

# Asia Pacific Journal of Clinical Medical Research

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## Asia Pacific Journal of Clinical Medical Research

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# Effectiveness of Research-Oriented Integrated Nursing Interventions on Cancer Pain Management in Chinese Hospitalized Oncology Patients: A Meta-Analysis

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**Abstract:** Objective: To systematically evaluate the effectiveness of research-oriented integrated nursing interventions on cancer pain management in hospitalized oncology patients in China. **Methods:** A computerized search of Chinese and English databases was conducted to identify relevant studies. Two researchers independently assessed the quality of included literature using the Newcastle-Ottawa Scale (NOS). Data were extracted and analyzed via Stata 14. A random-effects model was applied due to significant heterogeneity ( $I^2 > 50\%$ ). Sensitivity analysis and Egger's test were performed to assess bias. **Results:** 12 eligible studies (2014–2024) were included. Meta-analysis demonstrated that integrated nursing interventions significantly reduced cancer pain scores compared to routine care (SMD = -1.51, 95% CI: -1.90 to -1.12;  $I^2 = 84.8\%$ ), with superior efficacy. Subgroup-analyses revealed enhanced effects for “Nursing modes” (SMD = -2.11) and “cancer pain education” (SMD = -2.30). **Conclusion:** Research-oriented integrated nursing interventions significantly improve cancer pain management in Chinese hospitalized oncology patients, particularly through synergistic effects of “Nursing modes” and “cancer pain education.” However, implementation bias from “additive interventions” in teaching hospitals and high heterogeneity warrant attention. Future studies should optimize designs to enhance clinical applicability.

**Keywords:** Meta-Analysis; Hospitalized Oncology Patient; Cancer Pain Management; Nursing Intervention

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## 1.Introduction

In the medical and health industry, the objective demands of nursing research have contributed to the rapid development of clinical nursing academic research, providing fertile ground for the exponential growth of nursing research. With the development of the discipline and the vigorous momentum of scientific research, the literature on clinical intervention in nursing often shows a tendency of “research orientation”, and the utilitarian theory of scientific research and the theory of positive supremacy increasingly occupy the academic publishing market. “Research-oriented Integrated Nursing Intervention” refers to the integration of multidisciplinary resources and diverse nursing measures (such as medication, psychology, rehabilitation, etc.) within the framework of clinical Research. And an intervention model that is dynamically adjusted according to the individual needs of patients. In the field of cancer pain management, physical pain and the stress brought by pain constantly trouble cancer patients and are also topics that the academic community has never fully explored. This study aims to

systematically evaluate the effectiveness of research-oriented comprehensive nursing intervention in cancer pain management for inpatients with tumors in China through meta-analysis technology, with the expectation of expanding new horizons for clinical nursing research.

## 2.Methods

### 2.1 Literature Retrieval Strategy

7 databases, namely Pubmed, Science Direct, China National Knowledge Infrastructure (CNKI), Wanfang Journal Database, Cochrane Library and Embase, were retrieved by computer. The study adopted a systematic retrieval strategy of “subject terms + free terms”, and the retrieval period was from the establishment of the databases to January 29, 2025. The search terms in the English characters are “cancer patients/hospitalization cancer patients” and “cancer pain/cancer pain management/cancer breakthrough” “nursing” NRS score/VAS visual score/cancer pain score” intervention effect/influencing factors.

### 2.2 Inclusion and Exclusion Criteria for Literature and Data Extraction

Inclusion criteria for the study: ① The research subjects were inpatients with tumors in China who were diagnosed with cancer; ② The research reported the improved outcomes of patients' cancer pain scores under various nursing intervention measures or nursing management models; ③ The research types are cohort studies and case-control studies. Exclusion criteria: ① Literature that cannot completely extract the required data; ② The full text of the literature cannot be obtained for download. ③ Literature with a quality evaluation of “low quality”. Subsequently, the researchers conducted a detailed screening based on the inclusion and exclusion criteria, and screened the literature according to the screening sequence of article summaries - outcome measures - full text - data format. And complete the data extraction of the first author, publication year, research subjects, research categories, sample size, contents of nursing intervention or cancer pain management, NRS score (mean  $\pm$  standard deviation), and the evaluation results of literature quality of the included literature.

### 2.3 Literature Quality Evaluation

Researchers used the Newcastle-Ottawa Scale (NOS)<sup>[1]</sup> to conduct two rounds of independent quality evaluations on the literature of case-control studies and cohort studies respectively. The maximum score of the NOS scale is 9 points. The scoring criteria are as follows: a score lower than 5 is considered Low-quality literature, 5 to 6 is Medium-quality literature, and 7 to 9 is High-quality literature. This study only included the literatures with a NOS score of  $\geq 5$  points to ensure that the quality of the selected literatures achieved a qualified standard.

### 2.4 Technical Route

The study conducted a Meta-Analysis of the included literatures based on stata.14. If  $I^2 < 50\%$  and  $p > 0.05$ , the heterogeneity of the meta-results was relatively small, and the fixed-effect model was selected. If  $I^2 > 50\%$  and  $p \leq 0.05$ , it indicates that the heterogeneity of the Meta-results is relatively large. Then, a random effects model will be selected and the included literatures with greater influence will be excluded to complete the sensitivity analysis of the integrated results and explore the source of sensitivity clearly. When the number of original literatures included in the research institute more than 10, Egger's test should be conducted and a funnel plot should be drawn to evaluate the literatures' situation.

## 3.Result

### 3.1 Literature Search Results

The study obtained 4,073 literatures through preliminary literature retrieval. Based on the retrieval results, the researchers further completed the screening of article closure and extraction, obtaining 843 literatures. By deeply reviewing the full text of the literature, the researchers determined the research type and variable data format of the literature and excluded the results of 576 literatures. Meanwhile, based on the inclusion and exclusion criteria, 16 literatures were finally included and entered the meta-analysis stage.

### 3.2 Characteristics and quality Evaluation of Included literature

The research adopted a mode of independent double entry and double evaluation by two researchers to complete data extraction and literature quality evaluation. The contents of the inconsistent materials were confirmed after discussion. The final characteristics of the entered literature are as follows (see Table 1). A total of 16 literatures were included in the first

round of meta-integration of the research institute, involving 1,689 samples of Chinese cancer pain patients, including 845 in the intervention group and 844 in the control group. The integrated research types of the institute are mostly randomized controlled studies (RCTs, n=13), and the publication time span of the included studies is from 2014 to 2024. The research data are relatively recent.

Table 1 Summary of the characteristics of the literature included in the Meta-analysis

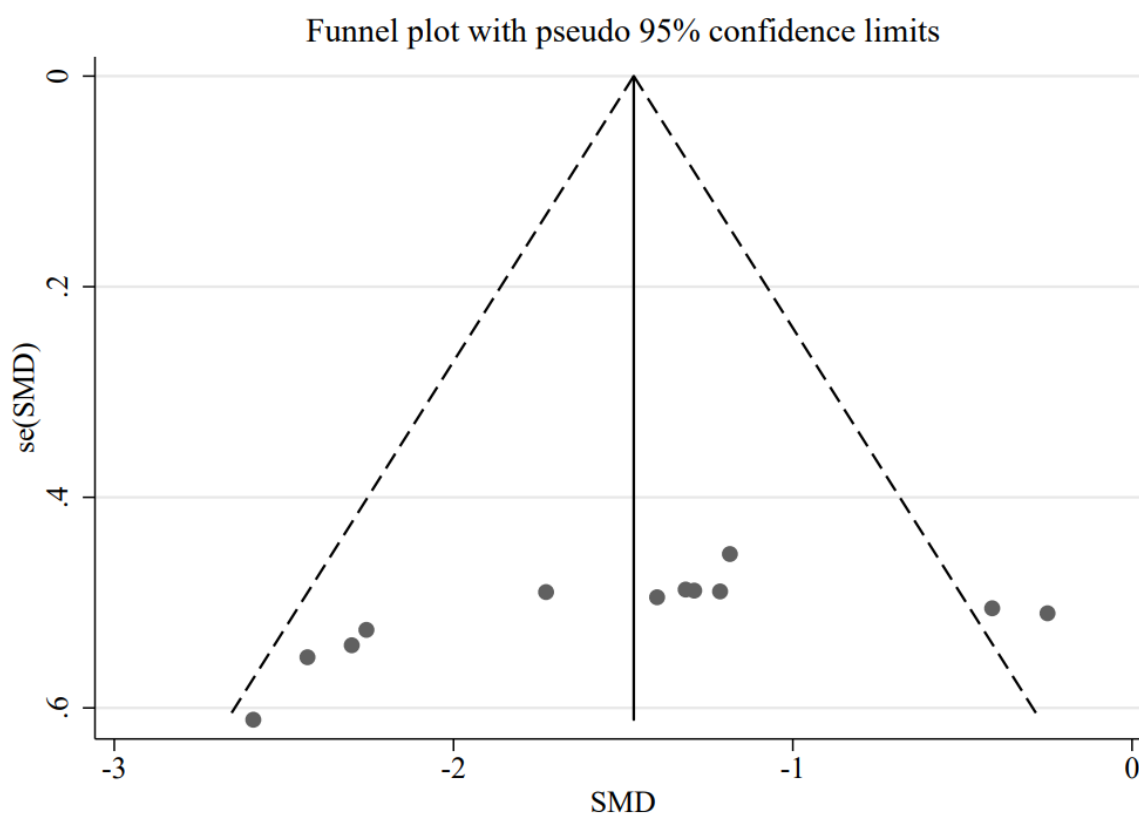
First author	Publication year	Research category	Type	C/T	M± SD		The core content of nursing intervention	Literature quality
					C	T		
Duan Lizhen et al. <sup>[2]</sup>	2023	Drug management	RCT	32/32	2.03±1.04	2.50±1.14	Drug titration care for the “5A” goal	Mid
Zou Junlian <sup>[3]</sup>	2020	Nursing mode	RCT	39/39	2.25±0.88	5.41±1.22	Comprehensive Nursing Management	Mid
Zhang Shufang et al. <sup>[4]</sup>	2014	Nursing mode	RCT	54/54	2.25±0.58	4.00±0.72	Comprehensive Nursing Management	High
Wang Cailian et al. <sup>[5]</sup>	2023	Psychological nursing	RCT	48/48	2.04±0.95	3.40±1.12	Mindfulness-based stress reduction therapy + Comprehensive care	High
Cui Fuli <sup>[6]</sup>	2019	Cancer pain education	RCT	55/55	2.30±0.50	4.60±1.00	Pain education + family education	Mid
Liu Zhu et al. <sup>[7]</sup>	2019	Traditional Chinese Medicine Nursing	RCT	50/50	3.62±0.28	4.11±0.35	Emotional and emotional harmony nursing combined with Lizhao Powder moxibustion	Mid
Wang Xiaohong <sup>[8]</sup>	2022	Cancer pain management	RCT	90/90	2.10±0.60	2.40±0.80	Specialized management of cancer pain	Mid
Liu Ping et al. <sup>[9]</sup>	2024	Psychological nursing	RCT	51/51	3.95±0.58	4.95±0.76	Family-style participation in dignity therapy	High
Wang Jiongqi et al. <sup>[10]</sup>	2024	Cancer pain management	NRCT	58/68	2.47±0.62	4.26±1.51	Full-process MDT cancer pain group management	Mid
Quan Xiaoting et al. <sup>[11]</sup>	2022	Traditional Chinese Medicine Nursing	RCT	120/110	3.01±0.49	5.02±0.35	Auricular point embedding + standardized management	Mid
Xue Shuzhi et al. <sup>[12]</sup>	2020	Nursing mode	MCS	50/50	2.87±0.64	3.98±0.86	Integrated nursing management of medical care	Mid
Wang Lihui et al. <sup>[13]</sup>	2022	Nursing mode	NRCT	60/60	2.45±0.53	4.12±0.74	Team working mode of cancer pain care strategy	Mid
Lu Tianyi et al. <sup>[14]</sup>	2023	Drug management	RCT	61/61	1.30±1.00	3.20±1.10	Drug treatment management services	Mid
Chen Xueling <sup>[15]</sup>	2019	Traditional Chinese Medicine Nursing	RCT	30/30	1.30±2.45	2.07±2.45	Traditional Chinese physical therapy + routine care	Mid
WeiChi Su <sup>[16]</sup>	2020	Cancer pain management	RCT	26/25	1.80±4.81	2.50±2.93	Comprehensive pain management strategy	High
Qiuling Zhao <sup>[17]</sup>	2023	Drug management	RCT	21/21	1.86±0.79	2.76±1.00	Wechat mini-program drug guidance service	High
Note:RCT: Randomized Controlled study; NRCT: Non-randomized Controlled study MCS: Paired Cohort Study MDT: Multidisciplinary Medical Collaboration Model.								

