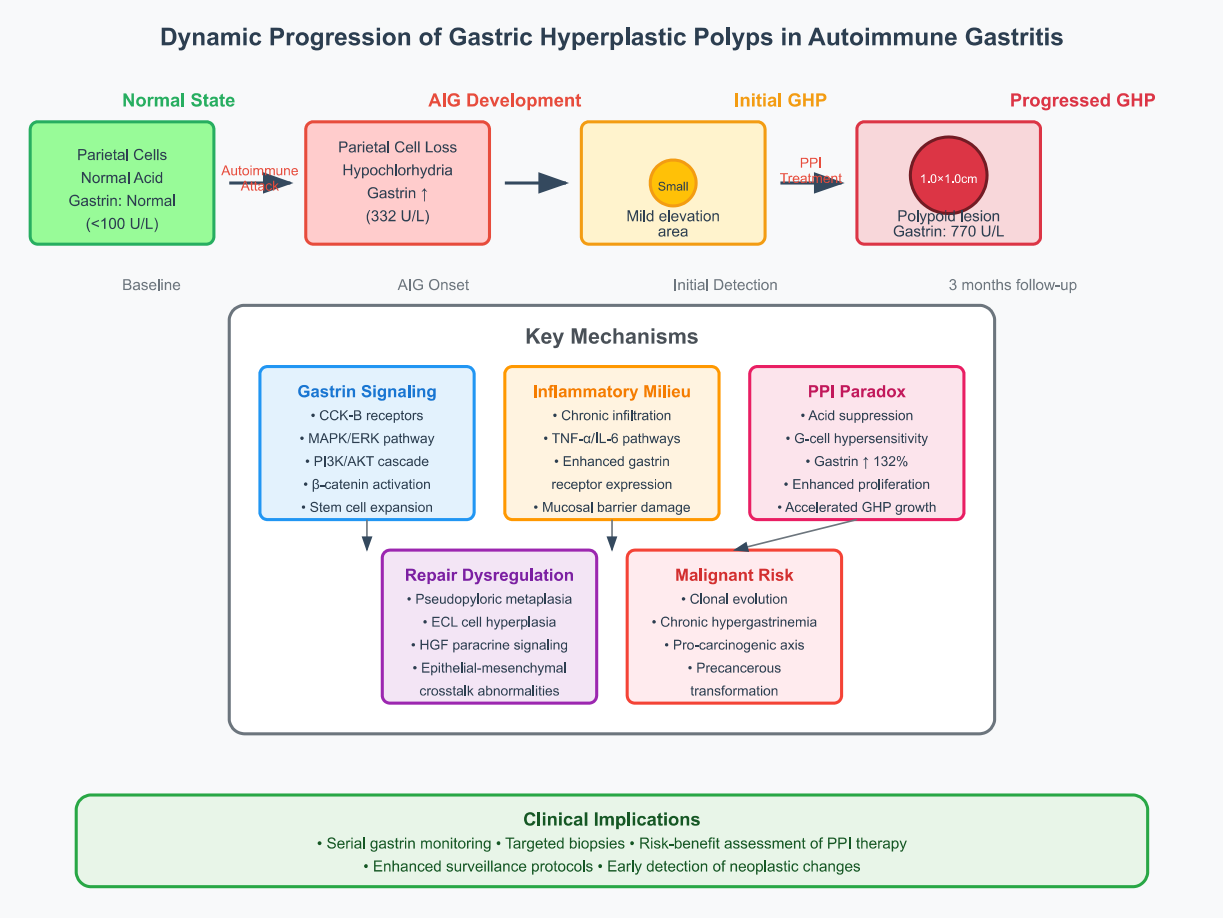
## Authors

**Yiming Song,** Jianing Yan

## Correspondence

419503527@qq.com (J. Yan)

## Graphical Abstract

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Case Report

Life Conflux

# Dynamic Evolution of Hyperplastic Polyps in the Setting of Autoimmune Gastritis: An In-Depth Interpretation of Gastrin-Driven Patho-mechanisms in a Case

**Yiming Song** [1], Jianing Yan [2*]

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## Abstract

Through longitudinal clinical follow-up of a 75-year-old male with autoimmune gastritis (AIG) complicated by gastric hyperplastic polyps (GHPs), this study systematically investigated the critical regulatory role of gastrin signaling networks in gastric mucosal remodeling. The case demonstrated that within the established pathological cascade of AIG (from parietal cell destruction to gastric acid deficiency and hypergastrinemia), GHPs exhibited short-term volumetric growth. This observation challenges traditional views about the indolent nature of hyperplastic polyps. Our findings highlight the dual role of proton-pump inhibitor (PPI) therapy in AIG management while alleviating mucosal inflammation, it paradoxically induces G-cell hypersensitivity that elevates gastrin levels beyond biological thresholds (332 increasing to 770 U/L), creating a critical driver for GHP progression. This case redefines clinical management paradigms for AIG complications and offers translational insights for gastrointestinal tumor surveillance.

**Keywords:** autoimmune gastritis; gastric hyperplastic polyp; proton-pump inhibitor

## Introduction

Autoimmune gastritis (AIG) has garnered significant research interest among chronic gastric disorders due to its distinct pathophysiological mechanisms. AIG initiates with autoimmune-mediated destruction of parietal cells, progressing through a characteristic sequence of hypochlorhydria, compensatory hypergastrinemia, and glandular atrophy - ultimately predisposing patients to pernicious anemia and gastric carcinogenesis [1,2]. GHPs, commonly associated with AIG, show higher prevalence in these patients compared to the general cohort [3]. These polyps typically develop in the context of chronic gastritis, Helicobacter pylori (H. pylori) infection, and mucosal repair processes, histologically characterized by foveolar epithelial hyperplasia, stromal edema, and inflammatory infiltration [4]. Notably, though GHPs generally exhibit indolent growth patterns, certain cases - particularly those complicated by AIG - may demonstrate accelerated progression as documented in our clinical observation.

This study reported a rapid progression of GHPs during the course of AIG in a 75-year-old male patient, which systematically reveals a central regulatory role of the gastrin signaling pathway by integrating endoscopic imaging, histopathology, and laboratory findings, providing a new perspective to understand the dynamic evolution of this type of lesion.

## Case Presentation

A 75-year-old male patient presented to our hospital with upper abdominal discomfort in 2023. He had no history of other systemic diseases and H. pylori eradication treatment. Endoscopic imaging evaluation revealed extensive mucosal atrophy in the greater curvature side of the gastric body (Figure 1A). A 1.2cm×1.0 cm elevated depressed lesion was detected in the upper part of the lesser curvature of the gastric body (Figure 1B). Biopsy results suggested it is a well-differentiated adenocarcinoma (Figure 2A). There was also extensive atrophy on the lesser curvature side of the gastric body with a mildly elevated area of the mucous membranes in the lower part (Figure 1C-D), designated by directional annotation markers. Histological examination showed significant proliferation in the gastric foveolar (Figure 2B). Marked atrophy was observed in the gastric angle, and the gastric antrum area was also atrophic (Figure 1E-F). Atrophic changes were present in the gastric antrum-sinus region (Figure 1E-F). Histology of the gastric antrum did not show significant atrophic intestinal

1 Department of Gastroenterology, the People's Hospital of Fenghua Ningbo, Ningbo, China

2 Department of Gastroenterology, The First Affiliated Hospital of Ningbo University, Ningbo, China

* Corresponding Author.

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**Figure 1.** The first endoscopic imaging evaluation. (**A**) Gastric body mucosa along the greater curvature demonstrating mucosal atrophy. (**B**) Depressed lesion with raised margins in the superior lesser curvature of gastric body. (**C-D**) Mild mucosal elevation in the inferior gastric body. (**E-F**) Atrophic changes observed at the gastric angle and antrum).

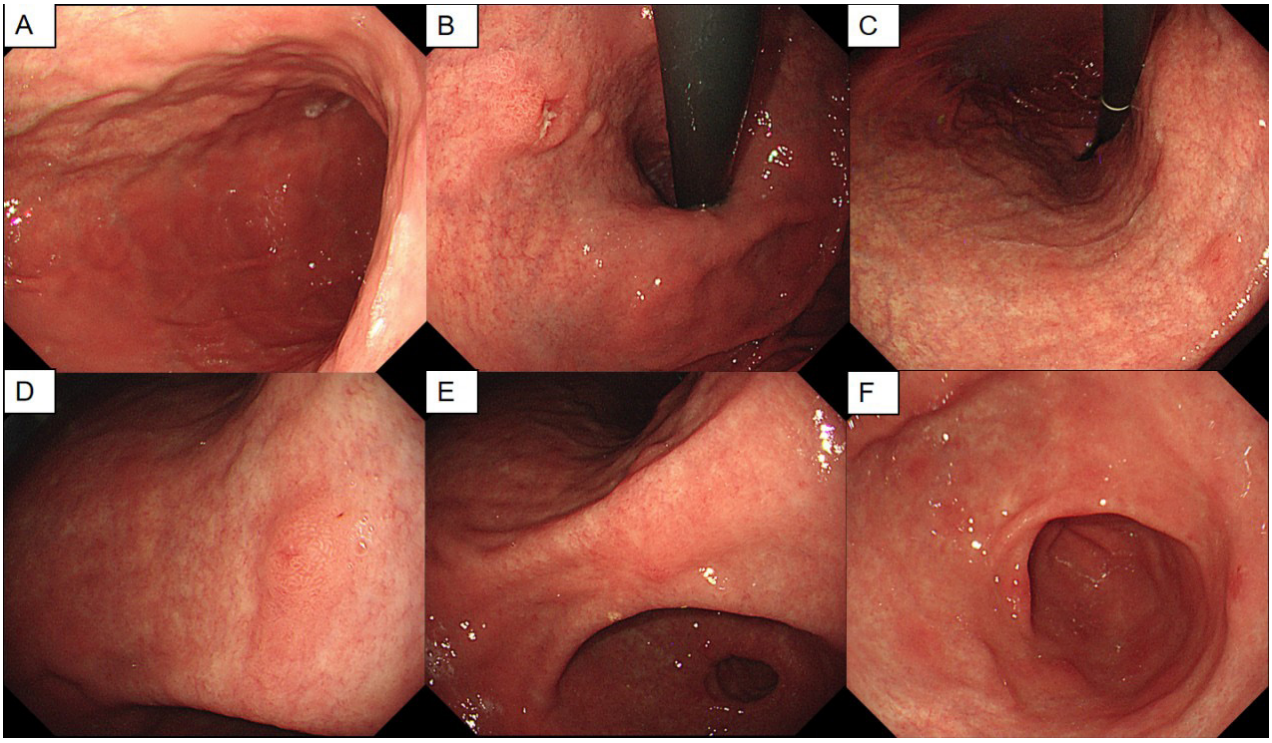
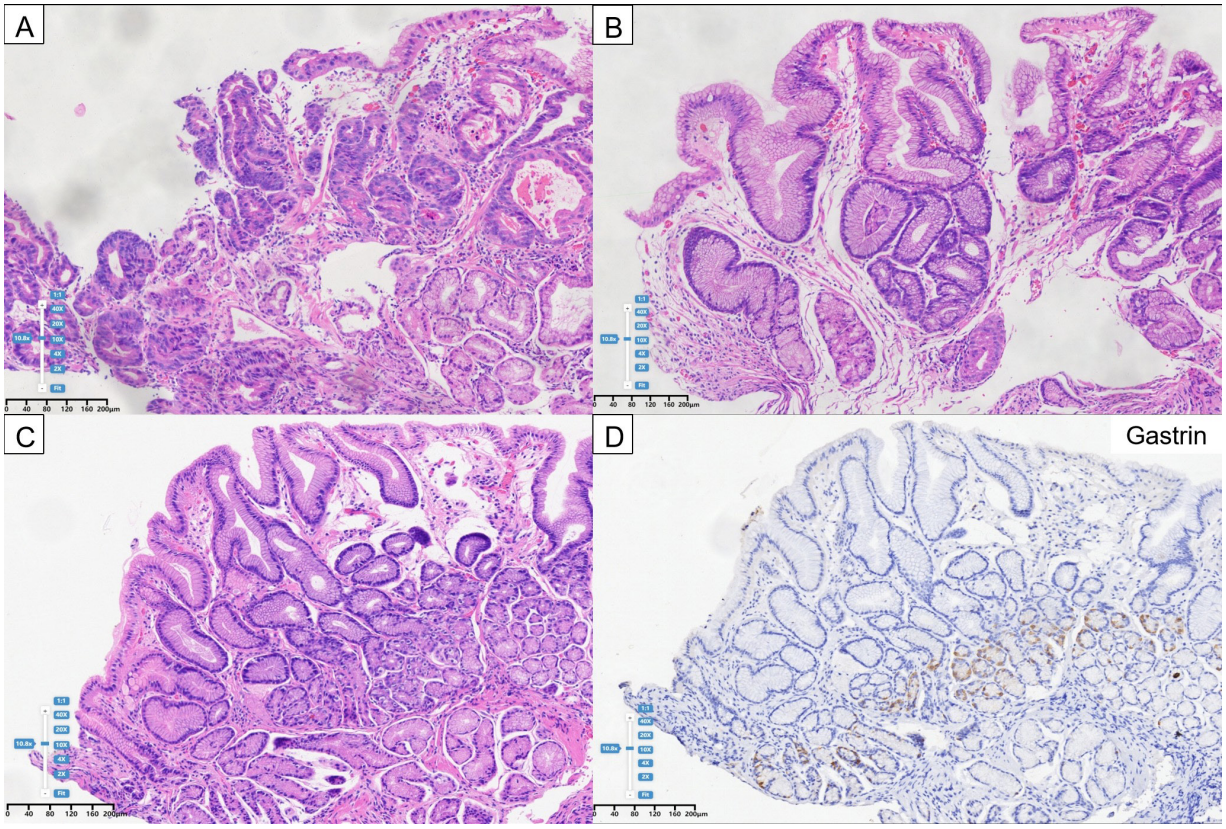



**Figure 2.** Pathological section. (**A**) Hematoxylin-eosin (HE) staining of superior lesser curvature lesion demonstrating well-differentiated adenocarcinoma. (**B**) HE-stained section from inferior lesser curvature showing foveolar hyperplasia. (**C**) Antral HE specimen revealing foveolar hyperplasia without atrophy or intestinal metaplasia. (**D**) Gastrin staining indicated abnormal proliferation of gastric antrum G cells.



epithelial chemotaxis features, but prolongation of the gastric foveolar was noted (Figure 2C). Gastrin staining indicated abnormal proliferation of gastric antrum G cells (Figure 2D). Serological tests revealed a serum gastrin level of 332 U/L, suggesting hypergastrinogenemia. Blood counts and vitamin B12 levels were normal.

Then, the patient underwent endoscopic submucosal dissection (ESD) and 3-month postoperative proton pump inhibitor (PPI) treatment in other hospitals (esomeprazole 20mg twice daily from September 4 to November 20 in 2023), and the treatment process was uneventful.

Last year the patient returned to our hospital for a gastroscopy review. Endoscopic imaging evaluation showed that the tumor in the upper part of the gastric body had been completely resected. However, extensive mucosal atrophy still persisted in the gastric body. A close - up view of the gastric body mucosa revealed the presence of Remnant oxyntic mucosa (Rom) (Figure 3A-C). Notably, the previously elevated area in the lower part of the gastric body has progressed to a 1.0 × 1.0 cm polypoid lesion (Figure 3D, E), designated by directional annotation markers. Extensive atrophy of the gastric antrum mucosa was still visible (Figure 3F). Histopathological examination of the gastric body mucosa showed mural cell destruction accompanied by deep lymphocytic infiltration and pseudo-pyloric glandular metaplasia (Figure 4A-B). H+/K+ATPase staining revealed mural cell destruction (Figure 4C), MUC6 staining demonstrated focal positivity (Figure 4D). Chromogranin A (CgA) staining indicated abnormal proliferation of neuroendocrine cells (Figure 4E). H. pylori staining revealed absence of H. pylori (Figure 4F).

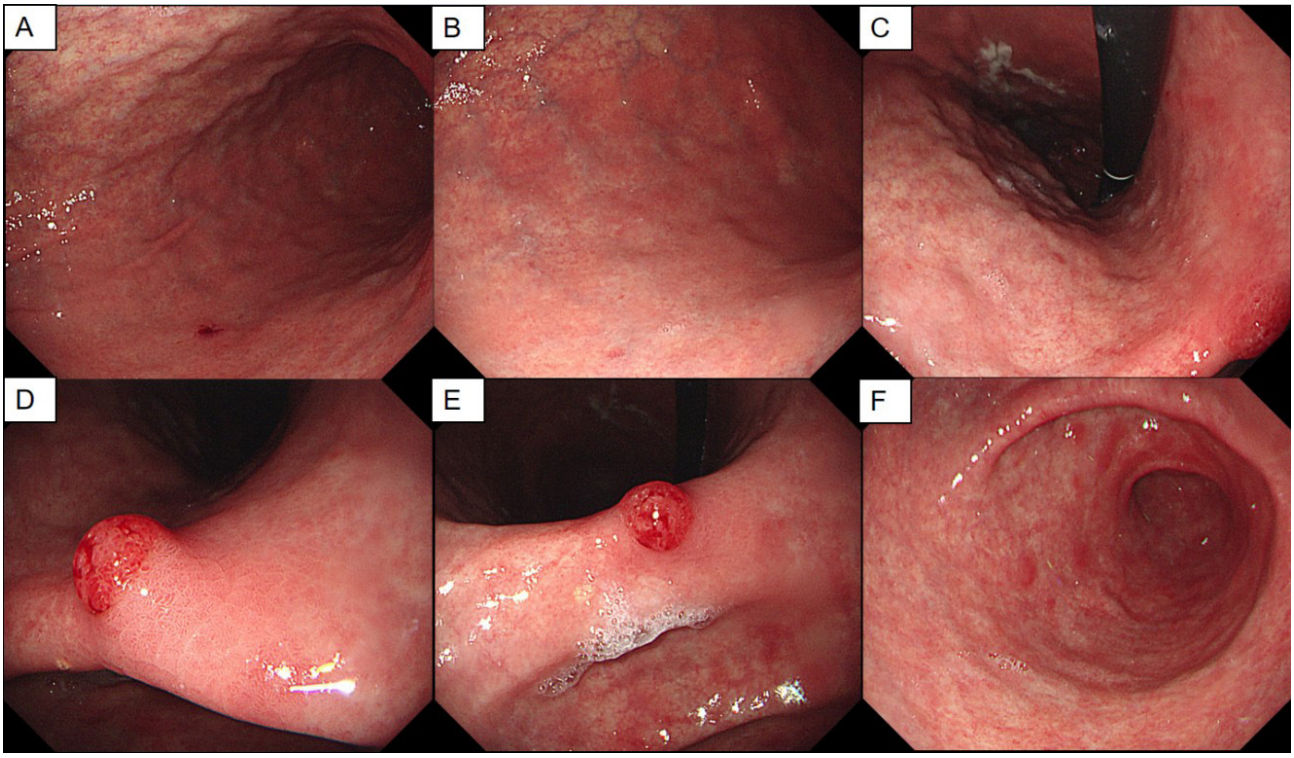
Serological tests revealed a positive outcome for the antiparietal cell anti-body and negative results for anti-intrinsic factor antibody. Gastrin levels spiked to 770 U/L, a 132% increase from the baseline (332 U/L vs. 770 U/L), which far exceeded the range of fluctuations commonly seen in patients with AIG (usually <500 U/L), suggesting the presence of additional stimuli such as PPIs.