### 2.3 Meta-Analysis Results

The Standardized Mean Difference (SMD for short) of the first round of Meta integration was -1.575 (95% CI: (-2.084 to -1.066)), indicating that the nursing intervention in the experimental group had a significant negative effect on the cancer pain score (NRS score), suggesting that the various reported control studies of cancer pain nursing intervention produced ideal positive results. The meta-integration results showed that  $I^2 = 94.0\% > 50\%$  and  $p \leq 0.05$ , indicating that the heterogeneity of the included studies was relatively large, and the random effects model was selected. Through the random effects model, the sources of research heterogeneity were sought. After excluding the studies of Wang Xiaohong<sup>[8]</sup>, Quan Xiaoting et al.<sup>[11]</sup>, and WeiChi Su<sup>[16]</sup>, the  $I^2$  decreased. Subsequently, any literature was excluded, and no significant change was found in the  $I^2$  level of the study. The meta-integration results tended to be robust.

After eliminating the literatures with high heterogeneity, a total of 12 literatures are now included for analysis. The funnel plots drawn based on stata.meta-st0012 basically show symmetry (Figure 1). Egger's test calculation results:  $t = -2.15$ ,  $p = 0.0217 > 0.05$ , which was not statistically significant. This indicates that there was no obvious bias in the results of the meta-analysis of the included literature. The Egger test scatter plot supports this result.

Figure 1 Meta-integrated funnel plot

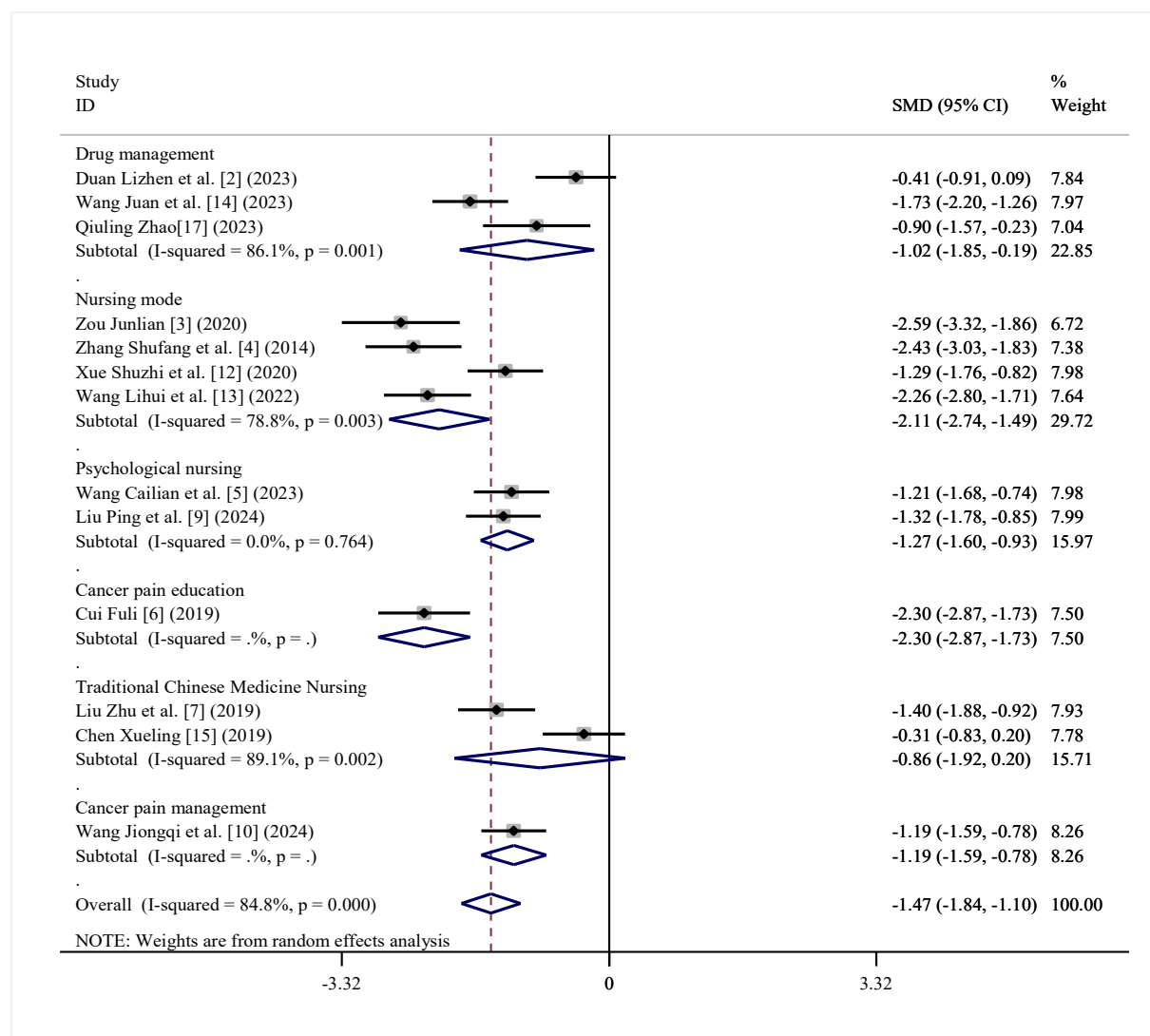


### 2.4 Meta-subgroup analysis

Since the research aims to evaluate the intervention effects of various studies in the field of cancer pain care intervention, the heterogeneity of the integration results of the meta-subgroups of each group is at a relatively high level ( $I^2 > 50\%$ ). The Meta-results shown in Figure 2 indicate that the final value of the total SMD is -1.51 (95% CI: -1.90 to -1.12), indicating that research-oriented comprehensive nursing intervention has a significant control effect on the cancer pain level (NRS score) of inpatients with tumors in China. Meanwhile, in the field of cancer pain nursing intervention, the lower-level classifications of “Drug management”, “Nursing mode”, “Psychological nursing”, “Cancer pain education”, and “Cancer pain management” presented superior results in the meta-integration (see Figure 2), and the original positive conclusions in the literature<sup>[2-17]</sup>. It has proved the effectiveness of relevant nursing intervention measures and nursing management models at the level of cancer pain control management for inpatients with tumors in China.



Figure 2 Forest plot of Meta-subgroup analysis



## 4. Discussion

### 4.1 Research-oriented comprehensive nursing intervention has achieved remarkable results in cancer pain intervention for inpatients with tumors in China

The Meta-integration results showed that the SMD of the effectiveness of research-oriented comprehensive nursing intervention in cancer pain intervention for inpatients with tumors in China was -1.51 (95% CI: -1.90 to -1.12), according to Cohen's criteria (Cohen's d) :  $SMD = -1.51 > 0.8$ , indicating a significant difference compared with the control group. The clinical value is clear and there is a sufficiently large effect level to support this conclusion. The meta-study by Xu Qianqian et al. (2016)<sup>[18]</sup> reported that the reducing effect of nursing intervention on the short-term pain score of cancer pain patients from 2004 to 2013 was  $WMD = -0.82$ , 95%CI(-1.11, -0.53), and the converted estimation result was  $SMD = -1.11$  (95%CI: -1.50, -0.72). The SMD effect value of research-oriented comprehensive nursing intervention was lower than that of the former study, revealing the following trends: The advancement of the nursing discipline and the development of medical technology have made the cancer pain nursing intervention system for inpatients with tumors in China more mature and scientific than it was ten years ago. The literature included in this study (2014-2024) builds on the past and pges on the future in terms of nursing intervention content, and adopts more scientific and effective intervention measures as the basic routine for constructing a research-oriented comprehensive nursing intervention system. ② The medical service system and nursing intervention have achieved high-quality development. Currently, the available analgesic drugs and relief measures for cancer

outbreak pain in hospitals have made progress compared to ten years ago. Postoperative analgesic pumps for cancer patients and cancer pain management models based on integrated medical and nursing care have been widely and deeply applied, and the satisfaction of inpatients with cancer is better. The reduction of SMD within a reasonable range further supports the universality of the conclusion that comprehensive nursing intervention is effective. Therefore, combined with the original literature, the content of research-oriented comprehensive nursing intervention has considerable practicality in the field of clinical cancer pain intervention and can achieve ideal analgesic benefit outcomes, which is worthy of promotion.

#### **4.2 The intervention effect of cancer pain in the subgroups of “Nursing mode” and “Cancer pain education” was higher than the overall level of the study**

The results of the subgroups “Nursing mode” and “Cancer pain education” were SMD: -2.11(-2.74,-1.49), with a weight of 29.72%, and SMD:-2.3(-2.87,-1.73), with a weight of 7.5%, respectively. This revealed that the research-oriented comprehensive nursing intervention in this category demonstrated superior effects in the field of cancer pain intervention. It is undeniable that the results of the two subgroups demonstrated the potential for enhancing the effectiveness of specific intervention strategies. However, considering other factors, in the five studies included in the “Nursing mode” and “Cancer pain education” subgroups, each literature intervention group “added” to the routine of tumor cancer pain. For instance, the “Nursing mode” subgroup, relying on the advantages of the management mode, highlighted more frequent and diverse characteristics of nursing assessment and intervention in the intervention content. This led to the fact that in the high-frequency output and feedback mode of the nursing system, the effect of the intervention group was bound to be better than that of the conventional control group. Researchers were also unable to provide reasonable blinding to clinical nurses, thereby resulting in differences in intervention intensity between the control groups. Therefore, for the “Nursing mode” subgroup, the superimposed effect of systematic objective results and implementation bias might be the reason why this subgroup is much higher than the overall level of the study. The “Cancer pain education” subgroup is considered to reveal an enhancement mechanism - cancer pain education intervention, through the knowledge-belief-practice path, invisibly promotes the improvement of patients’ self-management and self-efficacy. “Cancer pain education” forms a synergy mechanism by empowering patients and integrating with the existing systematic nursing intervention, creating the core driving force for subgroups to demonstrate superiority. Therefore, clinical nurses must attach importance to the limitations of the effectiveness of a single nursing measure and learn the profound value of the combined use of systematic cancer pain nursing intervention measures.

#### **4.3 Bias Effects and Limitations of Research-oriented Comprehensive Nursing Intervention in Teaching Hospitals**

Admittedly, the trend of “nursing research favoring positive results” created by the objective research demands in teaching hospitals has already become a clear academic impression in the academic circle. In addition to what has been mentioned earlier, in research-oriented comprehensive nursing intervention, the bias effects caused by directly “adding intervention” to the routine nursing of the department and the inability to blind clinical nurses in most cases, as well as the bias at the time of literature publication caused by human factors such as case screening and sample modification, also need to be taken seriously. Among the literatures included in this study, the heterogeneity level of the same type of studies is also at a relatively high level ( $I^2 > 50\%$ ), which indicates that the differences in the intervention data results among similar research designs are also relatively obvious. In the research-oriented clinical nursing process, patients will actively cooperate due to the perception of “being given special attention”, forming the “Hawthorne effect” which leads to an inflated intervention effect. The high effect size caused by the absence of blinding methods needs to be taken seriously. The additional nursing work pressure associated with the research institute and the additional demands arising from patients’ autonomous perception of the “group” and the awareness between groups have been forced to form the contradiction of “role conflict”, which will bring another type of bias to the intervention outcome. This brings new challenges to the evidence-based value of the research results.

### **5.Outlook**

Only by facing up to the utilitarian undertone of “positive preference” and deconstructing the path dependence of “additive intervention” can a balance point be found between the rigor of scientific research and the practicality of clinical practice.

Future nursing research should be “evidence growing from the bedside” rather than “hastily produced papers for the sake of papers”. This requires not only a self-revolution in methodology but also a deep awakening of academic culture - making research truly serve patients rather than being confined to journals.

## Conclusion

This meta-analysis demonstrates that research-oriented comprehensive nursing interventions significantly enhance cancer pain management for hospitalized oncology patients in China, yielding clinically meaningful improvements in pain outcomes. The intervention's efficacy is particularly amplified in subgroups employing structured “Nursing mode” frameworks and systematic “Cancer pain education” programs, where synergistic mechanisms—including standardized assessment protocols, patient empowerment through knowledge reinforcement, and integrated care delivery—collectively drive superior pain relief compared to conventional approaches.

However, implementation in teaching hospital settings reveals noteworthy methodological constraints: the inherent challenges of blinding clinical staff, intervention heterogeneity across studies, and potential inflation of effect sizes due to Hawthorne effects collectively introduce implementation bias and threaten validity. Furthermore, role conflicts arising from added nursing workloads and patients' perception of specialized attention warrant careful consideration in real-world application. While these interventions represent an advancement over historical nursing practices, future studies should prioritize pragmatic designs that minimize artificial and design biases, standardize intensity metrics across control groups, and evaluate sustainability beyond research contexts to strengthen translational impact.

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no

## Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# Clinical Translation of Molecular Biomarkers in Alzheimer's Disease: From Pathological Detection to Precision Medicine

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**Abstract:** Alzheimer's disease (AD), as the predominant form of neurodegenerative disorders, exerts a profound impact on the health of the global elderly population. For decades, the elucidation of AD pathogenesis and the development of diagnostic and therapeutic approaches have been the focus of extensive research. Over recent years, a fundamental shift has occurred in AD diagnostics—transitioning from reliance on clinical diagnosis alone to biomarker-supported frameworks. AD biomarker research has transitioned from postmortem histopathology to in vivo detection paradigms, enabling precision diagnosis and intervention. This review synthesizes recent advances in molecular biomarkers across three domains: Fluid biomarkers, Molecular imaging and Innovative detection platforms, and also evaluates the challenges and prospects of the clinical transformation of molecular markers for AD.

**Keywords:** Alzheimer's Disease; Biomarkers; Clinical Translation; Precision Medicine

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## 1. Introduction

Alzheimer's Disease (AD), the predominant form of neurodegenerative disorders, constitutes 60-70% of all dementia cases. The global AD population has reached over 50 million and is projected to rise to 152 million by 2050 <sup>[1]</sup>. This disease not only causes progressive cognitive decline but also imposes substantial socioeconomic burdens on affected families and healthcare systems. Traditional AD diagnosis relies primarily on clinical symptoms and neuropsychological assessments; however, by this stage, pathological changes are often irreversible <sup>[2]</sup>. Over the past decade, the diagnostic paradigm for AD has undergone a fundamental shift from purely clinical diagnosis towards biomarker-supported approaches, a transformation fully reflected in the Chinese Guidelines for the Clinical Application of Fluid Biomarkers in Alzheimer's Disease published in 2024 <sup>[3]</sup>.

The core value of molecular biomarkers lies in their ability to detect early AD pathological changes 10-20 years prior to the manifestation of clinical symptoms, thereby providing a critical window for intervention. According to the AT(N) research framework (Amyloid, Tau, Neurodegeneration), molecular biomarkers are primarily classified into three major categories:  $\beta$ -amyloid ( $A\beta$ ) deposition-related biomarkers, tau protein pathology biomarkers, and neurodegeneration/neuronal injury biomarkers <sup>[1-3]</sup>. The detection technologies for these biomarkers encompass cerebrospinal fluid (CSF) analysis, peripheral

fluid testing, and molecular imaging, collectively forming a multi-tiered detection system.

Recent breakthroughs in global AD research in 2025 across six key areas have propelled the diagnostic and therapeutic model from a “one-size-fits-all” approach towards personalized and precision medicine (“tailored to the individual”) <sup>[4]</sup>. Studies demonstrated that inhibition of the metabolic enzyme IDO1 can reverse cerebral glucose metabolic dysfunction in AD patients while concurrently restoring synaptic function; furthermore, they elucidated the molecular link between hearing loss and AD. Critically, CSF proteomic analysis has classified AD into five distinct subtypes, laying the groundwork for precision medicine <sup>[4]</sup>. These advances not only deepen our understanding of AD pathogenesis but also open new avenues for the clinical application of molecular biomarkers.

This review will systematically summarize the current clinical applications and recent advances in AD molecular biomarkers from four perspectives: CSF biomarkers, blood-based biomarkers, molecular imaging techniques, and emerging biomarkers/technologies. It will further explore the associated challenges and future research directions, aiming to provide a reference for clinicians and researchers.

## 2.Cerebrospinal Fluid Biomarkers

### 2.1 Clinical Application of Core Biomarkers

Cerebrospinal fluid (CSF), due to its direct reflection of the brain’s microenvironment, has long served as the “molecular repository” for AD biomarker detection. According to the 2024 Chinese Guidelines, core CSF biomarkers include A $\beta$ 42, A $\beta$ 40, phosphorylated tau protein (p-tau), and total tau protein (t-tau) <sup>[3]</sup>. These biomarkers accurately reflect the cerebral pathological alterations in AD: decreased A $\beta$ 42 concentration or a reduced A $\beta$ 42/40 ratio indicates cerebral A $\beta$  deposition, while elevated p-tau concentration reflects tau hyperphosphorylation and neurofibrillary tangle formation <sup>[3]</sup>.

In clinical practice, the diagnostic value of a single biomarker is limited, and multiplex biomarker testing has become the consensus. The guideline recommendation explicitly states: “Multiple CSF biomarkers can be used in combination for AD diagnosis, demonstrating superior diagnostic performance compared to individual CSF biomarkers. The CSF A $\beta$ 42/40 ratio and A $\beta$ 42/p-tau181 ratio exhibit better diagnostic performance for AD than A $\beta$ 42 alone (Evidence Level 1A)” <sup>[3]</sup>. This recommendation is grounded in substantial research evidence indicating that composite indices significantly enhance diagnostic specificity and sensitivity.