In summary, the patient was diagnosed with AIG through the following features: (i) Medical history: no history of H. pylori infection. (ii) Serology: PCA positive. (iii) Histopathology: histopathological examination of the gastric body mucosa showed mural cell destruction accompanied by deep lymphocytic infiltration and pseudo-pyloric glandular metaplasia with gastric neuroendocrine cell hyperplasia. Combined with the characteristic endoscopic and pathological changes, the patient was diagnosed with AIG combined with GHPs. This case is unique because the polyps in the lower region of the gastric body shows a short- term volume doubling phenomenon, which reveals a dynamic progression of AIG related mucosal lesions.

## Discussion

AIG is an organ-specific autoimmune disorder characterized by chronic inflammation of the gastric corpus mucosa and progressive glandular atrophy. Gastrin, a polypeptide hormone produced by antral G cells, plays a key physiological role in regulating gastric acid secretion and mucosal proliferation. In AIG, markedly elevated gastrin levels are closely linked to the development and progression of gastric hyperplastic polyps through the following mechanisms: dual activation of gastrin

**Figure 3.** The second endoscopic imaging evaluation. (**A**) Mucosal atrophy in gastric body greater curvature. (**B**) the gastric body mucosa revealed the presence of Remnant oxyntic mucosa. (**C**) Mucosal atrophy of superior lesser curvature. (**D-E**) Hyperplastic polyps in inferior lesser curvature; (**F**) Antral mucosal atrophy.

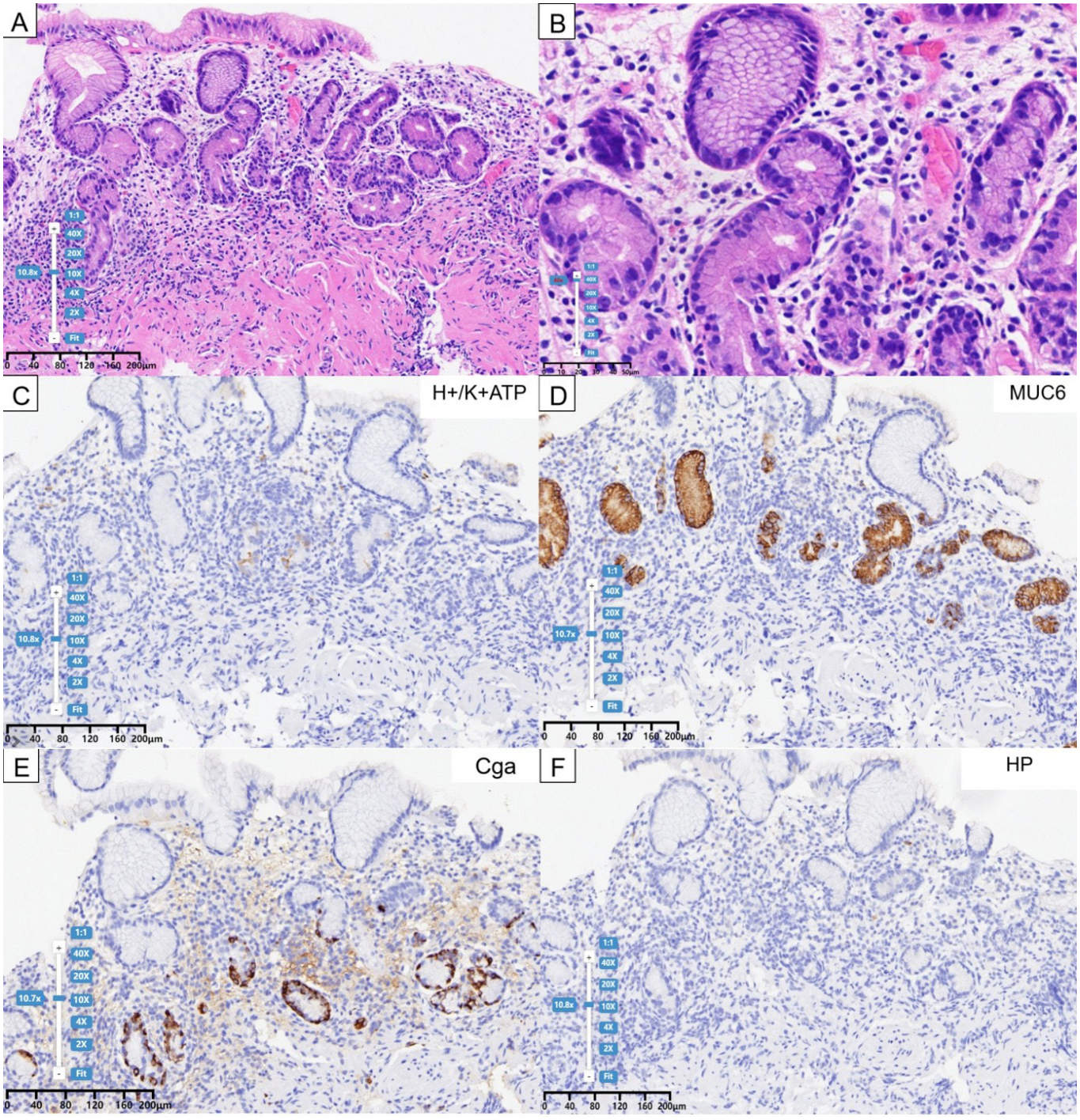


signaling pathways, inflammatory microenvironment and impaired mucosal repair, Paradoxical effects of PPI therapy and re-evaluating malignant transformation risks.

As a central regulator of gastric mucosal homeostasis, gastrin activates both MAPK/ERK and PI3K/AKT signaling cascades through CCK-B receptors, promoting epithelial cell proliferation and suppressing apoptosis [5]. In AIG, parietal cell loss leads to achlorhydria, which removes the normal negative feedback regulation of G cells and results in sustained hypergastrinemia demonstrated by 132% elevated levels in this case [6]. This pathological state induces abnormal β-catenin pathway activation, driving excessive expansion of gastric stem cell populations - a critical cellular basis for GHP formation [7].

Chronic inflammatory infiltration not only directly damages the mucosal barrier but also enhances gastrin receptor expression via TNF-α/IL-6-mediated pathways, creating a pro-proliferative

**Figure 4.** Pathological section. (**A**) HE-stained section from upper gastric body greater curvature showing dense lymphocytic infiltration, parietal cell loss, and pseudopyloric metaplasia. (**B**) High-definition magnified view of Image A. (**C**) H+/K+ATPase staining revealed that confirming parietal cell depletion. (**D**) MUC6 staining demonstrated focal positivity. (**E**) CgA staining exhibited neuroendocrine cell hyperplasia. (**F**) H. pylori staining revealed absence of H. pylori.



feedback loop [8,9]. The co-occurrence of pseudopyloric metaplasia and neuroendocrine cell hyperplasia observed in this case highlights dysregulated repair mechanisms: metaplastic epithelium loses normal secretory functions, while hyperplastic ECL cells exacerbate epithelial-mesenchymal crosstalk abnormalities through paracrine HGF secretion [10].

Pharmacologic interventions may worsen mucosal proliferation imbalances. While PPIs can alleviate AIG-related mucosal injury symptoms, their potent acid suppression exacerbates hypergastrinemia [11]. The post-treatment exponential gastrin elevation in this case suggests PPIs may potentiate proliferative signaling through G-cell hypersensitivity [12]. This observation warrants cautious risk-benefit assessment of PPI use during rapid GHP progression.

Although traditionally considered benign, our findings suggest potential clonal evolution within GHPs under chronic hypergastrinemia. Therefore, AIG-associated GHP surveillance should integrate serial gastrin monitoring and targeted biopsies to detect early neoplastic changes.

## Conclusion

This case delineates the dynamic progression of GHPs in AIG, highlighting that under PPI therapy, the central role of dysregulated gastrin signaling. The triad of persistent hypergastrinemia, chronic inflammatory milieu, and aberrant mucosal repair forms a "pro-carcinogenic axis" that facilitates the transition from benign hyperplasia to precancerous transformation. Future investigations should prioritize exploring gastrin receptor antagonists as therapeutic targets and elucidating epigenetic regulatory mechanisms underlying polyp malignant transformation, thereby establishing a scientific foundation for precision management of AIG-related complications.

## Abbreviations

AIG: autoimmune gastritis; CgA: Chromogranin A; ESD: endoscopic submucosal dissection; GHPs: gastric hyperplastic polyps; H. pylori: Helicobacter pylori; PPI: proton-pump inhibitor; Rom: Remnant oxyntic mucosa.

## Author Contributions

Yiming Song designed this study. Jianing Yan wrote the manuscript. The final manuscript has been approved by all authors.

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## Ethics Approval and Consent to Participate

Informed written consent was obtained from the patient for publication of this report and any accompanying images.

## Competing Interests

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data Availability

Not Applicable.

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# Causal Relationship between Chronic Gastritis and Sleep Apnea Syndrome: A Bidirectional and Two-step Mendelian Randomization Study

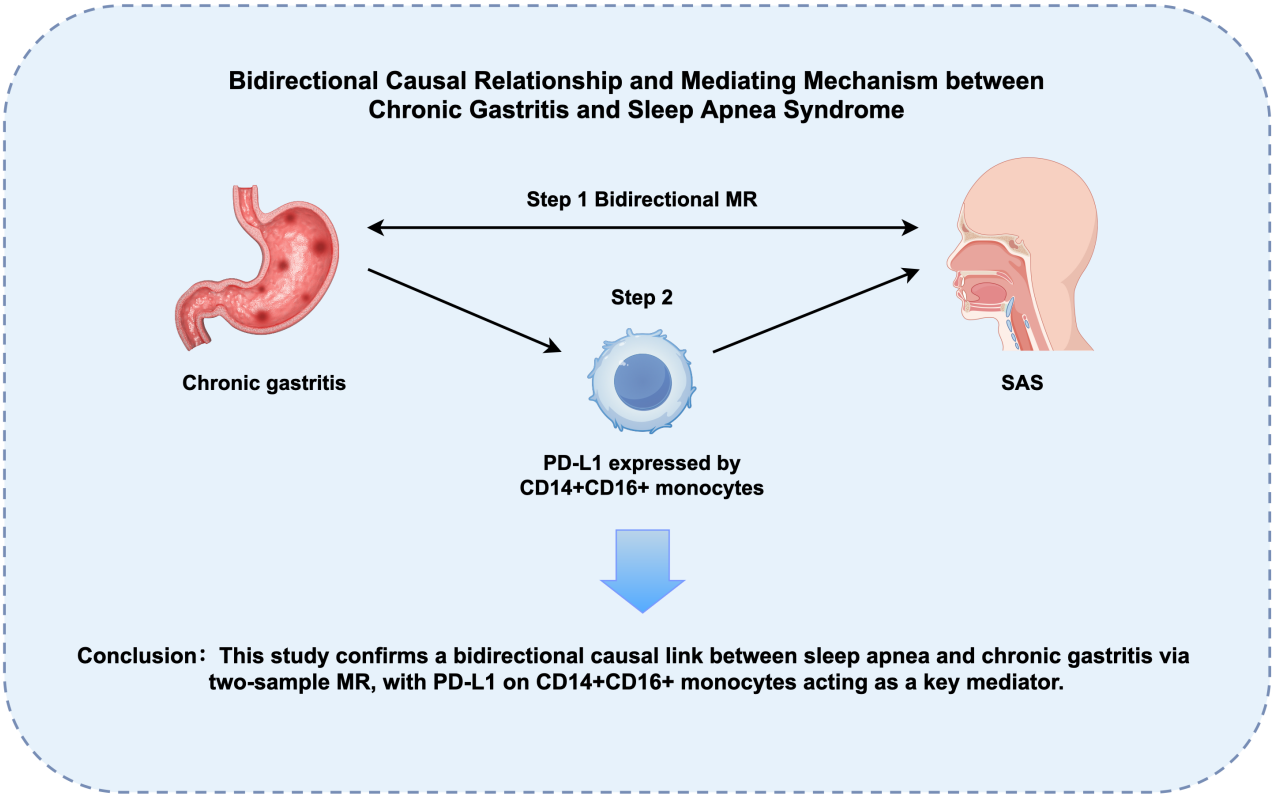

## Authors

**Ziyan Zhao,** Yanyu Zhu, Chang You, Yan Wang, Tianchi Zhuang, Yingqi Yang

## Correspondence

945998089@qq.com (T. Zhuang), muyoung@stu.njmu.edu.cn(Y. Yang)


## Graphical Abstract

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Research Article Life Conflux

# Causal Relationship between Chronic Gastritis and Sleep Apnea Syndrome: A Bidirectional and Two-step Mendelian Randomization Study

**Ziyan Zhao** [1], Yanyu Zhu [2], Chang You [3], Yan Wang [4], Tianchi Zhuang [5*], Yingqi Yang [4*]

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## Abstract

**Background:** Sleep apnea syndrome (SAS) and chronic gastritis (CG) are prevalent in middle-aged and elderly people. Although clinical observations suggest an association between the two diseases, the causal relationship between them has not been clarified. This study aims to explore the causal relationship between SAS and chronic gastritis and to elucidate the possible mediating mechanisms.

**Methods:** The causal relationship between SAS and CG was evaluated using five methods (primarily Inverse Variance Weighted (IVW), with others as sensitivity analyses). A two-step Mendelian randomization (MR) study, leveraging GWAS data, was performed to assess the mediating role of signatures in immune cell and inflammatory proteins. Sensitivity analyses were conducted to verify result robustness. The CAUSE framework was applied to address horizontal pleiotropy through comparison of causal and shared genetic models, adjusting for both linkage disequilibrium (LD)-related and independent pleiotropic effects. MR results followed STROBE-MR guidelines.

**Results:** Through two-sample MR analysis, we identified significant bidirectional causal relationships between sleep apnea syndrome (SAS) and chronic gastritis (forward: OR = 1.131, 95% CI: 1.031-1.240, p = 0.009; reverse: OR = 1.065, 95% CI: 1.007-1.128, p = 0.028). Simulation studies confirmed CAUSE's superior specificity in controlling false positives through its dual-model framework that explicitly accounts for pleiotropic pathways. Two-step MR analysis revealed that the changes in the level of PD-L1 expressed by CD14+CD16+ monocytes played a significant mediating role in the effect of chronic gastritis on SAS.

**Conclusion:** The bidirectional causal relationship between SAS and chronic gastritis was confirmed through two-sample and two-step MR analyses. Chronic gastritis may increase the risk of SAS through genetic signatures in immune cells, providing new perspectives for future research and aiding in the development of new prevention and treatment strategies.

**Keywords:** sleep apnea syndrome, chronic gastritis, a two-sample Mendelian randomization, a two-step Mendelian Randomization Study.

## Introduction

Sleep Apnea Syndrome (SAS) involves repeated episodes of partial or full blockage of the upper airway while sleeping[1, 2], affects millions worldwide and is associated with significant morbidity and mortality[3]. SAS is increasingly prevalent among the middle-aged and elderly, with estimates suggesting a prevalence rate of 20%–40% in people aged[4]. Chronic gastritis is a prevalent, long-term, serious, and subtle illness affecting humans[5]. It is estimated that over half of the global population suffers from this condition to some extent[6], implying that a significant number of people around the world, possibly in the hundreds of millions, could have chronic gastritis[7]. The intersection of these two conditions presents a fascinating area of study, with potential implications for the diagnosis and management of both disorders.

Despite the increasing prevalence of SAS and the well-documented impact of sleep disorders on gastrointestinal health[8, 9], the specific mechanisms linking SAS to chronic gastritis remain poorly understood. A comprehensive analysis of multiple clinical data and biological mechanisms established a causal link between SAS and chronic gastritis, gastric ulcer, inflammatory bowel disease, and fatty liver[10], working to elucidate the specific biological pathways involved in the interactions between SAS and gastrointestinal disorders. Another systematic review and Meta-analysis examined the association between SAS and various gastrointestinal disorders (GID), including gastroesophageal reflux disease (GERD) and inflammatory bowel disease (IBD)[11], which found that GERD is more prevalent in patients with SAS and that these GID disorders may exacerbate the manifestations of SAS. Nevertheless, there is insufficient evidence to support a causal relationship between SAS and chronic gastritis, and its underlying mechanisms remain unclear.

1 School of Rehabilitation Medicine, Nanjing Medical University, Nanjing, China
2 The First School of Clinical Medicine, Nanjing Medical University, Nanjing, China
3 School of Stomatology, Nanjing Medical University, Nanjing, China
4 The Second School of Clinical Medicine, Nanjing Medical University, Nanjing, China
5 School of Nursing, Nanjing Medical University, Nanjing, China
* Corresponding Author.

Mendelian randomization (MR) is extensively used in genetic and epidemiological studies, utilizing single nucleotide polymorphisms (SNPs) as natural instrumental variables (IVs) to suggest potential causal links between exposure factors and outcomes[12]. By utilizing identified genetic variations, Mendelian randomization (MR) analysis effectively mitigates the influence of confounding factors and potential reverse causation, thereby providing robust evidence for causal inference. This research employs MR analysis combined with two-step MR to explore the causal relationship between SAS and chronic gastritis, offering new insights into their intricate relationship.