It is noteworthy that different p-tau epitopes possess distinct clinical significance. Beyond the classical p-tau181, emerging biomarkers such as p-tau217, p-tau231, and p-tau205 demonstrate unique advantages <sup>[5-8]</sup>. Particularly, p-tau217 shows exceptional performance in differentiating AD from other neurodegenerative disorders <sup>[5]</sup>, while p-tau231 is considered an indicator of earlier pathological changes <sup>[7]</sup>. A pivotal 2025 study by the University of Pittsburgh team revealed that p-Tau262 and p-Tau356 levels are significantly elevated during the “preclinical phase” preceding neurofibrillary tangle (NFT) formation, a discovery that may further advance the AD diagnostic window <sup>[9]</sup>.

### 2.2 Auxiliary Value of Non-AD Specific Biomarkers

In addition to core AD biomarkers, neurofilament light chain protein (NfL) and glial fibrillary acidic protein (GFAP), serving as markers of neurodegeneration and neuroinflammation, respectively, hold significant auxiliary value in AD diagnosis and management <sup>[10-12]</sup>. The Chinese Guidelines state: “Non-AD specific CSF biomarkers such as NfL and GFAP indicate pathological changes in the brain, including neurodegeneration or inflammation, reflecting the severity of AD progression, but cannot be used alone for AD diagnosis (Evidence Level 1A)” <sup>[3]</sup>.

NfL, a marker of axonal damage, increases in concentration with AD progression and correlates significantly with the rate of brain atrophy and cognitive decline <sup>[13]</sup>. GFAP, reflecting astrocytic activation, is elevated early in AD and demonstrates outstanding predictive value for the conversion from mild cognitive impairment (MCI) to AD <sup>[13]</sup>. Furthermore, studies have found GFAP is significantly elevated in individuals with A $\beta$  positivity but normal cognition, suggesting its potential for ultra-early screening <sup>[14]</sup>.

### 2.3 Novel Biomarkers and Disease Subtyping

A landmark study employing CSF proteomic analysis of 419 AD patients and 187 healthy controls classified AD into five distinct molecular subtypes: Hyperplasticity Subtype, Innate Immune Activation Subtype, Choroid Plexus Dysfunction



Subtype, Blood-Brain Barrier Dysfunction Subtype, and RNA Dysregulation Subtype. This subtyping holds critical implications for individualized treatment <sup>[15]</sup>. For instance, patients with the Innate Immune Activation Subtype may benefit preferentially from anti-inflammatory therapies, while those with the Blood-Brain Barrier Dysfunction Subtype might require vasoprotective strategies. The Chinese Guidelines also note: “Fluid biomarkers can be used for pre-screening of clinical trial participants to exclude individuals with a low probability of AD pathology (Evidence Level 1A)” <sup>[3]</sup>.

### 3. Blood-Based Biomarkers: Breakthroughs in Non-Invasive Detection

#### 3.1 Advances in Plasma A $\beta$ and p-Tau Biomarkers

Although CSF testing is reliable, the invasiveness of lumbar puncture limits its widespread application. Blood-based biomarkers, leveraging the advantages of non-invasive collection, suitability for large-scale screening, and longitudinal monitoring, have become a major research focus in recent years. The 2024 Chinese Guidelines recommend: “Blood-based biomarkers utilizing highly sensitive detection methods may be used cautiously to support AD diagnosis; however, results should be confirmed whenever possible by CSF testing or PET imaging (Expert Consensus)” <sup>[3]</sup>.

Technological breakthroughs have been pivotal in advancing blood biomarkers (Table 1). The emergence of ultra-sensitive detection technologies such as Single Molecule Array (Simoa) and Immunoprecipitation coupled with Mass Spectrometry (IP-MS) has enabled the accurate quantification of extremely low concentrations of AD-related proteins in blood <sup>[16-20]</sup>. Notably, for p-Tau proteins, a highly sensitive assay developed by the University of Pittsburgh team in 2025 successfully detected plasma p-Tau262 and p-Tau356. These biomarkers can identify Tau pathology changes years earlier than current methods <sup>[9]</sup>.

Plasma p-tau217 is currently one of the most extensively studied blood biomarkers. Multiple large cohort studies demonstrate that plasma p-tau217 performs nearly as well as CSF testing in differentiating AD from other neurodegenerative disorders, achieving areas under the curve (AUC) exceeding 0.95 <sup>[21-23]</sup>.

Table 1: Performance Comparison of Major AD Blood Biomarker Detection Platforms

Technology Platform	Detection Principle	Sensitivity	Multiplexing Capacity	Throughput	Application Scenario
Simoa	Single Molecule Array	fg/mL	Low ( $\leq 6$ -plex)	Medium	Research & small-scale clinical use
NULISA	Nucleic Acid Signal Amplification	fg/mL	High (120-plex)	High	Large-scale screening & omics studies
Lumipulse®	Chemiluminescent Immunoassay	pg/mL	Low	High	Routine clinical testing
IP-MS	Immunoprecipitation-Mass Spectrometry	pg/mL	Medium	Low	Reference method & research

#### 3.2 Value of Peripheral Metabolic and Inflammatory Biomarkers

Beyond biomarkers directly reflecting AD core pathology, peripheral metabolites and inflammatory factors provide crucial information for early AD warning and subtyping. In April 2025, a study published in Chem by a Shanghai Jiao Tong University team pioneered the development of Molecularly Resolvable Surface-Enhanced Raman Spectroscopy Molecular Group Technology (MORE SERSome). This technology integrates laser desorption/ionization mass spectrometry with SERS, enabling molecular-level resolution of serum metabolic fingerprints <sup>[24]</sup>. Applying this method, the team identified significantly altered metabolites in AD patient serum, including ergothioneine and uric acid, and constructed a SERSome-Graph Convolutional Neural Network model achieving an impressive AD discrimination AUC of 91.5% <sup>[24]</sup>. These findings not only provide a novel technology for blood-based AD diagnosis but also reveal the role of metabolic dysregulation in AD pathogenesis.

Inflammatory biomarkers also play a significant role in AD risk assessment and subtype differentiation. A 2025 study

discovered that dark microglia are twice as abundant in the brains of AD patients compared to healthy individuals. These cells produce neurotoxic lipids that accelerate the loss of synaptic connections <sup>[25]</sup>. This neuroinflammatory change manifests as alterations in specific cytokine profiles in peripheral blood, offering clues for identifying the Innate Immune Activation AD subtype.

### 3.3 Application Scenarios and Optimization Directions for Blood Biomarkers

The core application scenarios for blood biomarkers include <sup>[3]</sup>: Large-scale population screening: The Chinese Guidelines state: “Plasma A $\beta$ 42/40 ratio, p-tau181, and p-tau217 can be used for screening AD patients.” Disease progression prediction: Plasma p-tau181, p-tau217, and A $\beta$ 42/40 ratio can predict the risk of AD progression. Therapeutic effect monitoring: Blood biomarkers are suitable for dynamic assessment of treatment efficacy in clinical trials.

However, blood biomarker testing still faces challenges related to pre-analytical variability (sample collection, storage conditions), analytical variability (differences across detection platforms), and biological variability (circadian rhythms, dietary influences). Standardization of pre-analytical workflows and harmonization of detection platforms represent critical future development directions. The Chinese Guidelines also cautiously note: “Combining blood biomarkers with AD risk factors, age, sex, APOE genotype, and neuropsychological assessments enhances AD diagnostic performance, albeit with a marginal effect” <sup>[3]</sup>.

## 4. Molecular Imaging: Visualizing Pathological Progression

### 4.1 Clinical Application of A $\beta$ -PET Imaging

Molecular imaging technologies have revolutionized the field by enabling non-invasive visualization of AD pathology in the brain, realizing “in vivo histopathology”. The formal clinical adoption of the A $\beta$ -PET tracer Florbetaben ([<sup>18</sup>F]) marks the entry of AD diagnosis and treatment into the era of visualized biomarkers. The core strength of A $\beta$ -PET lies in its ability to detect abnormal cerebral  $\beta$ -amyloid deposition 15-20 years prior to symptom onset <sup>[26, 27]</sup>. The clinical utility of A $\beta$ -PET extends beyond diagnosis to include treatment response monitoring, clinical trial participant screening, and treatment decision support. However, the current high cost of A $\beta$ -PET scans and the requirement for specialized PET equipment and radionuclide production facilities limit its widespread accessibility and adoption.

### 4.2 Advances in Tau-PET Imaging

Compared to A $\beta$ -PET, the development of Tau-PET imaging has been more challenging, primarily due to the complexity of tau isoforms and their intracellular localization. Tau-PET tracers offer a more accurate reflection of tau protein distribution and density. Research indicates that tau deposition exhibits a stronger correlation with cognitive dysfunction than A $\beta$  deposition, thereby conferring unique value to Tau-PET in predicting disease progression and assessing therapeutic efficacy <sup>[28-30]</sup>. Recent research found that combining Tau-PET imaging with CSF p-tau262/p-tau356 detection enables comprehensive monitoring of tau pathology, spanning from the soluble tau aggregate (STA) stage to neurofibrillary tangle formation <sup>[9]</sup>. This holistic monitoring capability provides critical technical support for tau-targeting disease-modifying therapies.

### 4.3 Comparative Analysis and Integrated Application of Multimodal Imaging

A $\beta$ -PET, Tau-PET, and FDG-PET (reflecting cerebral glucose metabolism) each have distinct emphases, forming a complementary approach in clinical practice:

- A $\beta$ -PET: High negative predictive value for ruling out AD diagnosis <sup>[31]</sup>.
- Tau-PET: Assesses disease stage and rate of progression <sup>[30]</sup>.
- FDG-PET: Reflects synaptic dysfunction and the extent of neurodegeneration <sup>[32]</sup>.

Future development focuses on integrating multiple molecular imaging techniques with fluid biomarkers, genetic testing, and digital biomarkers to construct a multimodal AD assessment system, enabling precise disease subtyping and personalized intervention.