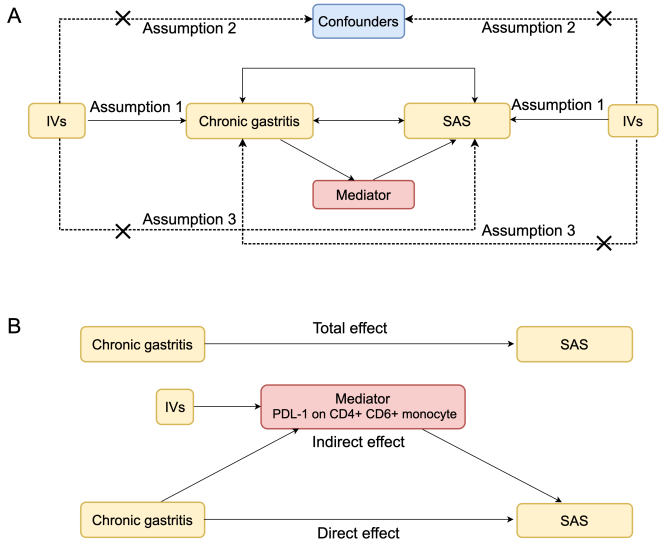
This research aims to investigate the causal relationship between SAS and chronic gastritis. By elucidating the mechanisms connecting SAS and chronic gastritis, it may contribute to understanding the pathogenesis and progression of the disease, as well as identifying potential biomarkers and treatment strategies[13, 14]. Further comprehensive research is necessary to understand the exact mechanisms connecting SAS with chronic gastritis.

## Methods

### Study Design

Initially, we conducted a bidirectional two-sample MR analysis to investigate the causal relationships between SAS and the likelihood of developing chronic gastritis. MR-CAUSE was performed to estimate the horizontal pleiotropy in the analysis. Two-step MR analysis was performed to assess the mediating role of signatures in immune cell and inflammatory proteins between the two diseases (Figure 1).

**Figure 1.** Flowchart of study design. (**A**) Three assumptions of MR analysis and (**B**) the flow of two-step MR analysis.




### GWAS data for SAS, chronic gastritis, and signatures in immune cell and inflammatory proteins

The data used in this study were all obtained from publicly available genome-wide association study (GWAS) datasets. The GWAS of the Systemic Autoimmune Syndrome (SAS) included summary statistics (GWAS IDs: finn-b-G6_SLEEPAPNO, SNPs =16,380,465) from a cohort of 16,761 SAS patients of European descent and 201,194 controls for comparison. The GWAS of chronic gastritis included summary statistics (GWAS IDs: finn-b-K11_CHRONGASTR, SNPs = 16,380,395), with a study sample comprising 5,213 cases and 189,695 controls from Europe desent [15]. The GWAS catalog (GCST90001391 to GCST90002121) provided summary statistics for signatures in immune cell, including data from 3,757 Europeans with 731 signatures in immune cell (SNPs= 22,000,000 approximately)[16]. Information on 91 inflammatory proteins was obtained from GWAS data, which included 14,824 participants of European descent (Zhao et al., 2023)[17].

### Selection of instrument variables

To identify IVs on a global scale, genome-wide significance levels ($p < 1e\text{-}05$) were employed as criteria to screen for IVs that are significantly linked to exposure factors. In all studies, by excluding SNPs within the 10,000 kb and $r^2 < 0.001$ threshold, three high linkage disequilibrium SNPs (rs10938398, rs3996329, rs9937053) were excluded to avoid confounding effects and guarantee the independence of the selected SNPs. These SNPs are strongly associated direct causes of chronic gastritis (visceral fat, obesity BMI, type 2 diabetes, heart disease, neuroticism, depression, caffeine intake, sleep apnea) and have robust clinical evidence necessitating exclusion as confounding factors. Therefore, these confounding factors need to be excluded. Then, the PhenoScanner was used to remove IVs associated with confounders. To avoid weak instrumental bias, the statistical intensity of IV was evaluated using the F-statistic. IVs are considered to have good statistical performance when the F-statistic exceeds 10. The IVs screening procedures and analytical approaches were implemented with reference to methodological frameworks outlined in Burgess et al. [18] and standardized protocols advocated by the International Mendelian Randomization Consortium[19].

### Two-Sample Mendelian Randomization Analysis

All data analysis was performed using the TwoSampleMR software package (version 0.6.8) in R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria) for two-sample Mendelian randomization analysis. In this study, the main approach to determine the causal relationship between SAS and chronic gastritis is the inverse variance-weighted method (IVW) using a random-effects model. A range of analysis methods, such as weighted median, MR-Egger, weighted mode, and simple mode methods, were employed to guarantee the reliability of the results and to detect possible horizontal pleiotropies. The IVW method takes into account the uncertainty of the individual SNP effect estimates, giving greater weight to those SNPs with a smaller standard error. MR-Egger explains the potential heterogeneity in IVs and can detect and correct bias to test for horizontal pleiotropy[20, 21]. The reliability of causality is enhanced when the results of all methods are roughly the same. This study estimates the causal effect of genetic prediction of SAS on chronic gastritis, represented as odds ratios (OR) and corresponding 95% confidence intervals (95% CIs). To adjust the p-values, both forward and reverse MR analyses were performed. A p-value of less than 0.05 was considered strong evidence of a causal

association. To evaluate the potential sample overlap bias between the GWAS datasets of sleep apnea (SAS) and chronic gastritis, this study employed the Mendelian Randomization with Latent Adjustment for Overlap (MRlap) [22]. This method quantifies the sample overlap ratio through a Bayesian probabilistic model and adjusts the causal effect estimates based on genome-wide genetic correlations. We also use the MR-CAUSE method to avoid false positive results caused by horizontal pleiotropy and strengthen the stable type of positive results[23]. To systematically address horizontal pleiotropy, the Causal Analysis Using Summary Effect Estimates (CAUSE) framework was employed to distinguish causal effects from confounding by comparing genetic architectures under competing causal and shared confounding models, accounting for both linkage disequilibrium (LD)-related and independent pleiotropic pathways. We followed the STROBE-MR (Strengthening the reporting of observational studies in epidemiology using mendelian randomization) guidelines for MR results reporting [24].

**Two-step Mendelian Randomization Analysis**

To systematically investigate the bidirectional mechanism of action between chronic gastritis and sleep apnea syndrome (SAS), this study used a two-step MR to characterize their interactions through immune cell features and inflammatory proteins[25]. The first step was to perform MR analysis between chronic gastritis and SAS to obtain the total causal effect. The second step was to evaluate the role of immune cell features or inflammatory proteins in the relationship between chronic gastritis and SAS, respectively. The total effect of chronic gastritis and SAS was decomposed into a direct effect (i.e., the effect of chronic gastritis on SAS without mediation) and an indirect effect (i.e., the effect of chronic gastritis on SAS through mediators). We calculated the effects of chronic gastritis on SAS, with and without the presence of signatures in immune cell and inflammatory proteins, to compare the outcomes and determine the existence of any mediating effects. We conducted inverse variance-weighted (IVW) analysis to evaluate the causal effects before and after the removal of intermediaries. Additionally, the weighted median method and MR-Egger were performed as complementary methods, and MR-Egger was used to identify and adjust for bias to assess potential horizontal pleiotropy, thereby reducing bias caused by horizontal pleiotropy [20]. We also performed a reverse pathway analysis, in which SAS was used as an exposure to identify immune cell signatures and inflammatory protein markers of its effects, and thus assessed the direct and indirect effects of SAS on chronic gastritis.

Sensitivity analysis

After the preliminary screening, we evaluated the heterogeneity of the Cochran's Q test of IVW and MR-Egger methods for the significant results, and when $p < 0.05$, significant heterogeneity was considered to be present [26]. Horizontal pleiotropy was evaluated using the MR-Egger regression intercept and MR-PRESSO. A p-value greater than 0.05 for the MR-Egger regression intercept suggests that horizontal pleiotropy can be disregarded. For MR-PRESSO, $p > 0.05$ after overall inspection shows that horizontal pleiotropy is negligible [27]. Repeat the analysis after eliminating the peripheral SNP, and compare the differences before and after deleting the outlier. To assess the stability of the results, a leave-one-out analysis was conducted by excluding each SNP individually, and the results were considered stable if the exclusion of any single SNP did not lead to significant changes [28]. Funnel plots were used to illustrate the distinct relationships between SNPs and were employed to evaluate bias in the findings. Reverse MR can assess if the genetic variation serving as an exposure trait also has a causal relationship with the outcome, affecting it via the exposure trait.

To validate the robustness of the main Mendelian Randomization (MR) analysis results, this study utilized three external datasets UK Biobank verification: ukb-b-6716 (n=342,000), ukb-b-12191 (n=408,000), and ukb-d-K11_CHRONGASTR (n=28,500). All validation cohorts had no overlap with the main samples, and the phenotype definitions were complementary to reduce bias from a single data source. We retained the instrumental selected in the main ($p < 1e\text{-}05$, F-statistic > 10) and removed SNPs in the validation cohorts that exhibited high linkage disequilibrium (LD) with the main analysis SNPs (10,000 kb, $r^2 < 0.001$), while also excluding SNPs with inconsistent exposure association directions. Statistical methods employed the inverse-variance weighted (IVW) method as the primary estimator, supplemented by the weighted median method and MR-Egger regression (testing for horizontal pleiotropy), with outlier SNPs removed using MR-PRESSO. Consistency was crossvalidated through IVW, MR-Egger, weighted median, and pleiotropy tests (e.g. MR-PRESSO), and heterogeneity was assessed using Cochran's Q ($p < 0.05$).

Through cross-validation using inverse variance weighted (IVW), MR-Egger regression, weighted median, and MR-PRESSO methods, we systematically assess the robustness of results. The statistical assumptions of different methods are mutually independent (e.g., IVW assumes no pleiotropy of instrumental variables, while MR-Egger allows for directional pleiotropy), and the consistency in effect direction and significance can effectively replace traditional multiple corrections [29]. The International Mendelian Randomization Study Guidelines indicate that when the main analysis (IVW) is consistent with sensitivity methods (such as weighted median, MR-Egger), additional corrections may be overly conservative and obscure true associations [30]. Several MR studies [30, 31] in similar designs did not use BH correction, instead relying on inter-method consistency as a robustness standard [31, 32].

MR-PRESSO was performed in R (version 4.4.1) using the MR-PRESSO software package (version 1.0), while the "forestplot" software package was used for graphical representation.

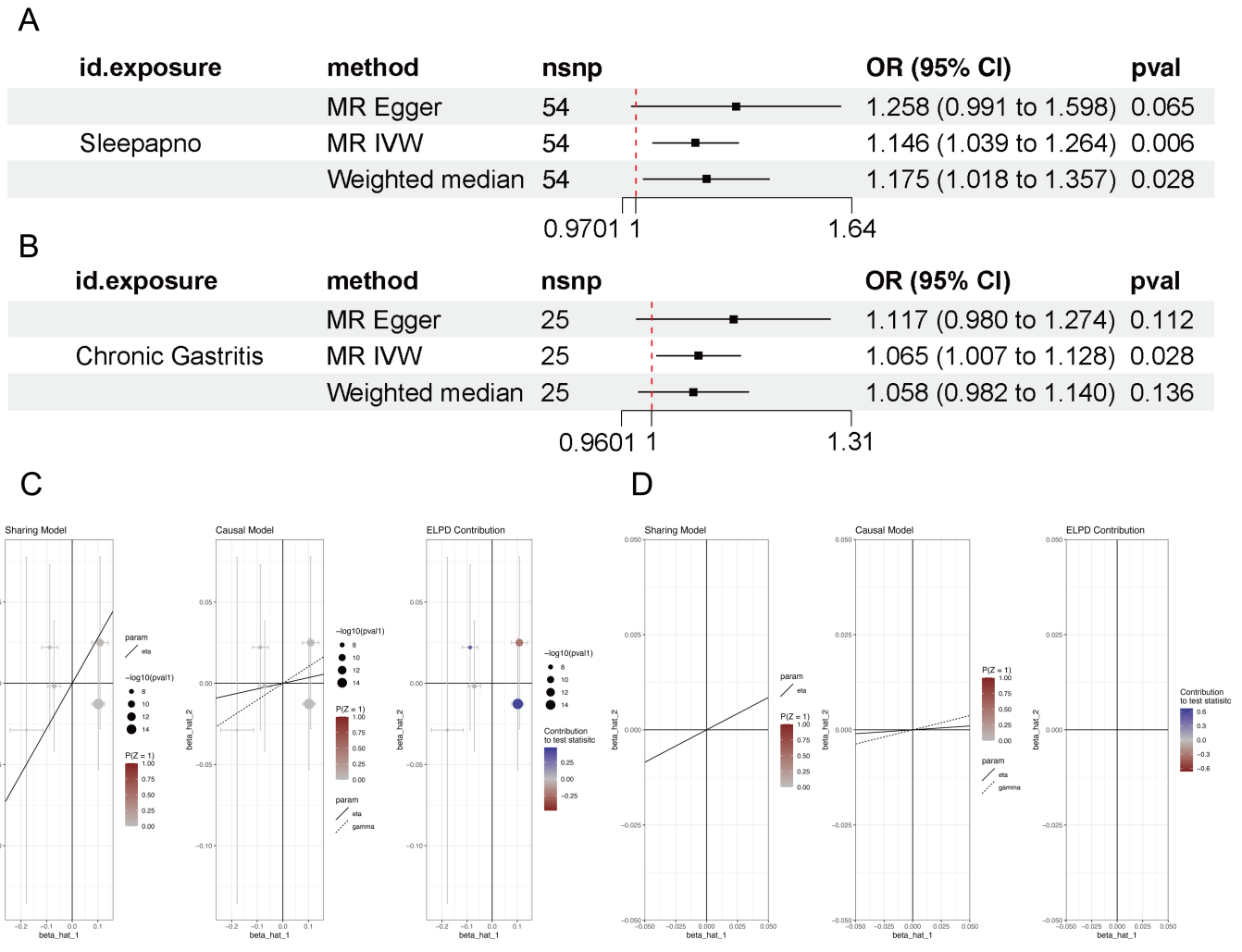
## Results

**Causal Relationship between Chronic Gastritis and Sleep Apnea Syndrome**

In a two-sample MR analysis, the IVW method analysis identified a notable association between SAS and an higher risk of chronic gastritis (OR = 1.131, 95% CI: 1.031-1.240, p = 0.009)(Figure S1, Table S1). We systematically screened confounding factors associated with chronic gastritis and SAS, and the PhenoScanner database was used to eliminate confounder-related SNPs. SNPs (rs10938398, rs3996329, rs9937053) significantly related to confounding factors of chronic gastritis (diabetes, excessive coffee drinking, heavy

alcohol consumption, long-term heavy smoking, non-steroidal anti-inflammatory drugs) were removed [33], but no SNPs significantly related to confounding factors were found in reverse MR. To test the robustness of the results, we also used 4 tests, including MR-Egger regression, weighted median method, simple mode method, and weighted mode method, and the positive causal relationship between SAS and chronic gastritis remained significant (Figure 2A). Then, we conducted a comprehensive and sufficient sensitivity test. In the heterogeneity test analysis, the P value of the Cochran's Q test was greater than 0.05, indicating the absence of significant heterogeneity (Table S2); the leave-one-out analysis further confirmed the stability of the primary outcome, indicating that no individual SNP overinfluenced the effect estimate (Figure S2); and the funnel plot indicated that the analysis was not significantly biased (Figure S3). To exclude the effect of horizontal pleiotropy, we performed an MR-Egger regression cut-off distance term test and an MR-PRESSO test (Table S3). The results showed that there was no significant horizontal pleiotropy (p > 0.05). The results of MR-Cause analysis showed that the causal model was superior to the shared model (delta_elpd < 0), and this method showed negative results under the causal model (p > 0.05) (Figure 2B), and the results were robust. In order to comprehensively assess the relationship between SAS and chronic gastritis, we used two-way MR to assess the inverse effect of chronic gastritis on SAS levels to explore whether chronic gastritis may also be a contributing factor of SAS. IVW results showed that chronic gastritis was associated with an increase in SAS levels (OR = 1.065, 95% CI: 1.007-1.128, p = 0.028) (Figure 2C) (Table S4). The sensitive test of reverse Mendelian randomization and the MR-CAUSE test have the same steps as before, and the results are stable without heterogeneity, horizontal pleiotropy, or bias (Table S5, S6). The above results indicate that there is a bidirectional causal relationship between SAS and chronic gastritis. The leave-one-out method and funnel plots both suggested that the data were reliable (Supplementary file). MRlap analysis revealed partial sample overlap between the SAS and chronic gastritisAS datasets (both derived from the FinnGen biobank), but its impact on causal effect estimates was statistically insignificant (forward: p_different = 0.688, reverse: p_different = 0.576), indicating that the research

**Figure 2.** (**A**) The forest plot of the potential positive causal relationship between SAS and chronic gastritis. (**B**) The forest plot of the potential reverse causal relationship between SAS and Chronic gastritis. MR: mendelian randomization. Results of MR-CAUSE Analysis. (**C**) MR-CAUSE test of Chronic gastritis caused by SAS. (**D**) MR-CAUSE test of SAS caused by chronic gastritis. The p-values from the sensitivity analysis are uncorrected for validation, and their consistency supports the robustness of the results.