## 5. Emerging Biomarkers and Technological Innovations

### 5.1 Exploration of Biomarkers in Alternative Biofluids

Beyond blood, research on AD biomarkers in more accessible biofluids like saliva, tears, and urine has progressed. The 2024



Chinese Guidelines state: “Potential AD biomarkers include lactoferrin and exosomal miRNA in saliva, eIF4E and miRNA in tears, and elevated formaldehyde and formic acid levels as well as AD-associated neuronal thread protein (AD7c-NTP) in urine”<sup>[3]</sup>. Notably, urinary AD7c-NTP testing has been implemented in some clinical settings in China, offering advantages of complete non-invasiveness and low cost, making it suitable for large-scale preliminary screening. Studies show significantly higher urinary AD7c-NTP levels in AD patients compared to healthy controls, correlating with CSF t-tau and A $\beta$ 42 levels. However, the Guidelines cautiously note: “The clinical value of biomarkers in saliva, tears, urine, and other biofluids for AD requires further evidence”<sup>[3]</sup>. Exosome technology holds particular promise for alternative biofluid analysis. Neuron-derived exosomes carry brain-specific proteins (e.g., A $\beta$ , Tau) and can be isolated from peripheral blood via immunocapture techniques, providing an opportunity for “liquid biopsy”<sup>[33]</sup>.

## 5.2 Metabolomics and Multi-omics Integration

Metabolomics, through the systematic analysis of small molecule metabolites (<1500 Da), reveals metabolic pathway dysregulation in AD pathology. The MORE SERSome technology developed by the Shanghai Jiao Tong University team overcame the technical bottleneck of conventional label-free Surface-Enhanced Raman Spectroscopy (SERS) for multi-analyte detection, achieving molecular-level resolution of serum metabolic fingerprints and demonstrating the significant potential of metabolomics for precise AD diagnosis<sup>[24]</sup>. Recent research also revealed that inhibiting the metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1) can reverse cerebral glucose metabolic dysfunction in AD patients while simultaneously restoring synaptic function<sup>[34]</sup>. This discovery not only identifies a novel therapeutic target for AD but also underscores the central role of metabolic dysregulation in AD pathogenesis.

## 5.3 Digital Biomarkers and Artificial Intelligence Integration

Digital Biomarkers, an emerging field, enable continuous monitoring and early warning of AD by collecting data on movement patterns, speech characteristics, and cognitive behavior via wearable devices and smartphones. Research shows that digital biomarkers based on keystroke dynamics can predict MCI risk up to 5 years in advance<sup>[35-37]</sup>.

Artificial Intelligence (AI) is being integrated into molecular biomarker research for:

- Multi-omics data integration analysis: Identifying AD subtype-specific molecular networks.
- Radiomics feature extraction: Identifying subtle patterns in PET/MRI images imperceptible to the human eye.
- Predictive model construction: Such as the SERSome-Graph Convolutional Neural Network model<sup>[4, 24]</sup>.

A major recent breakthrough in AD research is the achievement of molecular mechanism-based disease subtyping. This subtyping provides a biological foundation for constructing AI models, holding promise for realizing genuinely personalized precision medicine (“tailored to the individual”)<sup>[15]</sup>.

# 6. Clinical Translation Challenges and Future Prospects

## 6.1 Bottlenecks in Standardizing Biomarker Clinical Application

Despite significant advances in molecular biomarker research, their clinical application faces multiple challenges. Standardization is the primary bottleneck—variations in sample collection protocols, detection platforms, and interpretation criteria across different laboratories hinder the comparability of results. For instance, the coefficient of variation for CSF A $\beta$ 42 measurements can exceed 20% between platforms, potentially leading to clinical misdiagnosis<sup>[38]</sup>.

Balancing cost-effectiveness presents another challenge. The high cost of A $\beta$ -PET scans limits its accessibility. While blood testing is less expensive, its accuracy still requires improvement. The Chinese Guidelines advise: “Results from blood-based biomarkers should be confirmed whenever possible by CSF testing or PET imaging (Expert Consensus)”.

Ethical and psychosocial implications also warrant serious consideration. Early AD diagnosis may induce anxiety and stigma, particularly for disease stages lacking effective treatments. Establishing comprehensive genetic counseling and psychological support systems is a necessary prerequisite for the widespread adoption of molecular biomarkers.

## 6.2 AD Molecular Subtyping and Precision Intervention Strategies

The proposal of AD molecular subtyping lays the groundwork for precision interventions. Intervention strategies should be tailored to specific subtypes:

- Innate Immune Activation Subtype: Utilize anti-inflammatory therapies (e.g., anti-TREM2 antibodies).

- Blood-Brain Barrier Dysfunction Subtype: Employ vasoprotective agents and blood-brain barrier stabilizers.
- RNA Dysregulation Subtype: Apply small-molecule drugs targeting RNA splicing <sup>[15]</sup>.

In drug development, monoclonal antibodies have achieved major breakthroughs. In February 2025, the Washington University School of Medicine initiated a six-year AD prevention trial using remternetug (developed by Eli Lilly) to intervene in high-risk individuals 11-25 years before symptom onset. Based on the hypothesis that “intervening when amyloid-beta plaques are at their earliest stage can prevent symptoms”, this trial represents a paradigm shift from symptomatic treatment towards disease modification and ultimately disease prevention <sup>[39]</sup>.

Notably, the diabetes drug semaglutide has shown unexpected potential in AD research. Analysis of health records from nearly 1.1 million US patients with type 2 diabetes revealed that those treated with semaglutide for three years had an approximately 70% lower risk of developing AD compared to insulin users and a 40% lower risk compared to users of another GLP-1 receptor agonist <sup>[40]</sup>. This protective effect was more pronounced in women, offering a new perspective for metabolic intervention in AD.

### 6.3 Shift Towards Preventive Medicine Models

AD management is undergoing a fundamental transformation from symptomatic treatment to disease modification and, critically, primary prevention. The Washington University prevention trial, costing over \$130 million with approximately \$98.3 million donated by the National Institutes of Health (NIH), underscores the high priority placed on preventive strategies <sup>[39]</sup>.

Lifestyle interventions hold significant value in AD prevention. Research published in a Nature journal by the California Institute for Preventive Medicine demonstrated that patients with mild cognitive impairment showed improved cognitive function and favorable biomarker trends following a regimen of strict vegetarianism, personalized exercise, yoga-based stress management, and psychological support. This indicates that healthy lifestyles can reduce neuroinflammation and promote neuronal repair <sup>[41]</sup>.

## 7. Conclusion

The research and application of molecular biomarkers for Alzheimer’s Disease are undergoing a revolutionary transformation, driving the evolution of AD diagnosis and treatment from empirical medicine to precision medicine. While CSF biomarkers continue to be optimized as the gold standard, blood-based biomarkers are gaining prominence in screening and monitoring due to their non-invasive nature. Molecular imaging technologies enable in vivo visualization of pathological changes, and emerging fields like metabolomics, multi-omics integration, and digital biomarkers are opening novel research avenues.

Recent groundbreaking advances—ranging from IDO1-mediated metabolic regulation and AD molecular subtyping to the discovery of ultra-early biomarkers like p-Tau262/p-Tau356—have not only deepened our understanding of AD’s complex pathological network but also provided a scientific foundation for individualized interventions. Research from the University of Pittsburgh suggests that targeting soluble tau aggregates (STAs) before neurofibrillary tangle formation may represent a critical therapeutic window for halting AD progression <sup>[9]</sup>.

Future directions for AD molecular biomarkers include:

- Technological Innovation: Enhancing detection sensitivity, reducing costs, and developing point-of-care testing (POCT) devices.
- Multimodal Integration: Constructing a comprehensive assessment framework integrating fluid biomarkers, imaging, and digital biomarkers.
- Clinical Translation: Advancing biomarker-guided personalized treatment and prevention strategies.
- Real-World Validation: Demonstrating the practical utility of biomarkers in diverse clinical settings.

With the application of tracers like Florbetaben (<sup>[18F]</sup>) and the development of original technologies like MORE SERSome, Chinese researchers are making significant contributions to the field of AD molecular biomarkers. These innovative technologies, combined with international cutting-edge discoveries, will collectively propel AD management into a new era characterized by early screening, diagnosis, and intervention, ultimately achieving the historic transition from an incurable to a preventable and treatable disease.

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## Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# The Influence of Behavioral Lifestyle Factors on Recent Episodic Memory Retention Capacity in Young-Old Adults: An Empirical Investigation Leveraging the 2022 Wave Data from the China Family Panel Studies (CFPS)

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**Abstract: Objective:** This study aimed to examine the influence of behavioral lifestyle factors on recent episodic memory retention capacity among young-old adults (aged 60-69 years) in China. The findings provide scientific evidence to inform proactive strategies to mitigate cognitive decline risk within China's rapidly aging population. **Methods:** Utilizing data from the 2022 wave of the China Family Panel Studies (CFPS), a total of 2,772 adults aged 60-69 were included in the analytical sample. Recent episodic memory retention capacity (scored 0-5 points, based on self-reported assessment) served as the dependent variable. Six categories of behavioral lifestyle indicators (including exercise frequency, sleep quality, dietary patterns, etc.) were analyzed as independent variables. Associations were assessed using multivariate ordinal logistic regression models, controlling for relevant covariates. **Results:** Self-reported potential impairment in recent episodic memory was identified by 47.19% of respondents. Multivariate analysis revealed significant associations between behavioral lifestyle factors and memory retention capacity. Regular exercise (OR = 1.297, 95% CI: 1.118–1.504), meat consumption (OR = 1.765, 95% CI: 1.393–2.237), regular reading habits (OR = 1.599, 95% CI: 1.283–1.992), and internet use (OR = 1.413, 95% CI: 1.217–1.641) emerged as significant protective factors. Abnormal sleep duration was detrimentally associated with retention capacity (too short: OR = 0.728, 95% CI: 0.591–0.897; too long: OR = 0.810, 95% CI: 0.670–0.980). Significant associations were also observed for control variables: urban residence (OR = 1.270, 95% CI: 1.100–1.467), high school education or above (OR = 1.543, 95% CI: 1.293–1.841), and better self-rated health status (OR = 1.156, 95% CI: 1.089–1.227) were positively correlated with better memory retention. **Conclusions:** Optimal sleep duration, regular physical exercise, meat intake, habitual reading, and internet engagement positively predict self-assessed recent episodic memory retention capacity in Chinese young-old adults. These findings underscore the potential for multi-faceted lifestyle interventions to enhance cognitive health in aging populations. Specifically, strategies should encompass community-based sleep hygiene management, tailored nutritional interventions (especially promoting adequate protein sources like meat), enhanced digital literacy and internet accessibility programs, and the promotion of age-appropriate physical activity initiatives. Furthermore, implementing culturally responsive strategies adapted to urban-rural contexts – such as deploying “mobile cognitive health units” in rural areas and fostering digital reading platforms in urban settings – is recommended to optimize intervention effectiveness.