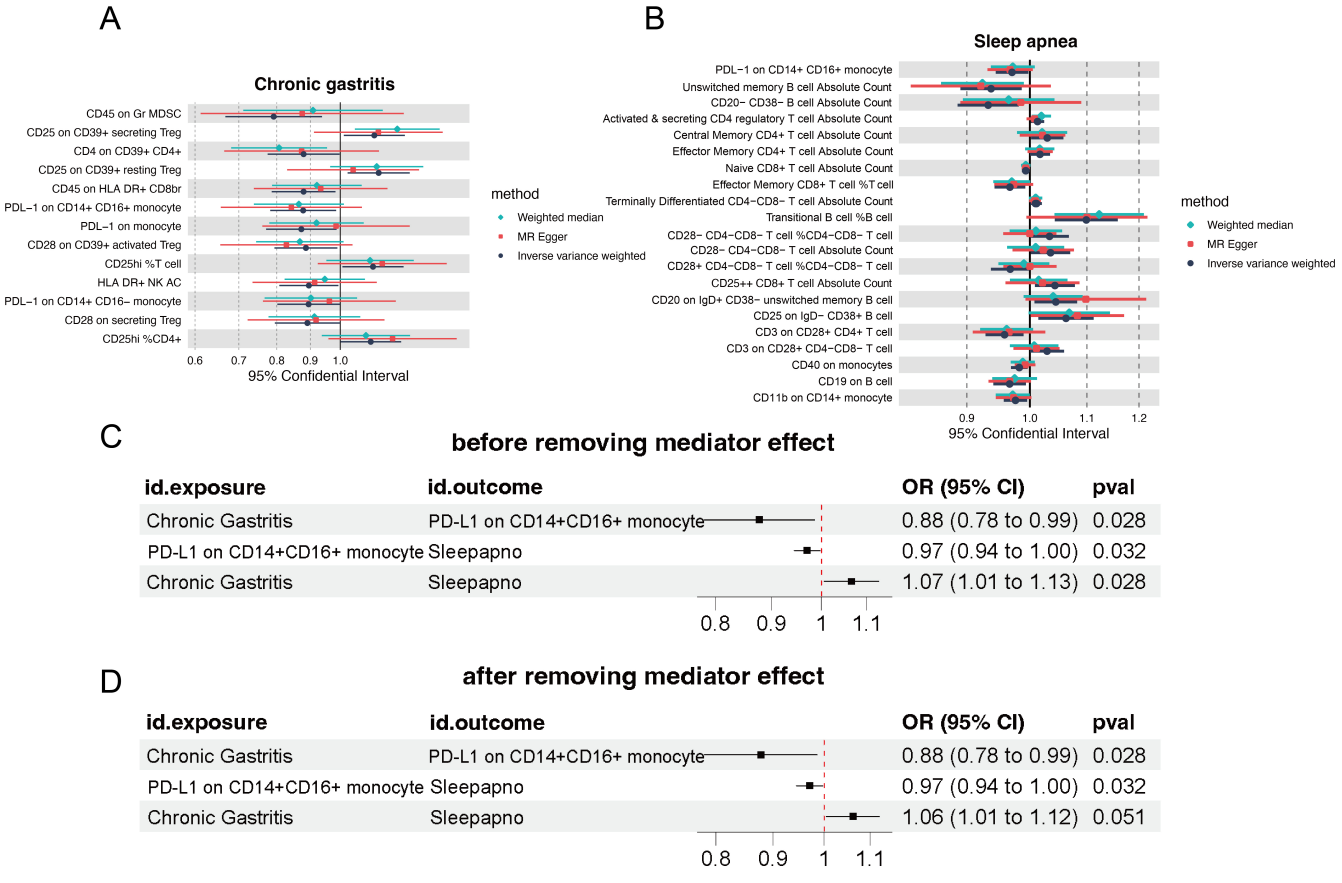
conclusions are not affected by overlap. Detailed analysis results are provided in the supplementary materials. This study validates the robustness of the main analysis results based on three independent subsets from the UK Biobank (ukb-b-6716, ukb-b-12191, ukb-d-K11_CHRONGASTR), all of which have no overlap with the main analysis sample and have complementary phenotype definitions. However, none of the three external datasets achieved statistical significance, yet we still believe that the main analysis results are scientifically reasonable: There are currently no other available datasets for sleep apnea syndrome (SAS), and the dataset for gastritis is relatively small with insufficient SNP numbers, but our original results have been validated through MRlap and various sensitivity tests, making them reliable.

## Mediating signatures in immune cell and inflammatory proteins in chronic gastritis triggering SAS

### Chronic gastritis increases the risk of SAS mediated by PDL-1 on CD14+CD16+ monocytes

Next, we explored the causal relationship between chronic gastritis and genetic markers of signatures in immune cell (Figure 3A) and between genetic markers of signatures in immune cell and SAS (Figure 3B) through three methods: MR Egger, IVW, and weighted median. The results were significant, indicating that chronic gastritis can increase the risk of SAS by changing genetic signatures in immune cell, especially PD-L1 expression on CD14+CD16+ monocytes. Finally, we estimated the direct effect of chronic gastritis on SAS after accounting for mediation via signatures in immune cell and inflammatory proteins (before removing: OR = 1.07, 95% CI: 1.01-1.13, p = 0.028; after removing: OR = 1.06, 95% CI: 1.01-1.12, p = 0.051) (Figure 3C) and found that the significance after removal of the immunomediator was significantly attenuated, revealing that chronic gastritis may increase the risk of SAS by upregulating PD-L1 levels on CD14+CD16+ monocytes. To exclude horizontal pleiotropy, we performed the MR-Egger regression intercept term test and the MR-PRESSO test, which showed that there was no significant horizontal pleiotropy (p > 0.05). In the heterogeneity test, the P-value of Cochran's Q was greater than 0.05, indicating the absence of significant heterogeneity. Leave-one analysis showed that no single SNP overinfluenced the effect estimate, further confirming the stability of the results. Through the above methods, it is shown that the changes of genetic markers of signatures in immune cell constitute a significant intermediary pathway between chronic gastritis and SAS, and chronic gastritis can increase the risk of SAS by activating immunity.

**Figure 3.** Mendelian Randomization Study for mediation analysis of genetic signatures in immune cell. (**A**) Causal relationship between chronic gastritis and genetic signatures in immune cell. (**B**) Causal relationship between genetic signatures in immune cell and SAS. (**C**) and (**D**) Mediation Mendelian randomization of PD-L1 on CD14+CD16+ monocytes. Two forest plots reveal the causal relationship between chronic gastritis and SAS before and after removing PD-L1 on CD14+CD16+ monocytes.



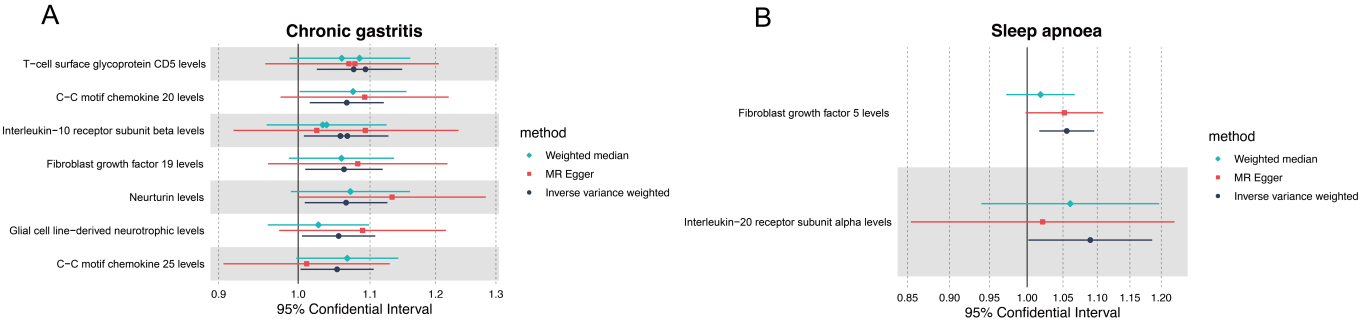
**No inflammatory protein were found to mediate chronic gastritis and SAS**

In further MR for mediation analysis, we also explored the causal relationship between the level of inflammatory proteins caused by chronic gastritis (Figure 4A) and the causal relationship between the level of inflammatory proteins leading to SAS (Figure 4B). The results showed that chronic gastritis caused T-cell surface glycoprotein CD5 levels, T-cell surface glycoprotein CD6 isoform levels, C-C motif chemokine 20 levels, Interleukin-10 receptor subunit alpha levels, Fibroblast growth factor 19 levels, Neurturin levels, Interleukin-10 receptor subunit beta levels, Glial cell line-derived neurotrophic factor levels, and C-C motif chemokine 25 levels. These nine inflammatory proteins increased. Fibroblast growth factor 5 levels and Interleukin-20 receptor subunit alpha levels are positively correlated with the incidence of SAS, but in our study sample, no specific inflammatory proteins was found to significantly mediate the relationship between chronic gastritis and SAS. This finding suggests that future research may pay more attention to exploring the non-inflammatory link between chronic gastritis and SAS and how to improve the comorbidity of the two diseases through comprehensive interventions.

**Mediating signatures in immune cell and inflammatory proteins in SAS triggering chronic gastritis**

In exploring the mediating pathways of SAS on chronic gastritis (CG), candidate inflammatory proteins (C-C motif chemokine 19 levels, Natural killer cell receptor 2B4 levels) and immune cell markers (CD11c+ monocyte %monocyte, etc.) did not reach statistical significance. Sensitivity analyses still indicate this null association. This suggests that there are no apparent immune inflammatory mediators mediating the effect of SAS on chronic gastritis, and other unmeasured mechanisms may exist.

**Figure 4.** Mendelian randomization study for mediation analysis of inflammatory proteins. (**A**) Causal relationship between chronic gastritis and inflammatory proteins. (**B**) Causal relationship between inflammatory proteins and SAS.




## Discussion

Sleep Apnea Syndrome (SAS) is a widespread disorder characterized by disruptions in breathing during sleep, manifested through recurrent episodes of complete (apnea) or partial (hypopnea) cessation of airflow, resulting in intermittent oxygen deprivation[33-35]. This condition is associated with symptoms such as intermittent hypoxemia, sleep fragmentation, systemic inflammatory responses, oxidative stress, and disruptions in physiological homeostasis[33]. Moreover, the inadequate oxygen supply can lead to dysbiosis of the intestinal microbiota, alterations in intestinal metabolites[36], and damage to the intestinal barrier due to insufficient mucosal oxygenation[37]. These symptoms may contribute to an increased incidence of chronic gastritis. Conversely, nocturnal awakenings, sleep deprivation, and sleep fragmentation induced by chronic gastritis may exacerbate the occurrence of SAS[38, 39] and impair the recruitment of upper airway dilators, potentially leading to further obstructive respiratory events[40]. Considering the limitations associated with time ranking in existing observational studies, this research elucidated the potential causal relationship between SAS and chronic gastritis as risk factors, utilizing a two-way, two-sample MR approach. Additionally, the study investigated the mechanistic pathways mediating the interaction between these conditions through two-step MR analysis.

Notably, our findings revealed, for the first time, that chronic gastritis mediates an increased risk of SAS via the expression of PD-L1 by CD14+CD16+ monocytes. CD14+CD16+ monocytes contribute to SAS-associated oxidative stress and induce low-grade systemic inflammation by infiltrating the endothelium and overexpressing PD-L1[41-43]. Furthermore, they inhibit the activation, proliferation, and cytotoxic activity of CD8+ T cells through the hypoxia-dependent PD-L1/PD-1 pathway[41, 44]. The primary rationale for the efficacy of low hypoglossal nerve stimulation therapy in treating SAS lies in its ability to markedly decrease the expression of PD-L1 and the proportion of CD16+ monocytes, which have notable pro-inflammatory effects. Additionally, it reduces the expression levels of TNF-α and IL-1β[45]. In the context of chronic gastritis, gastric dendritic cells located in the submucosa and intrinsic mucosa frequently express substantial amounts of PD-L1, thereby creating a cellular barrier that prevents T cells from infiltration and erosion of the gastric mucosa[46]. Similarly, monocytes may be extensively recruited and express PD-L1 in response to inflammatory stimuli, potentially exacerbating the severity of SAS. Nonetheless, there is currently no research indicating that gastritis influences SAS through PD-L1 expression by CD14+CD16+ monocytes, and the specific mechanisms involved remain to be elucidated.

This study presents several advantages, notably the utilization of association analysis based on clinical cross-sectional studies, which further elucidates the bidirectional causal relationship between SAS and chronic gastritis through MR

analysis. This approach effectively circumvents the ethical concerns associated with observational analyses, as well as the confounding effects and biases introduced by reverse causality. Furthermore, two-step MR was employed to investigate the potential mediation effect between SAS and chronic gastritis. This analysis confirmed the involvement of 91 inflammatory proteins and 731 signatures in immune cell, thereby establishing a foundation for future research endeavors.

Nevertheless, this study possesses certain limitations that warrant discussion. Firstly, although our investigation did not identify any guided pleiotropy, the possibility of potential pleiotropy remains, which is a common challenge in MR research and can introduce bias. Secondly, the MR study was performed on individuals of European descent, while the cross-sectional study involved a multi-ethnic Chinese cohort. Additionally, the GWAS sample size for chronic gastritis was relatively small. Future research focusing on a homogeneous ethnic group is necessary to mitigate potential confounding effects arising from population heterogeneity. Lastly, our study is essentially a statistical analysis and lacks experimental validation using blood samples from chronic gastritis.

## Conclusion

This study proved that there is a significant bidirectional causal relationship between SAS and chronic gastritis by two-sample MR. MR study for mediation analysis found that the level of PD-L1 expressed by CD14+CD16+ monocytes plays a key mediating role between chronic gastritis and SAS. These findings provide potential targets for the development of novel therapeutic strategies for chronic gastritis and SAS, and in particular, interventions targeting PD-L1 may help reduce the risk of both diseases.

## Abbreviations

95% CIs: 95% confidence intervals; CAUSE: Causal Analysis Using Summary Effect Estimates; CG: Chronic gastritis; GERD: Gastroesophageal reflux disease; GID: Gastrointestinal disorders; GWAS: Genome-wide association study; IBD: Inflammatory bowel disease; IVs: Instrumental variables; IVW: the Inverse Variance Weighted; LD: Linkage disequilibrium; MR:Mendelian Randomization Study; MRlap: the Mendelian Randomization with Latent Adjustment for Overlap; OR: Odds ratios; SAS: Sleep apnea syndrome; SNPs: Single nucleotide polymorphisms.

## Author Contributions

**Ziyan Zhao**: Methodology. Data curation, Writing - original draft, Writing - review & editing. **Yanyu Zhu**: Writing - review & editing. **Chang You**: Visualization. **Yan Wang**: Writing - review & editing. **Tianchi Zhuang**: Writing - review & editing: Conceptualization. **Yingqi Yang**: Conceptualization, Funding acquisition, Writing - review & editing. All authors read and approved the final manuscript.

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## Ethics Approval and Consent to Participate

Not applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

This study analyzed datasets that are publicly accessible. These datasets can be found at the following URLs: FinnGen (https://storage.googleapis.com/finngen-public-data-r9/summary_stats/finngen_R9_M13_OSTEONECROSIS.gz) and GWAS Catalog (https://www.ebi.ac.uk/gwas/downloads/summary-statistics).

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Review Article

Life Conflux

# The role of IL-17 family in the process of pulmonary fibrosis

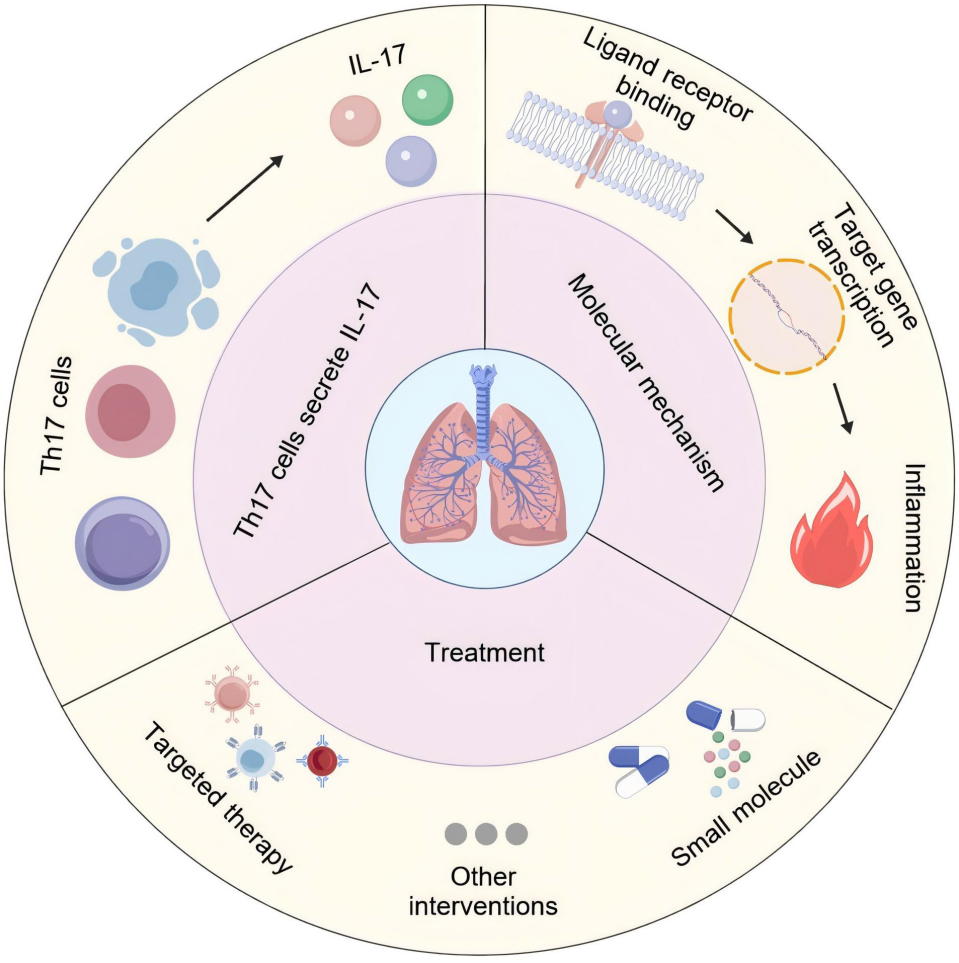
## Authors

**Ge Zhou,** Amin Li, Ruikai Wang

## Correspondence

kerywang1984@163.com (R. Wang)

## Graphical Abstract

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Review Article

Life Conflux

# The role of IL-17 family in the process of pulmonary fibrosis

**Ge Zhou** [1], Amin Li [2], Ruikai Wang [1*]

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## Abstract

Pulmonary fibrosis is a serious lung disease characterized by the destruction of alveolar structures and excessive proliferation of fibrous tissue. The interleukin-17 (IL-17) family consists of six members (IL-17A-17F), which play a crucial role in the occurrence and development of pulmonary fibrosis. The IL-17 family drives pulmonary fibrosis through multiple mechanisms such as pro-inflammatory cytokines, immune cell recruitment, and fibroblast activation. IL-17A is the core molecule, while other members participate in the disease process through synergistic or independent pathways. Targeting the IL-17 signaling axis provides a new strategy for the treatment of pulmonary fibrosis. This article summarizes the effects of IL-17 on pulmonary inflammation response and fibrosis process through literature review, as well as its possible involvement in signal transduction molecular mechanisms, and explores its potential as a therapeutic target, providing theoretical basis for future research aimed at regulating IL-17 expression and function to treat related diseases.

**Keywords:** interleukin-17 (IL-17) family; IL-17 receptor; pulmonary fibrosis

## Introduction

Pulmonary fibrosis is a chronic progressive lung disease characterized by fibrosis of lung tissue and scar formation. This disease can damage the alveolar structure, impair gas exchange function, and ultimately lead to respiratory failure [1]. From the perspective of histopathology, this disease presents obvious heterogeneity characteristics, specifically manifested as thickening of alveolar walls, infiltration of inflammatory cells, and abnormal proliferation of fibroblasts accompanied by a large amount of extracellular matrix deposition. Interstitial lung disease (ILD) and idiopathic pulmonary fibrosis (IPF) are the most severe and irreversible types of progressive pulmonary fibrosis [2].

In 2014, the US Food and Drug Administration (FDA) approved two drugs, pirfenidone and nintedanib, for the treatment of pulmonary fibrosis [3]. However, there is currently a lack of effective treatment options for pulmonary fibrosis, and available treatment methods can only delay the progression of the disease and cannot completely cure it. Therefore, a deeper understanding of the pathogenesis of pulmonary fibrosis and the search for new therapeutic targets have important clinical significance.

IL-17 is a cytokine secreted by Th17 cells and was initially found to be involved in the pathological processes of autoimmune and inflammatory diseases. The latest research confirms that IL-17 plays a crucial role in the occurrence and development of pulmonary fibrosis. This article will review the role of IL-17 in pulmonary fibrosis, providing reference for further research and treatment of pulmonary fibrosis.

## IL-17 cytokine family

### Overview

The IL-17 family consists of six structurally related cytokine members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F [4]. IL-17A is the first family member discovered and the most extensively studied cytokine. Researchers cloned the molecule using T-cell hybridoma technology in 1993 [5], and officially named it cytotoxic T lymphocyte associated antigen 8 (CTLA-8). IL-17B and IL-17C were identified through sequence similarity alignment in the expression sequence tag database [6]. IL-17D, IL-17E, and IL-17F were mainly discovered through sequence homology search of genomic DNA sequences.

The molecular weight distribution of members in this family is between 30-52 kDa, and they have overlapping but different biological activities. IL-17A is a homodimeric glycoprotein composed of 155 amino acids linked by disulfide bonds. Its molecular weight with IL-17E is approximately 30 kDA, while

1 Pulmonary and Critical Care Medicine, First Affiliated Hospital, Anhui University of Science & Technology (Huainan First People's Hospital), Huainan, China

2 Medical School, Anhui University of Science & Technology, Huainan, China

* Corresponding Author.

IL-17B, IL-17C, and IL-17F are approximately 40 kDA. IL-17D has the highest molecular weight at 52 kDA [7]. Except for IL-17B, all other members form homodimeric structures through disulfide bonds. Sequence analysis shows that IL-17F is most similar to IL-17A, with 55% sequence homology, and they often co express. The homology between IL-17B, IL-17C, and IL-17D is 23-29%, while the homology between IL-17E and IL-17A is only 17%, making it the most diverse subtype in the family (Table 1) [8].

**Table 1.** The molecular weight and sequence homology of IL-17 family members

| IL-17 Subtype | Size (kDA) | Sequence homology with IL-17A(%) |
|---|---|---|
| A | 35 | - |
| B | 41 | 29 |
| C | 40 | 23 |
| D | 52 | 25 |
| E | 34 | 17 |
| F | 44 | 55 |

### IL-17 cytokine family receptors

The IL-17 receptor (IL-17R) is a type I transmembrane protein consisting of an extracellular domain of 293 amino acids, a transmembrane domain of 21 amino acids, and a cytoplasmic tail of 525 amino acids. The IL-17 receptor family consists of five subunits, namely IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE, with IL-17RA being a common receptor [9]. IL-17A and IL-17F bind to the dimer IL17RA/RC complex, IL-17B and IL-17E bind to the dimer 17RA/RB complex, and IL-17C binds to the IL-17RA/RE complex. However, the subunits of IL-17RD specific heterodimers have not yet been determined [10]. IL-17 participates in chronic and persistent inflammation, autoimmunity, and maintaining epithelial integrity by binding to the IL-17 receptor. These receptors share a unique protein-protein interaction domain at their cytoplasmic tails, called the SEF/IL-17R (SEFIR) domain. NF-κB activator 1 (Act1) is an activator of NF-κB and a member of the SEFIR protein family. It is also a key component of IL-17 signaling transduction [11]. The IL-17 receptor binds to IL-17 family cytokines in the form of homodimers or heterodimers, and transmits signals through adaptor protein 1 (Act1) and E3 ubiquitin ligase tumor necrosis factor receptor associated factor protein 6 (TRAF6), leading to activation of nuclear factor kappa B (NF-κB), mitogen activated protein kinase (MAPK), and CCAAT/enhancer binding protein (C/EBP) pathways [12]. The signal transduction mediated by IL-17-Act1 leads to pro-inflammatory and neutrophil mobilization of cytokines and chemokines, including chemokine (C-X-C Motif) ligand- 1 (CXCL1), tumor necrosis factor (TNF), IL-6, and colony stimulating fctor 2 (CSF2) [13]. The IL-17A receptor is also commonly expressed in non hematopoietic cells, including epithelial cells and fibroblasts [14]. They play a crucial role in the development of pulmonary fibrosis and differentiation into myofibroblasts in EMT, leading to enhanced extracellular matrix deposition.

### Cell sources of IL-17 cytokine family

Although Th17 cells are generally considered the main source of IL-17, this cytokine is also produced by gamma delta T cells, cytotoxic T cells ($CD8^+$ alpha beta T cells), natural killer (NK) cells, invariant natural killer T cells (iNKT), innate lymphocytes (ILC), neutrophils, eosinophils and macrophages, known as type 17 cells [15]. IL-17A can be secreted by various cell types, including $Th17CD8^+$ T (Tc17), γδT17, Innate immunity and non hematopoietic cells. IL-17B originates from neutrophils, chondrocytes, neurons, stromal cells, intestinal epithelial cells and B cells (germinal and memory cells). IL-17C originates from epithelial cells. IL-17D comes from fibroblasts, colonic epithelial cells, brain, skeletal muscle, adipose tissue, heart, lungs and pancreas. IL-17E is produced by mast cells, alveolar macrophages, eosinophils, basophils, ILC2, dendritic cells, stromal cells, epithelial cells and Th2 cells secrete. IL-17F is composed of Th17, Lymphoid tissue inducer, NK, iNKT, Neutrophils, ILC3, and gamma delta T cells secrete (Table 2) [16].

**Table 2.** Cell sources of IL-17 cytokine family

| IL-17 Subtype | Cell sources |
|---|---|
| A | $Th17CD8^+$ T(Tc17), γδ T17, Innate immunity and non hematopoietic cells |
| B | neutrophils, chondrocytes, neurons, stromal cells, intestinal epithelial cells and B cells (germinal and memory cells) |
| C | epithelial cells |
| D | fibroblasts, colonic epithelial cells, brain, skeletal muscle, adipose tissue, heart, lungs and pancreas |
| E | mast cells, alveolar macrophages, eosinophils, basophils, ILC2, dendritic cells, stromal cells, epithelial cells and Th2 cells |
| F | Th17, Lymphoid tissue inducer, NK, iNKT, Neutrophils, ILC3 and gamma delta T cells |

Note: ILC2 type 2 innate lymphoid cells; NK natural killer; iNKT invariant natural killer T cells; ILC3 type 3 innate lymphoid cells

## The role of IL-17 in promoting inflammation and pulmonary fibrosis

### Acute Inflammation: Its Role in Pneumonia

Inflammation is the early stage of fibrosis. IL-17A participates in fibrosis development by regulating early pulmonary inflammation. In the pulmonary injury model induced by bleomycin (BLM), IL-17A expression is significantly upregulated, stimulating endothelial and epithelial cells to secrete pro-inflammatory factors such as Tumor Necrosis Factor alpha (TNF - α), IL-1, IL-6, Transforming Growth Factor

beta (TGF - β), while promoting the production of chemokines such as IL-8, CCL1, CXCL2, and CXCL5. These molecules mediate the recruitment of inflammatory cells to the alveolar surface, driving the inflammatory cascade and accelerating the progression of pulmonary fibrosis [17]. Multiple studies have shown that BLM stimulation triggers neutrophil infiltration through the IL-17-dependent pathway, significantly increasing IL-6 and IL-1 β levels, ultimately promoting abnormal collagen deposition [18]. Therefore, intraperitoneal anti-IL-17A blockade of IL-17A can alleviate acute inflammation and fibrosis characteristics in mice [19].

It is worth noting that clearing alveolar macrophages can downregulate the expression of IL-23 and IL-1 β, inhibit IL-17 activity, and alleviate early pulmonary inflammation and fibrosis caused by silica exposure [20]. In the IL-17 family, IL-17F has the highest sequence homology (55%) with IL-17A. It plays a key role in chronic inflammatory lung disease by inducing the production of IL-6 and chemokines in human tracheal epithelial cells, venous endothelial cells, and fibroblasts [21]. Further research has shown that IL-17C can stimulate Th17 cells to secrete a large amount of IL-17A, IL-17F, and IL-22, indicating its ability to enhance the effector function of Th17 cells. High expression of IL-17C in inflammatory tissues can exacerbate local inflammatory reactions [22]. The functional research of IL-17B is not yet sufficient, but existing evidence shows that some of its functions overlap with IL-17A and have pro-inflammatory properties under specific conditions, such as inducing peritoneal exudate cells and 3T3 cell lines to produce IL-6, IL-23, and IL-1 α, and promoting pulmonary fibrosis by synergistically stimulating Th17 cells to secrete neutrophil chemokines through TNF-α stimulation [23]. At present, the understanding of IL-17D is still limited. This molecule is mainly detected in activated B cells and resting $CD4^+$ T cells, and it participates in immune regulation by promoting the secretion of pro-inflammatory cytokines by endothelial cells [24].

**Chronic fibrosis: role in pulmonary fibrosis**

Idiopathic pulmonary fibrosis is a progressive pulmonary interstitial fibrosis of unknown etiology, typically fatal and associated with chronic inflammation and tissue repair dysfunction.

Research has revealed that the IL-17 signaling pathway regulated by IL-1 β and IL-23 plays a critical role in the early stages of disease. This pathway activates neutrophil inflammatory response and upregulates fibroblast matrix metalloproteinase expression, driving the progression of pulmonary fibrosis [25]. Clinical data shows that elevated neutrophil count in bronchoalveolar lavage fluid of IPF patients can serve as a predictive indicator of early mortality risk [26].

Further research has found that Th17 cells in IPF patients secrete abnormally high concentrations of TGF-β and IL-17A. In the mouse pulmonary fibrosis model induced by bleomycin, these cytokines significantly increased collagen deposition. The co culture experiment of human lung fibroblasts and Th17 cells also confirmed that it promotes the excessive generation of extracellular matrix components. It is worth noting that the pro fibrotic effect of IL-17 has cross disease commonalities: studies have shown that IL-17 is highly expressed in the bronchial mucosa of severe asthma patients and induces epithelial mesenchymal transition (EMT) in human small airway epithelial cells in vitro [27]. In addition, IL-17 mediates the EMT process of salivary gland epithelial cells in healthy individuals through an IL-22 dependent mechanism, revealing its important role in the progression of Sjogren's syndrome fibrosis [28].

**IL-17A**

The study of experimental animal models has confirmed the crucial role of IL-17A in regulating the complex interactions between lung inflammation and fibrosis. In the bleomycin induced pulmonary fibrosis model, the expression level of IL-17A was significantly increased. IL-17A exacerbates the pathological process of pulmonary fibrosis by activating inflammatory responses and promoting collagen production [29]. Experiments have shown that IL-17A receptors are widely expressed on the cell membrane surfaces of lung epithelial cells and fibroblasts, which are involved in the pathological process of epithelial mesenchymal transition associated with pulmonary fibrosis. In addition, these cells also regulate the transformation of fibroblasts into myofibroblasts and increase the deposition of extracellular matrix [30]. It is worth noting that the IL-17A co factor IL-22 exhibits antagonistic effects: It exerts anti-fibrotic effects by inhibiting the EMT process in the bleomycin model [31].

In addition, IL-17A promotes fibrosis by inhibiting autophagy activity in epithelial cells, while neutralizing IL-17A can enhance autophagy levels and accelerate collagen degradation in lung tissue [32]. In some studies, upregulation of IL-17R expression was detected in fibroblasts after treatment with bleomycin. The addition of exogenous IL-17A can promote the proliferation of fibroblasts and specifically induce the synthesis of α -smooth muscle actin (α-SMA) and collagen [33]. This process relies on signal transduction mediated by NF-κ B activator 1 (Act1): Act1, as a key adaptor protein for IL-17 receptor signaling, regulates fibrosis process by activating the NF- κ B pathway, which also plays an important role in autoimmune diseases [34].

**IL-17B**

The role of IL-17B in fibrotic diseases is still limited in current research, and its specific function has not been fully elucidated. Existing studies have shown that in a mouse model of bleomycin induced pulmonary fibrosis, the absence of IL-17B can significantly slow down the progression of fibrosis [35]. In addition, another study suggests that the outer membrane vesicles produced by symbiotic microorganisms can induce the expression of IL-17B, activate neutrophil chemotaxis genes and Th17 cell activation genes, ultimately exacerbating lung inflammation and fibrosis [2]. However, some studies support the possibility that IL-17A and IL-17B have similar roles in regulating inflammation and fibrosis. For example, IL-17B can stimulate peritoneal neutrophils, macrophages, and lymphocytes to produce IL-6, IL-23, and IL-1 α [23]. In addition, it also regulates the release of TNF-α and IL-1 β from human monocytes/macrophages. IL-17B promotes the recruitment of cells expressing chemokine receptors CXCR4 or CXCR5, while experimental intraperitoneal administration of recombinant human IL-17B confirms its chemotactic appeal to neutrophils, who subsequently release chemokines for other cells. Not only that, IL-17B can also work synergistically with IL-33 to regulate the immune response

mediated by helper T cells (Th) [36]. These findings suggest that IL-17B may promote fibrosis in the early stages of inflammation through its pro-inflammatory properties.

**IL-17C**

IL-17C is a multifunctional cytokine expressed in $CD4^+$ T cells, dendritic cells (DCs), macrophages, and epithelial cells, involved in the immune defense and inflammatory response of the lungs. These cells produce this interleukin during antibacterial activity, enhancing the inflammatory re sponse [37]. In pulmonary fibrosis and certain lung diseases, IL-17C can act in conjunction with IL-17A or independently mediate fibrosis signaling.IL-17C works by binding to a heterodimeric receptor complex composed of IL-17RA and IL-17RE subunits. Among them, IL-17RE is mainly distributed on the surface of epithelial cells and Th17 cells. Th17 cells produce IL-17A and IL-17F upon stimulation by IL-17C, indicating that IL-17C may regulate the initial stage of the inflammatory cascade [38]. In addition, IL-17C triggers the NF-κ B/Act1 signaling axis upon binding to receptors, thereby activating the MAPK pathway to regulate target gene expression [39]. In the study of idiopathic pulmonary fibrosis (IPF), lipopolysaccharide induced lung injury was used as the IPF model [40]. In this study, IL-17C was confirmed to play a crucial role in pulmonary inflammation by mediating epithelial injury, pro-inflammatory cytokine release, and neutrophil recruitment. Recently, it has been reported that IL-17C exacerbates pulmonary fibrosis damage by driving neutrophilic inflammation in a model of influenza induced lung inflammation induced by Haemophilus influenzae and cigarette smoke [41]. These findings collectively reveal the pivotal role of IL-17C in inflammation fibrosis transition.

**IL-17D**

Little is currently known about the receptor and function of IL-17D. Research has shown that IL-17D can be detected in B lymphocytes and resting $CD4^+$ T cells. Although IL-17D has a weak activation effect on immune cells, it can regulate the secretion of pro-inflammatory cytokines in endothelial cells [42]. At present, there is still limited understanding of the role of IL-17D in the regulation of pulmonary fibrosis, and further research is needed to elucidate the relationship between IL-17D induced inflammatory response and the development of pulmonary fibrosis.

**IL-17E**

Recent studies have shown that the levels of IL-17E and its receptor IL-17RB are elevated in lung tissue of IPF patients, particularly in alveolar epithelial cells and lung fibroblasts, showing significant enrichment. IL-17E drives pulmonary fibrosis by mediating EMT of alveolar epithelial cells and recruiting and activating pulmonary fibroblasts [43]. It is interesting that this is related to the release of IL-13, which exacerbates collagen deposition during the IPF process. Specifically, IL-25 promotes IL-13 release by activating type 2 innate lymphocytes (ILC2s), thereby triggering collagen deposition during IPF [44].

**IL-17F**

IL-17F and IL-17A have high sequence homology and are co expressed in the same cell population. However, there are significant differences in the roles of these two cytokines in fibrotic diseases [45]. IL-17A and IL-17F both have pro-inflammatory and pro fibrotic effects in pulmonary fibrosis, but IL-17A has a more significant effect. The effect of IL-17F may be relatively weak, but it may synergize with IL-17A in certain specific inflammatory environments. In addition, IL-17F transmits signals through the same IL-17RA/RC receptor complex, but with lower receptor affinity, resulting in weaker downstream signal intensity. Previous studies have shown that IL-17F activates neutrophils and regulates IL-17A related inflammatory gene expression through signaling components such as IL-17RA, Act1, and TRAF6, which have been shown to be key participants in IL-17A mediated inflammatory responses. Animal experiments have shown that specific overexpression of IL-17F in mouse lung tissue leads to infiltration of macrophages and lymphocytes, as well as increased mucus secretion. Although there is currently no direct evidence to suggest that IL-17F contributes to the progression of idiopathic pulmonary fibrosis , these observations related to IL-17A and inflammatory response suggest that IL-17F may be a potential effective target for treating IPF.

**The molecular mechanism of IL-17 in pulmonary fibrosis**

Pulmonary fibrosis is a complex pathological process with unknown etiology, which may be related to long-term exposure of the lungs to various types and sources of damaging factors. This process begins with damage to the alveolar epithelium, followed by a series of coagulation reactions, inflammatory reactions, and abnormal remodeling and regeneration of lung tissue, ultimately leading to progressive proliferation of fibrous connective tissue. Although studies have shown that environmental factors such as smoking and ionizing radiation may be key triggers [46], the potential molecular mechanisms underlying the development of these diseases are still not fully understood. At the same time, inflammation and its role in the proliferation phase are evident, and the intercellular regulatory system gradually shifts towards promoting collagen synthesis, fibroblast proliferation, activation, and differentiation. This process involves the epithelial mesenchymal transition mechanism and excessive deposition of extracellular matrix components [47].