**Keywords:** Young-Old Adults; Behavioral Lifestyle Factors; Episodic Memory Retention; Cognitive Health Promotion;

Aging Population; Cognitive Aging; China Family Panel Studies (CFPS)

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## 1.Introduction

China possesses the world's largest elderly population, exhibiting a distinctive aging profile characterized by unprecedented scale, accelerated pace, and significant regional disparities. Data from the Seventh National Population Census (2020) reveal pronounced growth in the older population compared to the previous decade: the proportion of citizens aged 60 and above increased by 5.44 percentage points, while the proportion aged 65 and above rose by 4.63 percentage points. Crucially, as of 2020, individuals aged 65 and above accounted for 13.50% of the total population. This figure far surpasses the international threshold defining an aging society (7%) by a substantial margin of 6.50 percentage points, highlighting the pronounced severity of China's aging phenomenon<sup>[1]</sup>. Young-Old Adults (aged 60–69 years) account for 55.83% of the total elderly population in China<sup>[2]</sup>. While this population generally exhibits relatively better overall health, impairments in cognitive function—notably, early-stage deterioration within the episodic memory system—have become increasingly salient. Episodic memory retention, a core cognitive process vulnerable to aging effects and thus a key target in cognitive aging research, encompasses an individual's neurocognitive capacity for the stable storage and accurate retrieval of specific, recently experienced spatiotemporal events (typically within minutes to days). Distinct from semantic memory, this process critically depends upon the rapid binding of novel information streams by the hippocampal-entorhinal cortical circuit and subsequent strategic organization of these memory traces under the executive control of the prefrontal cortex (PFC)<sup>[3]</sup>. Neuroimaging evidence indicates accelerated hippocampal atrophy among young-old adults (aged 60–69 years), reaching an annual rate of approximately 1.4%<sup>[4]</sup>. This rate is significantly higher than the observed 0.5% atrophy rate in middle-aged populations. Critically, the magnitude of atrophy exhibits a strong correlation with declining episodic recall performance<sup>[4]</sup>, establishing this decline as a sensitive early indicator of Alzheimer's disease pathology. As modifiable protective factors, behavioral lifestyles interact with the neurobiological substrates of memory retention through multiple pathways<sup>[5]</sup>. Drawing upon Stern's Cognitive Reserve Theory, such lifestyle factors may enhance neuroplasticity, potentially compensating for the cognitive decline risk associated with hippocampal atrophy. Supporting this, a large-scale 10-year follow-up study led by Jia Jianping and colleagues (n≈30,000) demonstrated that a healthy lifestyle profile can attenuate memory decline by up to 40%<sup>[6]</sup>. However, a notable research gap persists: population-specific evidence investigating these dynamics within China's young-old adult cohort remains scarce<sup>[7]</sup>.

Leveraging data from the 2022 wave of the China Family Panel Studies (CFPS), this research developed a multi-dimensional assessment framework encompassing exercise patterns, sleep quality, dietary habits, and other relevant behavioral domains. This framework was employed to systematically investigate the influence of distinct healthy lifestyle behaviors on recent episodic memory retention capacity among young-old adults (aged 60–69 years). The study aims to generate robust scientific evidence essential for formulating targeted health management interventions for older populations and, ultimately, contribute to optimizing their overall quality of life.

## 2.Participants and Methods

### 2.1 Study Participants

Data used in this study were derived from the 2022 wave of the China Family Panel Studies (CFPS), a national longitudinal survey database implemented by Peking University, providing data for researching various topics such as economic activities, educational attainment, family relationships, population migration, and physical-mental health of Chinese residents. Based on research needs, the following inclusion and exclusion criteria were established:

Inclusion criteria: ① Aged 60–69 years; ② Complete questionnaire responses.

Exclusion criteria: Questionnaires with logical inconsistencies.

Through the above criteria, a database consisting of 2,772 respondents was finally obtained.

## 2.2 Variable Construction

### 2.2.1 Dependent Variable

The dependent variable was the score of respondents' recent episodic memory retention capacity, measured by the question in the 2022 CFPS questionnaire: "How much can you remember about the main events that happened to you within the past week?" A higher score indicated stronger recent episodic memory retention capacity. The question had five response options: "Can remember only a little," "Can remember only some," "Can remember half," "Can remember most," and "Can remember completely," which were assigned scores 1–5, respectively.

### 2.2.2 Core Independent Variables

Relevant questions on behavioral lifestyle in the 2022 CFPS questionnaire were used as core independent variables.

#### 2.2.2.1 Exercise Frequency

Exercise frequency was measured by the item in the 2022 CFPS questionnaire: "How often have you participated in sports, fitness, or leisure activities in the past 12 months? (Excluding cycling/walking for commuting purposes only, including physical education classes)." The question had eight options: "Never," "Less than 1 time per month," "More than 1 time per month but less than 1 time per week," "1–2 times per week," "3–4 times per week," "5+ times per week," "Once a day," and "2+ times per day." Referencing the World Health Organization (WHO) recommendations for adult physical activity (recommending at least 150 minutes of moderate-intensity aerobic exercise per week)<sup>[7]</sup>, "1+ times per week" was defined as the basis for regular exercise. Thus, options with "1–2 times per week" or higher frequencies were categorized as "with exercise habits," while lower-frequency options (including never, less than 1 time per month, and more than 1 time per month but less than 1 time per week) were categorized as "without exercise habits."

#### 2.2.2.2 Nighttime Sleep Duration

Nighttime sleep duration was measured by the question: "How many hours do you sleep per day? (Excluding nap time)." Categorization referred to the Healthy China Action (2019–2030): 6–8 hours was classified as "normal," <6 hours as "too short," and >8 hours as "too long."<sup>[8]</sup>

#### 2.2.2.3 Dietary Habits

Dietary habits were measured using two questions in the 2022 CFPS questionnaire regarding meat and fruit-vegetable intake: "Have you eaten meat in the past week? (Including pork, beef, mutton, chicken, duck, etc., or aquatic products such as fish, shrimp, and shellfish)" and "Have you eaten fresh vegetables or fruits in the past week?" Both questions had "Yes" and "No" options, which were analyzed as two separate variables.

#### 2.2.2.4 Tobacco-Alcohol Behavior

Tobacco-alcohol behavior was measured by two questions: "Have you smoked in the past month?" and "Have you drunk alcohol 3+ times per week in the past month?" Both had "Yes" and "No" options, analyzed as separate variables.

#### 2.2.2.5 TV/Movie Watching Behavior

TV/movie watching behavior was measured by the question: "Generally, how many hours per week do you spend watching TV, movies, or other video programs through various means?" Weekly viewing time was converted to average daily duration. Respondents with >30 minutes/day were categorized as "with TV/movie watching habits," and ≤30 minutes/day as "TV/movie watching habits".

### 2.2.3 Control Variables

Seven potential confounders were considered as control variables: age, gender (male/female), usual residence (urban/rural), region (eastern China/mid-western China), subjective income level (lower/higher), presence of chronic diseases, and self-rated health status (poorer/better).

Subjective income level was measured by "How would you rate your income position locally? (1 = very low, 5 = very high)". Scores 1–2 were categorized as "lower," and 3–5 as "higher."

Self-rated health status was measured by "How do you think your health condition is?" Options ("very healthy", "healthy", "relatively healthy", "fair", "unhealthy") were categorized as "better" (very healthy/healthy/relatively healthy) and "poorer" (fair/unhealthy).



## 2.3 Statistical Methods

Statistical analyses were performed using SPSS 25.0. Categorical data were described using frequencies and percentages, and continuous data using mean  $\pm$  standard deviation. Univariate and multivariate ordinal logistic regression models were used to explore the effects of behavioral lifestyle on recent episodic memory retention capacity among younger older adults. A two-tailed  $p < 0.05$  was considered statistically significant.

## 2.4 Ethical Statement

This study used data from the CFPS, which were collected following relevant ethical regulations. The CFPS protocol was approved by the Biomedical Ethics Committee of Peking University (Approval No. IRB00001052-14010), and all participants provided written informed consent. The survey strictly protected respondents' privacy and rights.

## 3. Results

### 3.1 General Characteristics and Behavioral Lifestyle of Respondents

Among the 2,772 respondents, the mean age was  $64.58 \pm 2.97$  years. There were 1,432 males (51.66%) and 1,340 females (48.34%). A total of 1,345 respondents (48.52%) resided in urban areas, and 1,427 (51.48%) in rural areas. Education levels showed that 2,120 (76.48%) had a junior high school education or below, and 652 (23.52%) had a high school education or above. Additionally, 2,483 (89.57%) were married, and 289 (10.43%) were unmarried. For other general characteristics and current behavioral lifestyle status, see Table 1.

*Table 1 General Characteristics of Respondents*

Variables	n (%)
Gender	
Male	1432 (51.66)
Female	1340 (48.34)
Region	
Eastern Region	1198(43.22)
Mid-western Region	1574(56.78)
Usual Residence	
Urban	1345 (48.52)
Rural	1427 (51.48)
Educational Attainment	
High School and Above:	652 (23.52)
Junior High School or Below	2120(76.48)
Marital Status	
Unmarried	289 (10.43)
Married	2483 (89.57)
Self-rated Income Level	
Low	742 (26.77)
High	2030 (73.23)
Presence of Chronic Diseases	
No	1944 (70.13)
Yes	828 (29.87)
Self-rated Health Status	
Unhealthy	1044 (37.66)

Variables	n (%)
Healthy	1728 (62.34)
Exercise Habits	
No	1738 (62.70)
Yes	1034 (37.30)
Nighttime Sleep Duration	
Normal Sleep (6–8h)	1991(71.83)
Short Sleep (<6h)	339(12.23)
Long Sleep (>8h)	442(15.94)
Meat Consumption in Past Week	
No	281 (10.14)
Yes	2491 (89.86)
Fresh Vegetable/Fruit Consumption in Past Week	
No	55 (1.98)
Yes	2699 (98.02)
Smoking Status in Past Month	
No	1991 (71.83)
Yes	781 (28.17)
Weekly Alcohol Consumption $\geq 3$ Times in the Past Month	
No	2345 (84.60)
Yes	427 (15.40)
Noon Break Habits	
No	1896(68.40)
Yes	876(31.60)
TV/Movie Watching Habits	
No	1054 (38.02)
Yes	1718 (61.98)
Book Reading in Past 12 Months	
No	2435(87.84)
Yes	337(12.16)
Internet Use	
No	1652(59.60)
Yes	1120(40.40)

### 3.2 Recent Episodic Memory Retention Capacity Scores and Univariate Ordinal Logistic Regression Results

Among 2,772 respondents, the mean score for recent episodic memory retention was  $2.59 \pm 1.30$  (out of 5). Specifically, 782 respondents (28.21%) “can remember only a little”, 526 (18.98%) “can remember only some”, 781 (28.17%) “can remember half”, 422 (15.22%) “can remember most”, and 261 (9.42%) “can remember completely” about major events that occurred in the past week.

Univariate ordinal logistic regression showed that gender, region, residence, education level, chronic disease status, self-rated health, exercise habits, sleep quality, meat consumption, fresh vegetable/fruit consumption, smoking, alcohol consumption, TV/movie watching habits, reading behavior, and internet use were significantly associated with recent episodic memory retention capacity (all  $P < 0.05$ ) (Table 2).

Table 2 Univariate Ordinal Logistic Regression Results for Subjective Recent Episodic Memory Retention

Variables	$\beta$	SE	Wald $\chi^2$	OR (95%CI)	P
Age	-0.017	0.011	2.39	0.983 (0.961 ~ 1.005)	0.138
Gender (Reference: Female)					
Male	0.291	0.068	18.31	1.338 (1.171 ~ 1.529)	<0.001
Region (Reference: Eastern China)					
Mid-western China	0.227	0.069	10.82	1.254 (1.096 ~ 1.435)	<0.001
Usual Residence(Reference: Rural)					
Urban	0.520	0.068	58.48	1.683 (1.471 ~ 1.924)	<0.001
Educational Attainment(Reference: Junior High School or Below)					
High School and Above	0.851	0.081	110.38	2.342 (1.999 ~ 2.744)	<0.001
Marital Status(Reference: Unmarried)					
Married	0.167	0.109	2.35	1.182 (0.954 ~ 1.465)	0.126
Self-rated Income Level	-0.010	0.030	0.11	0.990 (0.934 ~ 1.049)	0.732
Self-rated Health Status	0.182	0.028	42.25	1.200 (1.137 ~ 1.267)	<0.001
Presence of Chronic Diseases(Reference: No)					
Yes	-0.168	0.074	5.15	0.846 (0.732 ~ 0.977)	0.023
Exercise Habits(Reference: No)					
Yes	0.563	0.071	62.89	1.756 (1.529 ~ 2.016)	<0.001
Nighttime Sleep Duration(Reference: 6–8h)					
Short Sleep (<6h)	-0.399	0.105	14.44	0.671 (0.546 ~ 0.824)	<0.001
Long Sleep (>8h)	-0.470	0.094	25.00	0.625 (0.520 ~ 0.751)	<0.001
Meat Consumption in Past Week(Reference: No)					
Yes	0.871	0.115	57.36	2.389 (1.909 ~ 2.990)	<0.001
Fresh Vegetable/Fruit Consumption in Past Week(Reference: No)					
Yes	0.567	0.249	5.19	1.763 (1.082 ~ 2.874)	0.023
Smoking Status in Past Month(Reference: No)					
Yes	0.250	0.075	11.11	1.284 (1.108 ~ 1.488)	<0.001
Weekly Alcohol Consumption $\geq 3$ Times in the Past Month(Reference: No)					
Yes	0.290	0.094	9.52	1.336 (1.111 ~ 1.607)	0.002
Noon Break Habits(Reference: No)					
Yes	-0.039	0.073	0.29	0.962 (0.834 ~ 1.109)	0.593
TV/Movie Watching Habits(Reference: No)					
Yes	0.184	0.070	6.91	1.202 (1.048 ~ 1.378)	0.009
Book Reading in Past 12 Months(Reference: No)					
Yes	0.973	0.104	87.69	2.646 (2.158 ~ 3.243)	<0.001
Internet Use(Reference: No)					
Yes	0.669	0.070	91.36	1.952 (1.702 ~ 2.239)	<0.001

### 3.3 Multivariate Ordinal Logistic Regression Results

The regression model identified exercise habits, nighttime sleep duration, meat consumption, reading behavior, and internet use as significant predictors of recent episodic memory retention (all  $P < 0.05$ ). Specific findings included: individuals with regular exercise habits had 1.297-fold higher odds of better memory retention than those without regular exercise habits (OR=1.297, 95%CI:1.118–1.504); compared to those with normal sleep duration (6–8 hours), both short sleep (<6 hours, OR=0.728, 95%CI:0.591–0.897) and long sleep (>8 hours, OR=0.810, 95%CI:0.670–0.980) were associated with reduced memory ability; respondents who consumed meat in the past week had 1.765-fold higher odds of better memory retention than non-consumers (OR=1.765, 95%CI:1.393–2.237); those who read books in the past 12 months had 1.599-fold higher odds of better memory retention than non-readers (OR=1.599, 95%CI:1.283–1.992); internet users in the past month had 1.413-fold higher odds of better memory retention than non-users (OR=1.413, 95%CI:1.217–1.641).

The model also revealed residence, education level, and self-rated health as significant influencing factors (all  $P < 0.05$ ): urban residents had 1.270-fold higher odds of better memory retention than rural residents (OR=1.270, 95%CI:1.100–1.467); individuals with high school education or above had 1.543-fold higher odds of better memory retention than those with junior high school education or below (OR=1.543, 95%CI:1.293–1.841); For every one-point increase in self-rated health scores, there was a 1.156-fold increase in the likelihood of better memory (OR = 1.156, 95% CI: 1.089–1.227) Details are presented in Table 3.

Table 3 Multivariate Ordinal Logistic Regression Results for Recent Episodic Memory Retention Capacity

Variables	$\beta$	SE	Wald $\chi^2$	OR (95%CI)	P
Age	-0.017	0.011	2.39	0.983 (0.961 ~ 1.005)	0.138
Gender (Reference: Female)					
Male	0.291	0.068	18.31	1.338 (1.171 ~ 1.529)	<0.001
Region (Reference: Eastern China)					
Mid-western China	0.227	0.069	10.82	1.254 (1.096 ~ 1.435)	<0.001
Usual Residence(Reference: Rural)					
Urban	0.520	0.068	58.48	1.683 (1.471 ~ 1.924)	<0.001
Educational Attainment(Reference: Junior High School or Below)					
High School and Above	0.851	0.081	110.38	2.342 (1.999 ~ 2.744)	<0.001
Marital Status(Reference: Unmarried)					
Married	0.167	0.109	2.35	1.182 (0.954 ~ 1.465)	0.126
Self-rated Income Level	-0.010	0.030	0.11	0.990 (0.934 ~ 1.049)	0.732
Self-rated Health Status	0.182	0.028	42.25	1.200 (1.137 ~ 1.267)	<0.001
Presence of Chronic Diseases(Reference: No)					
Yes	-0.168	0.074	5.15	0.846 (0.732 ~ 0.977)	0.023
Exercise Habits(Reference: No)					
Yes	0.563	0.071	62.89	1.756 (1.529 ~ 2.016)	<0.001
Nighttime Sleep Duration(Reference: 6–8h)					
Short Sleep (<6h)	-0.399	0.105	14.44	0.671 (0.546 ~ 0.824)	<0.001
Long Sleep (>8h)	-0.470	0.094	25.00	0.625 (0.520 ~ 0.751)	<0.001
Meat Consumption in Past Week(Reference: No)					
Yes	0.871	0.115	57.36	2.389 (1.909 ~ 2.990)	<0.001

Variables	$\beta$	SE	Wald $\chi^2$	OR (95%CI)	P
Fresh Vegetable/Fruit Consumption in Past Week (Reference: No)					
Yes	0.567	0.249	5.19	1.763 (1.082 ~ 2.874)	0.023
Smoking Status in Past Month(Reference: No)					
Yes	0.250	0.075	11.11	1.284 (1.108 ~ 1.488)	<0.001
Weekly Alcohol Consumption $\geq 3$ Times in the Past Month(Reference: No)					
Yes	0.290	0.094	9.52	1.336 (1.111 ~ 1.607)	0.002
Noon Break Habits(Reference: No)					
Yes	-0.039	0.073	0.29	0.962 (0.834 ~ 1.109)	0.593
TV/Movie Watching Habits(Reference: No)					
Yes	0.184	0.070	6.91	1.202 (1.048 ~ 1.378)	0.009
Book Reading in Past 12 Months(Reference: No)					
Yes	0.973	0.104	87.69	2.646 (2.158 ~ 3.243)	<0.001
Internet Use(Reference: No)					
Yes	0.669	0.070	91.36	1.952 (1.702 ~ 2.239)	<0.001

## 4. Discussion

### 4.1 Current Status Analysis of Respondents' Recent Episodic Memory Retention

In this study, the mean score for recent episodic memory retention capacity among respondents was  $2.59 \pm 1.295$  (out of 5). Individuals who “can remember only a little” or “can remember only some” about major events that occurred in the past week were classified as having subjective cognitive decline (SCD). The prevalence of possible SCD among young-old adults in this study was 47.19%, nearly half. Data from CHARLS (China Health and Retirement Longitudinal Study) 2015 showed that 30%–40% of rural Chinese older adults aged 60 or above reported subjective memory decline (SCD), lower than the proportion in this study<sup>[9]</sup>. Cognitive ability is closely associated with mental health. Psychological factors such as anxiety and depression can exacerbate older adults' subjective perception of their own cognitive decline<sup>[10]</sup>. The COVID-19 pandemic may have further amplified such emotional issues, leading to an increase in the reporting rate of subjective cognitive decline (SCD) among young-old adults in 2022.