The cytokines of the IL-17 family have pleiotropy and exert effective and diverse functions in vivo through classical and non classical signaling pathways [48]. Classical signal transduction induces transcription and post transcriptional mechanisms, which involve autoimmunity, hypersensitivity reactions, and metabolic reprogramming in lymphoid tissue. Non classical signal transduction collaborates with other receptor systems to regulate tissue repair and regeneration. According to classical concepts, pulmonary fibrosis is a gradual process that transitions from the initial injury stage to the inflammatory response stage, followed by the repair, regeneration, and remodeling of lung tissue. Genetic susceptibility factors, such as polymorphisms in Toll like receptor interacting protein genes, may participate in disease initiation by inhibiting the TGF-β signaling pathway [49].

IL-17 binds to the IL-17 receptor and initiates signal transduction by recruiting the adaptor protein Act1 through the SEFIR domain. Act1 rapidly recruits and ubiquitinates TNF receptor associated factor 6 , activates various downstream pathways, and induces transcription of IL-17A target genes

[50]. These pathways include: TRAF6/TAK1/NF-κB pathway, TRAF6/MAPK/AP-1 pathway, and Act1/TRAF6/C/EBP pathway (Figure 1) [51].

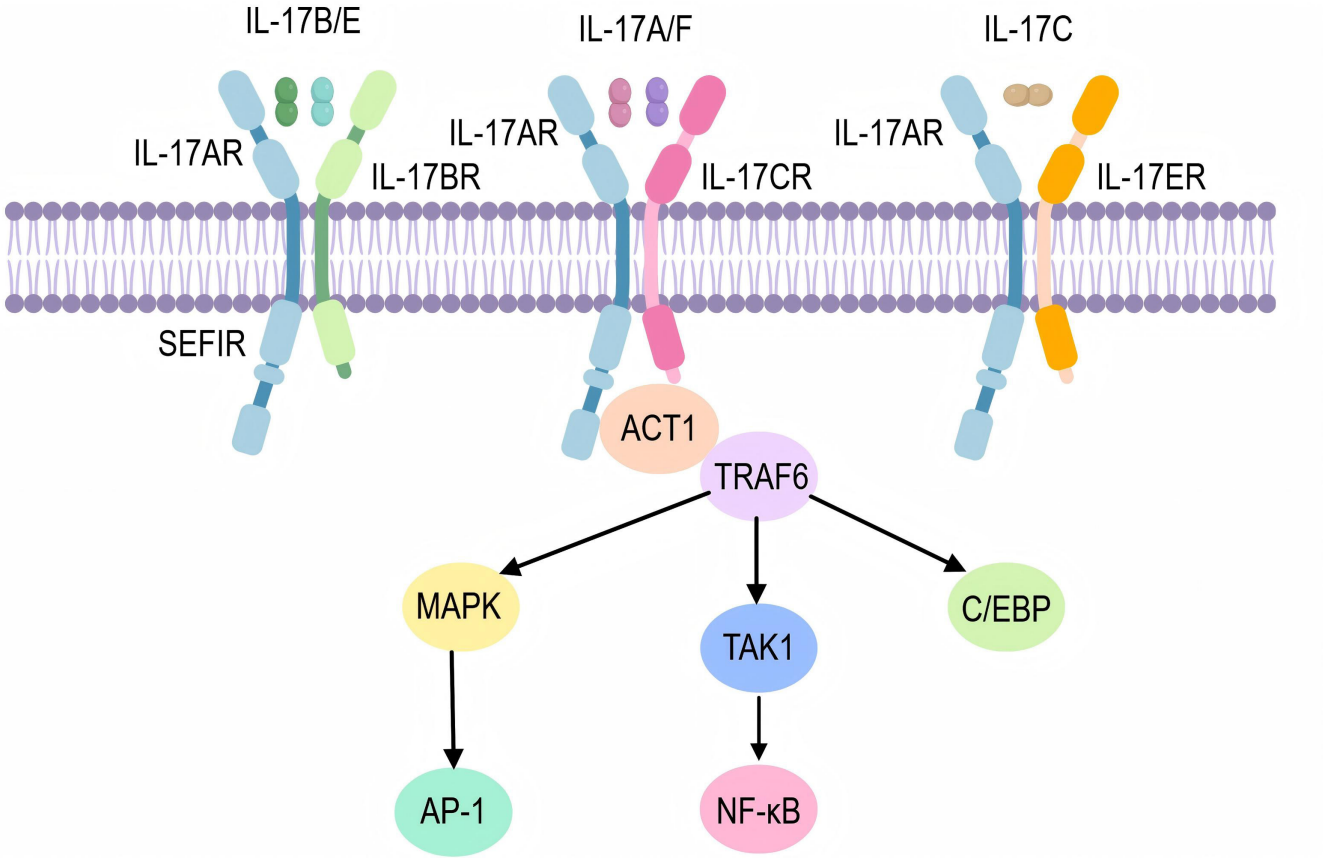
Act1 is a non redundant activator of IL-17RA dependent signaling, acting as a lysine-63 (K63) E3 ubiquitin ligase, recruiting and ubiquitinating TRAF6, activating the complex of transforming growth factor beta activated kinase 1 (TAK1) and nuclear factor kappa B inhibitor kinase (IKK) [52]. Then, IKK phosphorylates the NF-κ B subunit, and the labeled I κ B is hydrolyzed by proteases, exposing the nuclear localization signal of NF-κ B. This signal is released and binds to the target gene to promote its transcription. In the nucleus, NF-k B directly or in combination with activator protein-1 (AP-1) induces transcription of target genes [53]. After activation of these signaling pathways, they first promote the expression of antimicrobial peptides, chemokines, as well as cytokines such as TNF-α, IL-1 β, IL-6, and IL-23 in host cells. Subsequently, these inflammatory mediators recruit pro-inflammatory immune cells such as neutrophils and Th17 cells, ultimately forming a persistent inflammatory positive feedback loop [54].

**Targeted therapy**

Given the strong pro-inflammatory effect of IL-17, drugs targeting IL-17 or IL-17R are potential therapeutic candidates for inflammatory autoimmune diseases [55]. Clinical trials have shown that antibodies targeting IL-12p40 (ustekinumab), IL-17A (sukinumab and izumab), IL-17A and IL-17F (bimekizumab), IL-17RA (brodalumab), and IL-23 (guselkumab, tildrakizumab, and risankizumab) are effective in treating moderate to severe psoriasis [56]. Yiqizhu monoclonal antibody is a humanized IgG4 monoclonal antibody that specifically binds to IL-17A to block its interaction with receptors and inhibit the release of pro-inflammatory cytokines. Research has found that dual antibodies targeting IL-17A/F (compared to monoclonal antibodies) show better efficacy in treating psoriasis than single target inhibitors, but these drugs have poor therapeutic response in rheumatoid arthritis (RA) and multiple sclerosis (MS) [57].

Further research reveals that abnormal activation of the Act1-SHP2 complex leads to sustained IL-17 signaling and triggers treatment resistance. Animal experiments have shown that SHP2 inhibitors and small molecules that disrupt Act1-SHP2 interactions (such as Igulamide) can effectively alleviate inflammatory responses in mouse MS and RA models [58]. In the exploration of coronavirus disease 2019 (COVID-19 ) treatment, patients treated with netakimab monoclonal antibody showed reduced lung lesion size and decreased oxygen therapy demand, and improved survival rates [59]. However, in another study, netakimab treatment reduced C-reactive protein levels and improved some clinical parameters, but did not reduce the need for mechanical ventilation or improve the survival rate of COVID-19 patients

**Figure 1.** IL-17 molecular mechanism pathway




Note: Act1 NF-κB activator 1; AP-1 activator protein-1; C/EBP CCAAT/enhancer-binding protein; MAPK: mitogen-activated protein kinase; NF-κB nuclear factor-kappaB; TRAF6 tumor necrosis factor receptor associated factor 6; TAK1 transforming growth factor-beta-activated kinase 1.

[60]. These and other studies suggest that transient inhibition of IL-17 may be a therapeutic option for controlling excessive inflammation during acute viral infections.

In the field of respiratory diseases, IL-17 is involved in the pathogenesis of corticosteroid resistant neutropenic asthma. The experimental model of allergic asthma in mice has confirmed that anti-IL-17 monoclonal antibody can reverse neutrophil infiltration in the lungs [56]. Clinical observations have found that IL-17 levels and Th17 cells are increased in patients with neutropenic asthma [61]. Although clinical trials have not yet demonstrated a significant positive effect of blocking the IL-17 pathway on human asthma, patient stratification in future trials may improve outcomes [62].

However, all monoclonal antibodies targeting the IL-17-IL-17R pathway and approved for treatment have some drawbacks, such as requiring injection administration, poor tissue permeability, and various adverse reactions that escalate immune system inflammatory responses. To this end, researchers are developing novel oral small molecule drugs (SMD) that regulate immune responses by blocking IL-17A/IL-17RA protein interactions [63]. These small molecule drugs have advantages in terms of production cost, delivery convenience, and potential higher efficacy.

## Conclusion

IL-17 plays an important role in the occurrence and development of pulmonary fibrosis. It can promote inflammatory response and fibrosis process, becoming a potential therapeutic target. At present, there are multiple treatment methods targeting IL-17 under research, but their efficacy and safety still need further clinical trial verification. Future research needs to further explore the mechanism of IL-17 in pulmonary fibrosis, search for more effective treatment methods, and provide new ideas and strategies for the treatment of pulmonary fibrosis.

## Abbreviations

Act1: NF-κB Activator 1; Act1-SHP2: Act1-Tyrosine Phosphatase; AP-1: Activator Protein-1;α-SMA: alpha-smooth muscle actin; BLM: Bleomycin ; CTLA-8: Cytotoxic T lymphocyte Associated Antigen 8; C/EBP: CCAAT/Enhancer-Binding Protein; CXCL1/2/5: Chemokine (C-X-C Motif) Ligand 1/2/5; CSF2: Colony Stimulating Factor 2; CCL1: Chemokine (C-X-C Motif) Ligand 1; CXCR4/5: Chemokine receptor 4/5; COVID-19: Coronavirus Disease 2019; DC: Dendritic Cells; EMT: Epithelial Mesenchymal Transition; FAD: Food and Drug Administration; IL-17: Interleukin 17; IL-17A/B/C/D/E/F: Interleukin 17A/B/C/D/E/F; IL-17RA/RB/RC/RD/RE/RF: IL-17 receptor A/B/C/D/E/F; ILD: Interstitial Lung Disease; IPF: Idiopathic Pulmonary Fibrosis; IL-17R: The IL-17 receptor; IL-1/6/8/23: Interleukin 1/6/8/23; iNKT: invariant Natural Killer T cells; ILC: Innate Lymphocytes; ILC2/3: Type 2/3 Innate Lymphoid Cells; IL-12p40: Interleukin 12 Subunit p40; IKK: Inhibitor Of KappaB Kinase ; MAPK: Mitogen-Activated Protein Kinase; MS: multiple sclerosis; NF-κB: Nuclear; Factor-KappaB; NK: Natural Killer; RA: Rheumatoid Arthritis; SMD: small molecule drugs; TRAF6: Tumor Necrosis Factor Receptor Associated Factor 6; TNF: Tumor Necrosis Factor; TNF-α: Tumor Necrosis Factor alpha; TGF-β: Transforming Growth Factor beta; TAK1: Transforming growth factor-beta-activated kinase 1

## Author Contributions

Ge Zhou(First Author): writing original draft, prepare, create, or express the content for publication, especially in writing the initial draft, including substantive translation.

Amin Li: writing review and editing, prepare, create, or express the content for publication, especially in writing the initial draft, including substantive translation.

Ruikai Wang(Corresponding Author): supervision, supervise and lead the planning and execution of research activities.

All authors read and approved the final manuscript.

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## Ethics Approval and Consent to Participate

Not Applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

Not Applicable.

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# Tinnitus and Risk of Mortality in Normal-Hearing U.S. Adults: A Weighted Study Adjusted for Covariate Balance

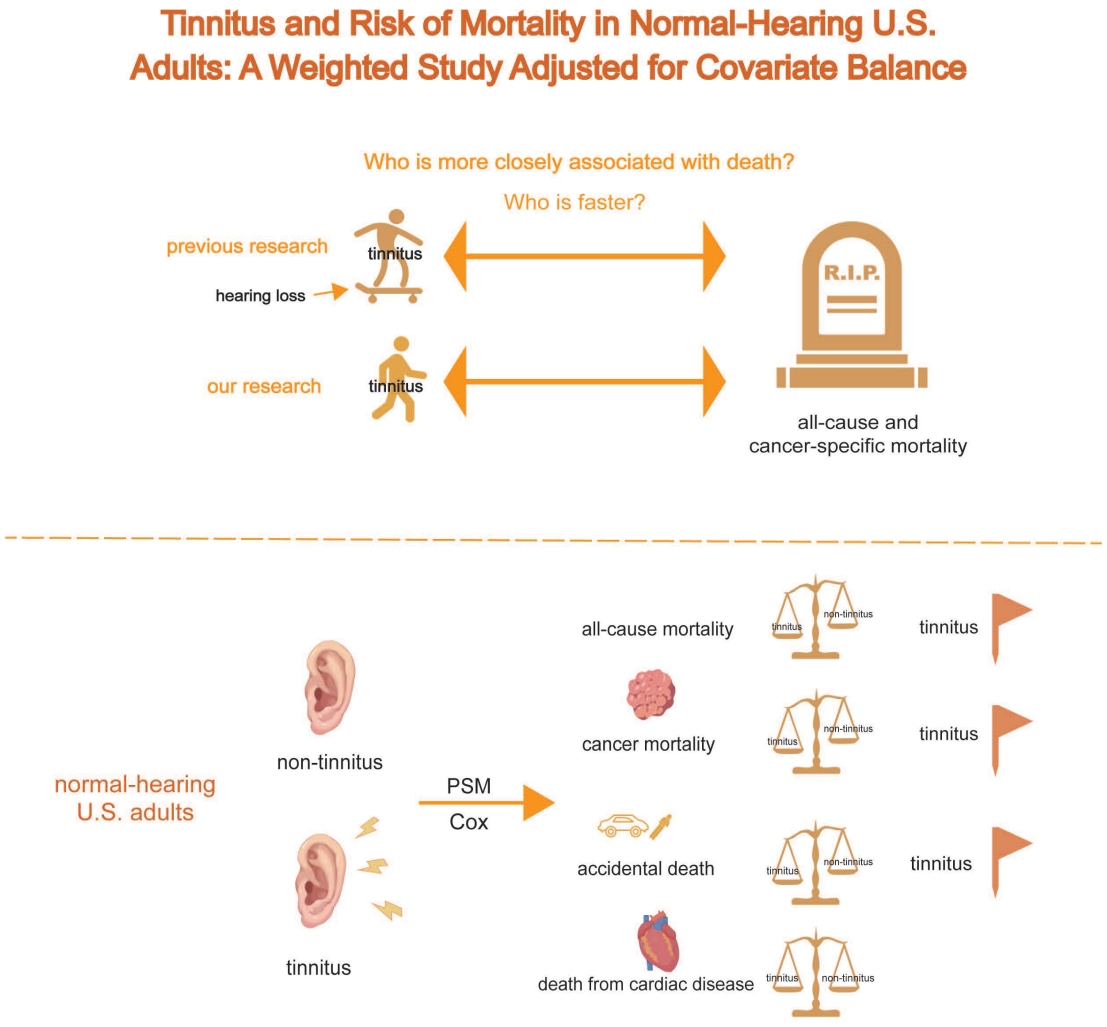

## Authors

**Yuchen Zhang,** Yanqiang Zhang, Ziyue Fu, Chuanlu Shen, Kaile Wu, Yehai Liu

## Correspondence

liuyehai@ahmu.edu.cn (Y. Liu), wukaile@ahmu.edu.cn (K. Wu)


## Graphical Abstract

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# Tinnitus and Risk of Mortality in Normal-Hearing U.S. Adults: A Weighted Study Adjusted for Covariate Balance

**Yuchen Zhang** [1,2†], Yanqiang Zhang [1,2†], Ziyue Fu [1,2], Chuanlu Shen [1,2], Kaile Wu [1*], Yehai Liu [1*]

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## Abstract

**Background:** Tinnitus and hearing loss are both prevalent chronic conditions that can significantly impair quality of life. While prior studies have explored the association between tinnitus and mortality, many have failed to isolate the effect of tinnitus from that of coexisting hearing loss—a frequent comorbidity—which may have introduced confounding and biased the results.
**Objectives:** To investigate the association between tinnitus and both all-cause and cause-specific mortality among U.S. adults aged 20-69 years with normal hearing.
Design and Methods:We conducted a population-based cross-sectional analysis using data from five cycles of the National Health and Nutrition Examination Survey. Adults aged 20-69 years with audiometrically confirmed normal hearing were included. Tinnitus status was self-reported. Key covariates included demographic characteristics, comorbid conditions etc.We implemented propensity score matching and multivariable-adjusted Cox proportional hazards models to estimate the hazard ratios (HRs) for all-cause and cause-specific mortality.
**Results:** Tinnitus was found to be significantly associated with an increased risk of all-cause mortality (HR: 1.84; 95% CI: 1.38-2.44). A particularly elevated risk was observed for cancer-specific mortality (HR: 2.11; 95% CI: 1.26-3.58). Importantly, these findings persisted after adjusting for multiple covariates in the matched cohort, highlighting the robustness of the results derived from a balanced comparison.
**Conclusions:** Among U.S. adults with normal hearing, tinnitus was independently associated with increased risks of all-cause and cancer-specific mortality. These findings underscore the importance of recognizing tinnitus as not merely a benign symptom, but a potential marker of broader health risks, even in individuals without hearing impairment.
**Keywords:** tinnitus; mortality; balance; normal hearing; National Health and Nutrition Examination Survey (NHANES)

## Introduction

Tinnitus is characterized by the perception of sound in the absence of an identifiable external source and is widely recognized as both a somatic and psychological symptom that may signal underlying health disturbances [1]. Due to the clinical heterogeneity of tinnitus in terms of etiology and symptoms, as well as a series of accompanying symptoms such as insomnia, irritability, depression, anxiety, hearing difficulties, and hearing allergies [1,2], patients who visit the hospital with tinnitus as their chief complaint may be biased from the actual tinnitus population. But according to the results of most current studies, the rate of tinnitus is 10-15%, which increases with age [3-5].
Tinnitus, as a highly prevalent and widely symptomatic disease, can cause a huge burden on patients and affect their lives, and the probability of tinnitus patients requiring disability pension is far more than normal people, also causing a huge burden on socioeconomic [6]. Although most patients with tinnitus are able to cope with the distress caused by tinnitus, a subset of patients believe that tinnitus has severely affected their lives [3]. Damage to any part of the hearing pathway can lead to tinnitus, as can some non-hearing disorders [7], so there is currently no appropriate therapy for tinnitus due to the complexity of its pathogenesis [1]. In addition, research has elaborated that tinnitus is a risk factor for depression [8,9], cardiovascular disease [10], cognitive disorders [11], glaucoma [12], and other diseases, which illustrates that tinnitus may affect the organism more severely than we thought. The most frequent comorbid symptom among tinnitus sufferers is hearing loss.
Hearing loss is an extremely prevalent accompanying symptom in the patients with tinnitus [13], and the vast majority of patients with tinnitus have varying degrees of hearing loss, but some researchers have found that approximately one-third of people with tinnitus have hearing thresholds within normal range [14]. Hearing loss has an effect on all-cause and cause-specific mortality in patients, and the exclusion of hearing loss as a confounding bias is necessary when examining the relationship between whether a patient has tinnitus and

1 Department of Otolaryngology, Head and Neck Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui Province, 230022, P. R. China
2 Anhui Medical University, Hefei, Anhui Province, 230022, P. R. China
† These authors have contributed equally to this work and share the first authorship
* Corresponding Author.

mortality [15]. In terms of pathology, hearing loss leads to significant white matter changes, and white matter changes are considered to be one of the manifestations of tinnitus. White matter integrity in patients with chronic tinnitus is both directly affected by age and mediated by hearing loss [16].

The development of hearing loss negatively affects higher auditory processing abilities, and when hearing problems other than hearing loss are present, individuals with normal hearing may have increased compensatory attentional resource mobilisation, as opposed to individuals with hearing loss who show a loss of compensatory network connectivity involving multisensory integration [17]. From the molecular biology point of view, studies have identified tinnitus as a distinct disorder independent of hearing difficulties [18].

Historically, this phenomenon has been largely overlooked in research, resulting in insufficient consideration of hearing loss—a common comorbid symptom of tinnitus—in studies investigating the association between tinnitus and other health outcomes. This omission may have introduced potential bias into previous findings. To minimize such confounding effects, the present study exclusively included participants with tinnitus who demonstrated normal hearing function.

In several past studies, investigators have found that, in addition to hearing related factors [19], obesity [20], smoking [21], gender [22], mental stress [3, 23], hyperlipidemia [24], and cardiovascular disease[25] are risk factors for tinnitus. Considering the above, tinnitus appears to be intricately linked with various systemic conditions, exerting health effects that may extend beyond current understanding.

To gain deeper insights into the relationship between tinnitus and broader health outcomes, as well as its potential prognostic implications, we sought to eliminate the confounding influence of hearing loss. Therefore, this study specifically focused on individuals with tinnitus and normal hearing to assess its association with all-cause and cause-specific mortality. Our findings aim to inform targeted screening and management strategies for this unique patient population in clinical settings.

## Methods

### Study Population

Participants in this study were derived from the National Health and Nutrition Examination Survey (NHANES) (https://www.cdc.gov/nchs/ ), an ongoing program designed to assess the health and nutritional status of a nationally representative sample of the U.S. population. NHANES employs a continuous, multistage, stratified probability sampling design across 2-year cycles, initiated in 1999 and approved by the National Center for Health Statistics Institutional Review Board.

We utilized data from five survey cycles (1999-2004, 2011-2012, and 2015-2016), selecting those that provided complete information on tinnitus-related variables as well as relevant covariates. All data—encompassing demographic, socioeconomic, and health interview responses, audiometric assessments, and laboratory test results—were collected by trained health professionals according to standardized protocols. Appropriate sample weights, as recommended in the official NHANES documentation, were applied to ensure nationally representative estimates during statistical analyses.

To reduce age-related confounding and improve cognitive assessment consistency, we restricted our analytic sample to adults aged 20 to 69 years, thereby excluding individuals whose cognitive function may not have fully developed (young adults) or may have declined due to aging (older adults). The study was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. As the analysis was based on publicly available, de-identified NHANES datasets, it was deemed exempt from institutional review board oversight and did not require individual informed consent.

Through December 31, 2019, the National Center for Health Statistics supplied mortality statistics connected to the National Death Index. The underlying cause of death was classified following the International Classification of Diseases, 10th Revision (ICD-10). Cardiac disease mortality was defined as death from cardiac disease (ICD-10 codes I00-I09, I11, I13, and I20-I51), while cancer mortality was classified as malignant neoplasms (ICD-10 codes C00-C97). The follow-up duration was calculated as the number of months from the date of completion of the tinnitus questionnaire to the date of death or until December 31, 2019, whichever occurred first. To minimize the potential impact of reverse causality, individuals who died within the first 24 months of follow-up were excluded from the analysis.

Through questionnaire surveys, an assessment was made of the existence and severity of tinnitus. The steps for selecting patients with tinnitus have been used in many literatures [26,27]. Tinnitus was classified as present or absent based on a yes or no answer to the following questions, “In the past 12 months, have you ever had ringing, roaring, or buzzing in your ears?”/ “In the past 12 months, have you been bothered by ringing, roaring, or buzzing in your ears or head that lasts for 5 minutes or more?”.

To exclude the impact of hearing on tinnitus, we limited our study to tinnitus patients with normal hearing. Selection steps for patients with normal hearing have also been verified [28]. Low frequency hearing loss (LFHL) was defined by the pure-tone average (PTA) of hearing thresholds at 500, 1000, and 2000 Hz, speech frequency hearing loss (SFHL) by the PTA of hearing thresholds at 500, 1000, 2000, and 4000 Hz, and high frequency hearing loss (HFHL) by the PTA of hearing thresholds at 3000, 4000, 6000, and 8000 Hz. According to the World Health Organization criteria, normal hearing was defined as an unassisted PTA of less than 25 dB in the better hearing ear. We included data from participants who had normal hearing at all three frequencies.

In terms of covariates, only age was reported as a continuous variable. NHANES reported any poverty income ratio (PIR) higher than 5 as 5. Self-reported sociodemographic characteristics were described in the following categories: sex (male/female), race/ethnicity (non-Hispanic white, non-Hispanic black, and Hispanic or other), marital status (married, never married or living with partner, divorced or separated, and widowed), education level (less than high school, high school or equivalent, and college or above), and family income (PIR: <1.30, 1.30-3.49, or ≥3.5). Using measured height and weight, we worked out balance (yes, no), body mass index (BMI) and classified it into three categories (<25, 25-30, ≥30). Lifestyle behaviors such as smoking history (former, never, and now), drinking history (mild, moderate, heavy, former, and never), and

physical activity (total metabolic equivalent <450, 450-749.9, ≥750) were also variables of interest. In addition, we recorded the presence or absence of the following diseases at any time during the participant's lifetime: congestive heart failure (CHF), hypertension, diabetes mellitus (DM), coronary cardiac disease, stroke, heart attack, hyperlipidemia, and cancer.

### Statistical Analysis

In accordance with the analysis guidelines of the NHANES, we performed all analyses with the use of sample weights, stratification, and clustering with a complex sampling design to account for sampling bias. In the final included normal hearing population with complete data, the continuous variables were described by MD (SD), and the categorical variables were described by the total number of subjects (weighted percentage), using Chi-square independent test and one-way analysis of variance, respectively. The propensity score matching and cox regression model adjusted for all factors were used to analyze the correlation between the existence or absence of tinnitus and mortality. In the propensity score analysis, cox regression was used to build a model to estimate the presence of tinnitus by the following variables: age, sex, race/ethnicity, marital status, education level, PIR, balance, BMI, smoking, drinking, physical activity, CHF, hypertension, DM, coronary heart disease, stroke, heart attack, hyperlipidemia, and cancer. The matching cohorts of tinnitus users and non-tinnitus users were established using 2:1 matching with 0.05 caliper distance and the nearest neighbor matching algorithm without displacement. Standardized mean differences (SMD) and p-values were calculated for all included variables to assess balance between the matched cohort of individuals with tinnitus and the cohort without tinnitus. Cox regression analysis was adjusted for all variables to estimate the hazard ratio (HR) and 95% confidence interval (CI) between tinnitus and mortality in the unmatched and matched cohorts, respectively. Individuals with a pertinent medical background for the outcome of each examined condition were excluded in the cause-specific mortality study (eg, cancer mortality excluded previously diagnosed cancer).

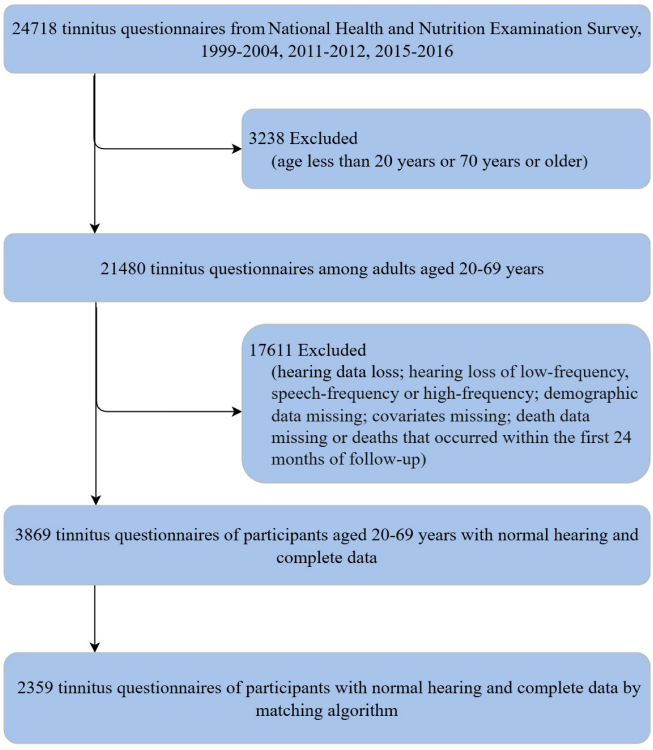
In addition, stratified analyses and interaction analyses were performed to check if there were differences in the associations between different age, sex, PIR, BMI, physical activity, and smoking status. All analyses were conducted based on R (version 4.1.3), and a p-value less than 0.05 was considered statistically significant.

## Results

### Participants Characteristics

The process of participant selection is illustrated in Figure 1. Among the 24,718 individuals who completed the tinnitus questionnaire across the selected NHANES cycles, 21,480 were between 20 and 69 years of age. After excluding participants with abnormal hearing thresholds, missing data on key covariates or mortality outcomes, and those who died within the first 24 months of follow-up, a final analytic cohort of 3,869 participants with normal hearing was established. Table 1 showed the demographic and clinically relevant data of the cohort. The tinnitus group had a higher mean (SD) age (38.88 [0.43] years vs 38.02 [0.27] years) and a higher proportion of men (42.47% [301] vs 43.75% [1266]) than the non-tinnitus group. Individuals with tinnitus exhibited lower levels of physical activity and higher smoking rates compared to those without tinnitus. Additionally, they had a higher prevalence of hypertension, hyperlipidemia, and stroke. After applying propensity score matching, a well-balanced cohort of individuals with and without tinnitus was created (Table 1).

**Figure 1.** Flowchart of this study.




### Tinnitus and Mortality

As shown in Figure 2, subjects with tinnitus an increased chance of dying from all causes and from cancer. After adjustment for demographic characteristics, lifestyle, and chronic diseases, compared with those without tinnitus, the HR for all-cause mortality was 1.84(95% CI, 1.38 to 2.44) in the unmatched cohort and 1.82(95% CI, 1.32 to 2.49) in the matched cohort. The HR for cancer mortality were 2.11 (95% CI, 1.26 to 3.58) and 2.33 (95% CI, 1.27 to 4.30), respectively. As can be seen, these associations were more significant in the cohort after matching. In addition, tinnitus was linked to a higher risk of accidental death (unmatched cohort: HR, 3.03; 95%CI, 1.47-6.29; matched cohort: HR, 2.80; 95% CI, 1.11-7.07) and death from other causes (unmatched cohort: HR, 1.46; 95% CI, 0.87-2.46; matched cohort: HR, 1.62; 95% CI, 0.91-2.88). In contrast, tinnitus did not affect the probability of death from cardiac disease (unmatched cohort: HR, 1.75; 95%CI, 0.95-3.24; matched cohort: HR, 1.27; 95% CI, 0.65-2.49). Most subgroups retained these connections. The correlation between tinnitus and all-cause mortality was stronger among participants with a BMI ≥ 30 (unmatched cohort: HR, 2.36; 95%CI, 1.60-3.50; matched cohort: HR, 2.08; 95% CI, 1.32-3.27) and BMI 25-30 (unmatched cohort: HR, 2.96; 95%CI, 1.72-5.09; matched cohort: HR, 2.65; 95% CI, 1.47-4.80) (Figure 3).

**Table 1.** Baseline characteristics of tinnitus in subjects with normal hearing

| | Unmatched | | | Matched | | |
|---|---|---|---|---|---|---|
| variable | No tinnitus | Tinnitus | P value | No tinnitus | Tinnitus | P value |
| Respondents, No. | 3058 | 811 | | 1556 | 803 | |
| Balance | | | 0.07 | | | 0.91 |
| yes | 185(5.13) | 63(7.37) | | 122(6.26) | 60(7.14) | |
| no | 583(21.86) | 150(20.96) | | 301(22.15) | 150(21.15) | |
| not record | 2290(73.01) | 598(71.67) | | 1133(71.58) | 593(71.71) | |
| Age, mean (SD) | 38.02(0.27) | 38.88(0.43) | 0.06 | 38.65(0.35) | 38.80(0.42) | 0.79 |
| Sex | | | 0.5 | | | 0.42 |
| Female | 1792(56.25) | 510(57.53) | | 902(55.44) | 504(57.54) | |
| Male | 1266(43.75) | 301(42.47) | | 654(44.56) | 299(42.46) | |
| Race and ethnicity | | | < 0.0001 | | | 0.89 |
| Hispanic or other | 1022(21.17) | 256(16.22) | | 441(16.54) | 255(16.36) | |
| Non-Hispanic Black | 706(12.79) | 148( 9.33) | | 305(9.89) | 146(9.29) | |
| Non-Hispanic White | 1330(66.04) | 407(74.44) | | 810(73.56) | 402(74.35) | |
| Marriage status | | | 0.21 | | | 0.96 |
| divorced or separated | 328(10.60) | 114(13.66) | | 190(11.90) | 110(13.38) | |
| married | 1644(54.91) | 407(52.51) | | 827(53.94) | 405(52.67) | |
| never married / living with partner | 902(28.68) | 241(27.59) | | 450(28.45) | 239(27.66) | |
| widowed | 60(1.36) | 22(1.89) | | 36(1.63) | 22(1.91) | |
| not recorded | 124(4.44) | 27(4.34) | | 53(4.08) | 27(4.38) | |
| Educational level | | | 0.23 | | | 0.92 |
| college or above | 1578(60.45) | 421(58.18) | | 796(59.18) | 418(58.22) | |
| high school or equivalent | 1191(35.38) | 319(38.45) | | 625(37.17) | 315(38.39) | |
| less than high school | 289(4.18) | 71(3.36) | | 135(3.65) | 70(3.39) | |
| PIR | | | 0.03 | | | 0.83 |
| <1.3 | 748(18.61) | 228(23.41) | | 418(20.66) | 221(22.73) | |
| 1.3-3.49 | 1287(39.18) | 329(37.37) | | 632(39.45) | 328(37.69) | |
| ≥3.5 | 1023(42.20) | 254(39.23) | | 506(39.89) | 254(39.58) | |
| BMI | | | 0.1 | | | 0.73 |
| <25 | 1020(37.47) | 289(39.49) | | 529(38.29) | 285(39.18) | |
| 25-30 | 1076(33.47) | 245(28.14) | | 506(30.97) | 245(28.40) | |
| ≥30 | 962(29.07) | 277(32.36) | | 521(30.74) | 273(32.42) | |
| Physical activity | | | 0.74 | | | 0.81 |
| <450 | 1293(43.60) | 351(45.23) | | 710(46.84) | 348(45.24) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 450-749.9 | 1123(33.22) | 287(32.87) | | 527(31.04) | 282(32.67) | |
| ≥750 | 642(23.18) | 173(21.90) | | 319(22.13) | 173(22.10) | |
| Smoke | | | 0.01 | | | 0.9 |
| former | 554(19.35) | 183(22.11) | | 317(20.99) | 181(22.26) | |
| never | 1795(55.39) | 391(46.47) | | 810(48.56) | 391(46.89) | |
| now | 709(25.26) | 237(31.42) | | 429(30.45) | 231(30.85) | |
| Alcohol use | | | 0.002 | | | 0.72 |
| former | 392(10.81) | 133(13.53) | | 234(12.56) | 130(13.51) | |
| never | 446(12.47) | 83(8.87) | | 196(10.64) | 83(8.95) | |
| now | 2034(71.37) | 568(75.32) | | 1075(74.02) | 563(75.25) | |
| not record | 186(5.35) | 27(2.28) | | 51(2.78) | 27(2.30) | |
| Congestive heart failure | | | 0.04 | | | 0.4 |
| no | 3037(99.51) | 799(98.57) | | 1544(99.59) | 795(99.00) | |
| yes | 21(0.49) | 12(1.43) | | 12(0.41) | 8(1.00) | |
| Hypertension | | | 0.22 | | | 0.87 |
| no | 2292(76.26) | 573(73.99) | | 1135(74.96) | 569(73.99) | |
| yes | 766(23.74) | 238(26.01) | | 421(25.04) | 234(26.01) | |
| DM | | | 0.03 | | | 0.73 |
| yes | 186(4.57) | 64(6.29) | | 117(5.57) | 60(6.05) | |
| no | 2572(92.46) | 684(92.06) | | 1311(92.22) | 680(92.29) | |
| not record | 300(2.96) | 63(1.65) | | 128(2.21) | 63(1.66) | |
| coronary heart disease | | | 0.57 | | | 0.75 |
| no | 3035(99.21) | 799(98.92) | | 1546(99.32) | 791(98.91) | |
| yes | 23(0.79) | 12(1.08) | | 10(0.68) | 12(1.09) | |
| Hyperlipidemia | | | 0.85 | | | 0.83 |
| no | 1061(35.33) | 269(34.90) | | 522(34.94) | 266(34.85) | |
| yes | 1997(64.67) | 542(65.10) | | 1034(65.06) | 537(65.15) | |
| Stroke | | | 0.1 | | | 0.38 |
| no | 3031(98.99) | 795(98.20) | | 1539(98.82) | 788(98.35) | |
| yes | 27(1.01) | 16(1.80) | | 17(1.18) | 15(1.65) | |
| Heart attack | | | 0.12 | | | 0.79 |
| no | 3028(99.00) | 796(98.26) | | 1538(98.76) | 789(98.33) | |
| yes | 30(1.00) | 15(1.74) | | 18(1.24) | 14(1.67) | |
| Cancer | | | 0.03 | | | 0.53 |
| no | 2947(95.62) | 758(92.86) | | 1482(94.59) | 752(93.05) | |
| yes | 111(4.38) | 53(7.14) | | 74(5.41) | 51(6.95) | |

Abbreviations: NA, not applicable; SMD, standardized mean difference; PIR, poverty income ratio; BMI, body mass index; DM, diabetes mellitus.

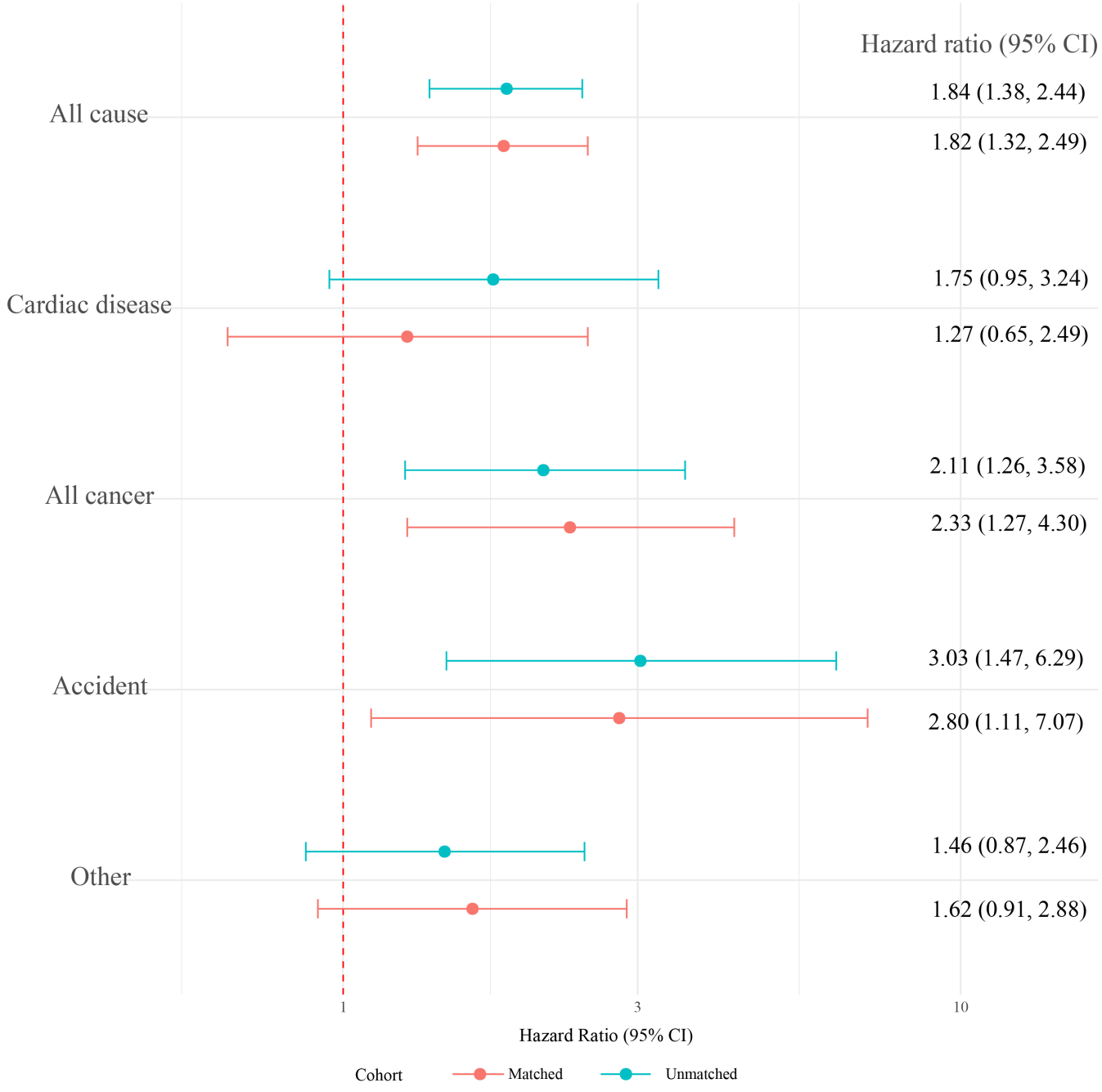
The association between tinnitus and cancer mortality was stronger among people with no balance (unmatched cohort: HR, 5.70; 95%CI, 2.23-14.55; matched cohort: HR, 5.01; 95% CI, 1.93-13.48) and female (unmatched cohort: HR, 2.31; 95%CI, 1.15-4.61; matched cohort: HR, 2.64; 95% CI, 1.35-5.17) (Figure 3).

## Discussion

This study investigated the relationship between tinnitus and its associated risk factors, as well as the impact of tinnitus on mortality from various diseases. The findings offer valuable insights for the clinical screening and management of tinnitus patients with normal hearing.

In this national representative US adult cohort study, we observed that subjects with tinnitus had a higher rate of dying from all causes and cancer, and accidental death, but tinnitus did not affect the rate of death from cardiac disease. In the course of subgroup analysis, we found that the relevance between tinnitus and all-cause mortality was greater in participants with a BMI ≥30 and 25-30. The correlation between tinnitus and cancer mortality was stronger in female and people with no balance.

**Figure 2.** Adjusted Hazard Ratios of all-cause and cause-specific mortality comparing tinnitus with no tinnitus, National Health and Nutrition Examination Survey, 1999-2004, 2011-2012, 2015-2016.

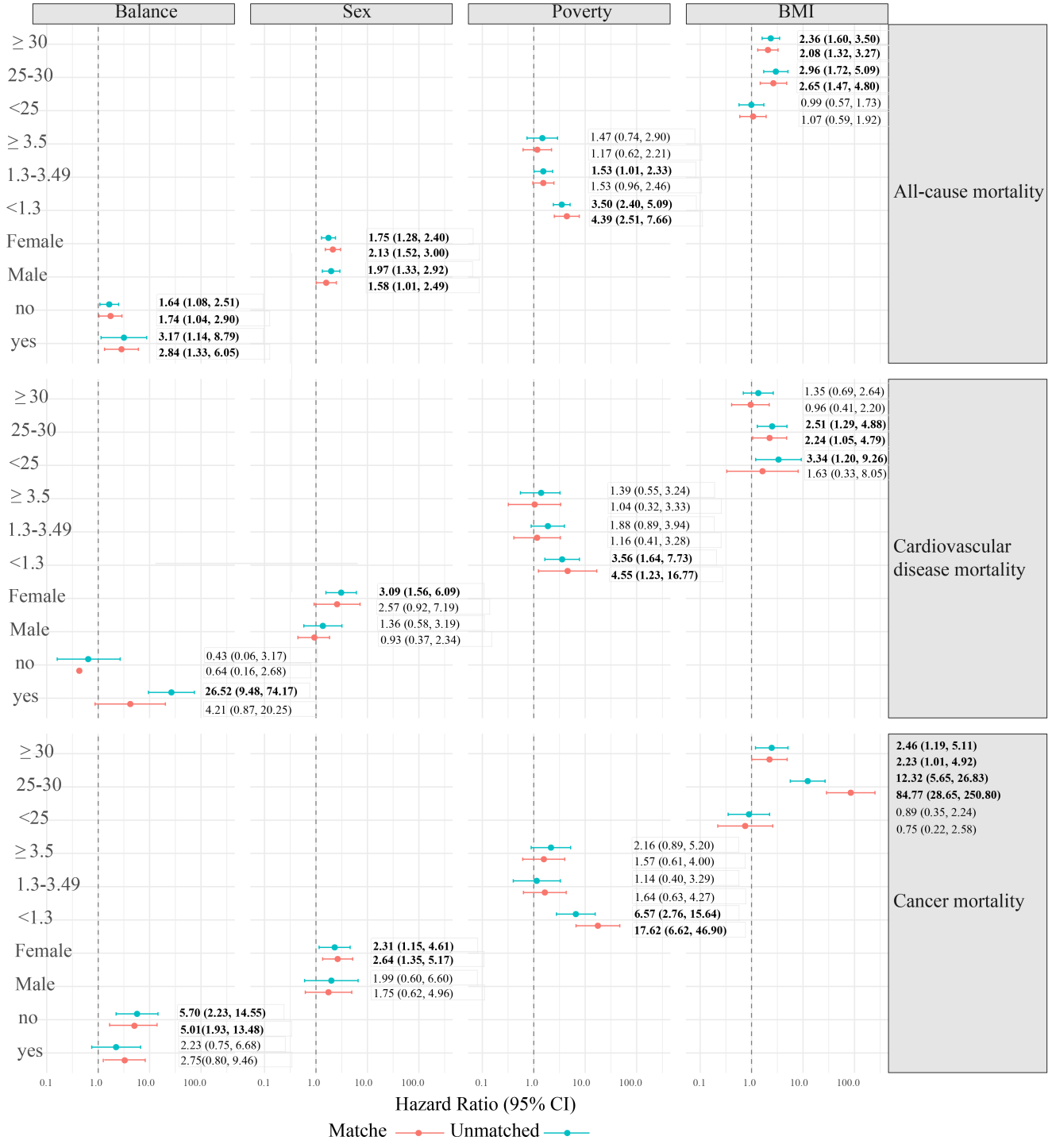


Tinnitus, one of the three major otologic challenges, has been closely associated with increased suicide rates in many countries [1, 29]. Therefore, the screening and therapy of patients with tinnitus is particularly important. The main symptoms of drug ototoxicity are tinnitus, hearing loss and balance disorders [31]. The relationship between hearing loss, balance disorders and all-cause and cause-specific mortality has been systematically addressed in studies [32,33]. Research on the relationship between tinnitus and all-cause and cause-specific mortality is still in a blank state.

In addition, it has been shown that hearing loss is related to an elevated risk of all-cause and cause-specific mortality [32], and a study by Martz E et al. found that those with tinnitus had lower cause-specific mortality than those without tinnitus, but it did not control for the confounding factor of hearing loss [34]. To eliminate the effect of hearing loss on tinnitus, this study compared tinnitus with all-cause and cause-specific mortality only in the normal hearing population.

Tinnitus patients have deteriorated neuropsychological ability

**Figure 3.** Stratified Hazard Ratios and 95% CIs for all-cause and cause-specific mortality according to tinnitus.



and lower sense of social identity, which affects socialization, decision-making and work ability, which in turn leads to a higher risk of death [35-37]. In terms of neuropsychological regulation, clinical studies have shown that tinnitus patients exhibit signs of impaired the hypothalamic–pituitary–adrenal axis (HPA axis) and higher levels of mental stress compared to non-tinnitus patients [38-40]. The deterioration of the ability to relax and activate the parasympathetic nervous system in individuals with tinnitus can have some negative physical and psychological effects [41], such as dysregulation of the body's stress system, depression, and sleep disturbances, all of which are related with an elevated risk of death [42].

From the perspective of social identity, some studies have found that due to the persistence of tinnitus, patients' sleep quality also can be impacted, which affects their social activities and productivity [43]. In addition, for people in some professions such as musicians, the impact of tinnitus on their careers is tremendous. Tinnitus may make them incompetent for work and their sense of social identity declines so rapidly that they are more likely to end up in demoralization or even suicide [44].

Therefore, while treating tinnitus, we need to pay attention to patients' mental health issues and provide them with the necessary psychological guidance and support. We should provide comprehensive treatment to help patients return to a healthy state and improve their quality of life and social identity.

Patients with tinnitus have a higher risk of death from cancer than those without tinnitus. On the one hand, cancer patients become anxious or even depressed because of cancer, and anxiety and depression can in turn trigger or aggravate tinnitus [45]. Therefore, patients with tinnitus indicate higher levels of anxiety and depression, and both anxiety and depression and tinnitus itself put cancer patients at higher risk of death [46].

On the other hand, ototoxicity can also cause tinnitus. Ototoxicity caused by cancer chemotherapy drugs such as cisplatin and vincristine can have a detrimental effect on quality of life [47]. Patients develop a fear of social life and of worsening tinnitus. Patients with mild and moderate-severe tinnitus fear that chemotherapy with drugs such as cisplatin will further aggravate the symptoms of tinnitus and thus become skeptical or even refuse drug treatment for cancer [48]

In this study, the correlation between tinnitus and all-cause mortality was stronger in participants with a BMI ≥30 and 25-30. Although the underlying mechanisms between tinnitus and mortality are not known, several causes can be considered. First, tinnitus may interact with psychosocial factors in patients and is associated with poor health outcomes, including cognitive deficits, decrease in social activities, mental health impairment [49, 50], and reducing ability to perform activities of daily living. Obese populations may be more inclined to reduce necessary activities, which further exacerbates the harm caused by obesity to the organism. Second, the study by Wade, K. H. indicated a causal relationship between higher BMI and all-cause and cause-specific mortality [51]. In addition, it has also been shown that weight loss after diet and physical activity interventions relieved tinnitus symptoms and improved quality of life in tinnitus sufferers [52, 53]. Combined with this study, we can speculate that perhaps maintaining an appropriate BMI has a positive effect on reducing both tinnitus morbidity and mortality, which provides new ideas for managing diet and physical activity in tinnitus patients.

The correlation between tinnitus and cancer mortality is stronger in women. During chemotherapy in cancer patients, estrogen levels are higher in women compared to men and fluctuate significantly with environmental factors and the menstrual cycle. Fluctuations in estrogen levels may lead to vasodilatory dysfunction [54], which may affect the blood supply to the inner ear and aggravate tinnitus. In addition, estrogen level change may mediate chemotherapeutic drug resistance [55]and promote cancer disease progression [56] leading to increased cancer mortality.

Cancer patients without balance have increased risk of falls compared to the rest of the population. On the one hand, people who suffer from balance disorders and tinnitus have a greater fear of falling and reduce their daily activities [57,58], thereby increasing the risk of cancer [59], all of which increase the risk of death [60]. On the other hand, in patients with neurological cancers, balance disorders may be a sequela of the cancer itself or secondary to treatment [61]. As neurological cancer patients deteriorate, the metabolism of fluid in the inner ear [62], which leads to labyrinthine disorders, results in severe balance disorders, tinnitus, etc. Tinnitus is also a marker of further deterioration of neurological cancer in patients with balance disorders [63]. The correlation between tinnitus and cancer mortality is stronger in those without balance.

**Strengths and limitations**

A major strength of this study is the exploration of the relationship between tinnitus and all-cause and cause-specific mortality only in a normal hearing population, which eliminated the effect of hearing loss on tinnitus. Second, this study uses a large, representative sample of NHANES, a research program that is prospective and based on enrollment to obtain high-quality information. Third, the application of propensity score matching (PSM) analysis to adjust for important demographic variables is also an advantage of this study. Considering the significant disparities in the number of tinnitus and non-tinnitus couples, PSM reduces the effects of bias and confounding variables in the study to allow reasonable comparisons between the matched cohort of individuals with tinnitus and the cohort without tinnitus. Last but not least, subgroup analyses of gender, marital status, and education level were conducted to address the issue of heterogeneity in this study.

There are still some limitations in our study. First, because the study was observational, the causal relationship between the presence of tinnitus and the severity of tinnitus and all-cause and cause-specific mortality could not be determined. Second, although we adjusted for confounders as much as possible, there may still be some confounders that we did not take into account. Third, our study population was US adults between the ages of 20 and 69, and the findings may not be applicable to children and older adults >69 years of age.

Future studies should evaluate the mechanisms that link tinnitus with total and cause-specific mortality in order to individualize the treatment of tinnitus patients with normal hearing, thereby improving the quality of life and reducing mortality in tinnitus patients.

## Conclusion

Among U.S. adults with normal hearing, tinnitus was independently associated with increased risks of all-cause and cancer-specific mortality. By employing propensity score matching to ensure covariate balance, this study enhances the validity of the observed associations and reduces the influence of potential confounders. These findings underscore the importance of recognizing tinnitus as not merely a benign symptom, but a potential marker of broader health risks, even in individuals without hearing impairment. Clinicians and public health professionals should consider systematic evaluation and follow-up in this population, particularly focusing on mental health, cancer screening, and accident prevention. Future research is needed to elucidate the underlying mechanisms of the association between tinnitus and mortality in participants with normal hearing and to explore the relationship between tinnitus severity and mortality in people with normal hearing.

## Abbrevations

SD: standard deviation; HR: hazard ratio; 95%CI: 95%confidence interval; NHANES: National Health and Nutrition Examination Survey; STROBE: Strengthening Reporting of Observational Studies in Epidemiology; ICD-10: International Classification of Diseases, 10th Revision; LFHL: Low frequency hearing loss; PTA: pure-tone average; Hz: hertz; SFHL: speech frequency hearing loss; HFHL: high frequency hearing loss; dB: decibel; PIR: poverty income ratio; BMI: body mass index; CHF: congestive heart failure; DM: diabetes mellitus; MD: mean deviation; SMD: standardized mean differences; eg: exempli gratia; Vs: versus; HPA axis: hypothalamic–pituitary–adrenal axis; PSM: propensity score matching; NA, not applicable.

## Acknowledgements

Not applicable.

## Author Contributions

Yuchen Zhang: Conceptualization, Software; Yanqiang Zhang: Methodology; Data curation; Ziyue Fu: Writing-Original draft; Kaile Wu, Yehai Liu: Software, Validation; Chuanlu Shen: Writing- Reviewing and Editing.

## Ethics Approval and Consent to Participate

NHANES was approved by the National Center for Health Statistics Research Ethics Review Board, and consent from all participants was documented. All methods were performed in accordance with the relevant guidelines and regulations of NHANES.

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## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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