### 4.2 The Impact of Behavioral Lifestyle on Respondents' Recent Episodic Memory Retention

This study employed a sequential analytical approach. First, univariate ordinal logistic regression analyses were conducted to identify preliminary associations between behavioral lifestyle factors and recent episodic memory retention capacity. Subsequently, multivariate ordinal logistic regression modeling was implemented to adjust for potential confounders and establish statistically independent predictors. The multivariate model established that nighttime sleep duration, regular exercise engagement, meat consumption frequency during the preceding week, consistent reading habits, and internet utilization were significant independent factors associated with respondents' recent episodic memory retention performance. The model showed that respondents with normal sleep duration had better recent episodic memory retention than those with short or long sleep durations. Multiple studies have also demonstrated a U-shaped relationship between sleep duration and memory ability, indicating that both insufficient (<6 hours) and excessive (>8 hours) sleep are harmful to memory. The underlying mechanism may involve sleep disorders impairing the function of the cerebrospinal fluid lymphatic system (glymphatic system), thus reducing the clearance efficiency of beta-amyloid proteins<sup>[11]</sup>. Meanwhile, increased cortisol secretion and circadian rhythm disorders caused by insufficient sleep may also lead to cognitive decline<sup>[12-13]</sup>. Prolonged sleep duration disrupts the secretion rhythms of melatonin and cortisol. The function of the hippocampus is highly dependent on circadian rhythm regulation, and circadian dysregulation can lead to decreased neural plasticity and memory processing

ability<sup>[14]</sup>.

The model also showed that individuals with regular exercise habits had better recent episodic memory retention than those without. Exercise increases blood flow to the hippocampus and promotes hippocampal neurogenesis. Studies have shown that the brain volume of regular exercisers (particularly in the hippocampus) is significantly larger than that of sedentary individuals, and the annual atrophy rate of the hippocampus is reduced<sup>[15]</sup>. Additionally, exercise can also reduce oxidative stress levels in the brain, promote mitochondrial biogenesis, inhibit mitochondria-mediated excessive cell apoptosis, maintain mitochondrial fission-fusion balance, and enhance mitochondrial autophagic activity, thereby improving recent episodic memory retention<sup>[16]</sup>.

Respondents who consumed meat in the past week had better recent episodic memory retention than non-consumers. The cognitive protective effect of meat primarily stems from its nutritional components, including vitamin B12, iron, zinc, and proteins: Vitamin B12 maintains myelin integrity, and its deficiency leads to elevated homocysteine, directly damaging neurons<sup>[17]</sup>. Iron and zinc are involved in neurotransmitter synthesis: iron serves as a cofactor for the rate-limiting enzyme in dopamine synthesis, while zinc regulates hippocampal synaptic plasticity. High-quality proteins provide more essential amino acids, serving as substrates for neurotransmitter synthesis<sup>[18]</sup>.

Respondents with reading habits had better recent episodic memory retention than non-readers, with the mechanism being that reading enhances functional connectivity between the default mode network and language centers<sup>[19]</sup>. It can also continuously activate the frontal-temporal network, improve neural circuit efficiency, and form “cognitive reserve” to buffer damage from aging, thereby enhancing cognitive ability<sup>[20-21]</sup>.

Respondents who used the internet had better recent episodic memory retention than non-users. Internet use exerts a protective effect on memory by promoting information processing speed and working memory capacity, providing continuous cognitive stimulation, and enhancing brain neuroplasticity—thereby improving memory encoding and retrieval abilities<sup>[22]</sup>. Neuroimaging studies provide evidentiary support for this, with relevant images showing increased gray matter volume in the anterior cingulate cortex of frequent internet users, suggesting enhanced neural reserve<sup>[23]</sup>.

### 4.3 The Impact of Control Variables on Respondents' Recent Episodic Memory Retention

The regression model also showed that control variables such as place of residence, educational attainment, and self-rated health status also influenced respondents' recent episodic memory retention.

Urban respondents demonstrated better recent episodic memory retention than rural respondents. Possible reasons include: Urban areas have superior medical resources, facilitating older adults' access to chronic disease screening and early intervention. Data from CHARLS 2011-2015 also show that the prevalence of cognitive impairment among older adults in rural China is significantly higher than in urban areas, which may be associated with inadequate management of chronic diseases such as hypertension and diabetes<sup>[24]</sup>. Poor control of chronic diseases accelerates hippocampal atrophy, damaging the neural substrate of episodic memory. Additionally, urban areas provide richer cognitive activation scenarios (e.g., libraries, community courses, digital devices), continuously stimulating the prefrontal-hippocampal circuitry. In contrast, rural older adults have narrower social circles and monotonous cognitive activities, which may lead to weakened neurosynaptic plasticity<sup>[25]</sup>. Meanwhile, rural older adults often face higher physical labor burdens and economic stress, with chronic stress leading to elevated cortisol levels that inhibit hippocampal neurogenesis. In contrast, urban older adults typically enjoy more comprehensive retirement security and lower psychological stress loads.

Respondents with high school education or above had better recent episodic memory retention than those with junior high school education or below. Individuals with high school education or above build more complex neural network connectivity through long-term education, which provides functional compensation during Alzheimer's pathological invasion and delays subjective memory decline. Neuroimaging studies have shown that at the same degree of brain atrophy, individuals with higher education can score 30% higher on cognitive tests<sup>[26]</sup>. Additionally, individuals with higher education are more inclined to maintain cognitive activities such as reading and internet use. Such lifelong learning behaviors can continuously stimulate neuronal dendrite proliferation and maintain synaptic transmission efficiency<sup>[25]</sup>.

Respondents with better self-rated health also had higher recent episodic memory retention. This may be because those

with good self-rated health generally have better control of chronic diseases, and reduced vascular risk factors help protect hippocampal microcirculation, avoiding ischemic neuronal death<sup>[25]</sup>. Individuals in better health generally have more energy and willingness to engage in cognitive stimulation and social activities, which helps maintain their memory abilities.

#### 4.4 Research Advantages and Limitations

The study has the following advantages: In terms of data representativeness, the study is based on the national authoritative database CFPS (China Family Panel Studies), and the sample covers urban and rural young-old populations, so the conclusions have high external validity. In terms of variable multidimensionality, the study simultaneously incorporates multidimensional indicators such as exercise habits, sleep quality, dietary behavior, smoking/drinking behaviors, and TV/movie viewing behaviors, comprehensively analyzing the impact of behavioral lifestyles on subjective recent episodic memory retention.

The limitations of this study are as follows: First, the cross-sectional design makes it difficult to determine the causal relationship between behavioral lifestyles and subjective recent episodic memory retention, which needs to be verified by longitudinal studies. Second, variables such as self-rated health and income may be affected by social desirability bias or different understandings of health standards, affecting the accuracy of the results. At the same time, the subjective memory assessment (dependent variable) is subjective and may be affected by emotions, expectations, etc.

#### 4.5 Suggestion

Based on the research conclusions, this study proposes the following practical recommendations: ① Community health services should strengthen sleep health management by establishing sleep disorder screening clinics, promoting cognitive-behavioral therapy, and forming senior mutual sleep monitoring groups to improve sleep quality among young-old adults<sup>[27-28]</sup>. ② Implement precision nutritional interventions by introducing “cognitive-friendly meals” in community canteens (each meal contains 40g lean meat and 200g dark-colored vegetables), and enhance awareness of the link between nutrition and cognitive function<sup>[29]</sup>. ③ Further promote internet penetration among older adults by encouraging the introduction of elder-friendly internet systems and sports facilities, establishing online fitness platforms for seniors, and promoting scientific fitness programs<sup>[30-31]</sup>. ④ Implement urban-rural differentiated cognitive function promotion policies: establish “mobile cognitive health stations” in rural areas equipped with simple brain health screening devices to monitor the risk of hippocampal atrophy in older adults<sup>[32]</sup>. Construct a digital reading cultural ecosystem for older adults in urban areas to promote the development of digital reading among the elderly<sup>[33]</sup>.

### Conclusion

Adequate sleep duration, regular exercise, meat intake, reading, and internet use can positively predict recent episodic memory retention among young-old adults. It is recommended to enhance memory function in older adults through: Community-based sleep health management and precision nutritional interventions (e.g., “cognitive-friendly meals”); Promoting internet accessibility and age-appropriate physical activities; Implementing urban-rural differentiated strategies, including “mobile cognitive health stations” in rural areas and digital reading promotion in cities.

These multidimensional interventions integrate lifestyle modification with targeted policies to address memory decline across diverse elderly populations.

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no

### Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# The Efficacy of Guizhi Fuling Capsule or Kuntai Capsule Combined With Diane-35 and Metformin in Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis

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**Abstract: Background:** Polycystic ovary syndrome (PCOS) is one of the most common reproductive endocrine metabolic diseases. Combined use of metformin and diene-35 has better curative effect in regulating serum hormone level (LH, FSH, T and E2) than using metformin alone. Traditional Chinese medicine (TCM) can also be used to treat PCOS. According to some studies, the combined use of metformin and diene-35 and TCM have achieved better curative effect than combining metformin and diene-35 in the treatment of patients with PCOS. **Methods:** Computerized searches of the science, Medline, VIP, Wan Fang and China HowNet (CNKI) databases were conducted to identify eligible randomized controlled trials (RCTs) from the data obtained up to March 1, 2022. The Cochrane Collaboration risk of bias tool was used to assess the risk of bias in individual RCTs, and R software(version 4.0.3) was used for data statistical analysis. **Results:** Nine RCTs involving 1035 patients were included. Comparing to D+M, significant reduce of LH( mean difference [MD]: -1.93, 95% confidence interval [CI]: -3.44, -0.42;Unit:U/L P < 0.01; I2=89%)、 T(MD: -1.44, 95%CI -2.59, -0.30;Unit:nmol/L P < 0.01; I2=98%) and significant increase of E2(MD: 31.43, 95%CI 24.54, 38.33;Unit:pmol/L P < 0.01; I2=96%) were shown in TCM+D+M. Comparing to D+M, TCM+D+M group has higher ovulation rate(RR 1.14 95%CI 1.07,1.22; P=0.42; I2=0%) and higher pregnancy rate(RR 1.29 95%CI 1.15,1.44; P=0.37; I2=7%). There is no significant difference between the two therapies in FSH changes (MD: -1.00, 95%CI -2.27, 0.28;Unit:U/L P < 0.01; I2=95%).Subgroup analysis showed that compared with the Guizhi Fuling capsule group, the Kuntai capsule group had more FSH reduction and E2 increase more. In other outcome indicators, the two subgroup did not show significant differences.**Conclusion:** Kuntai Capsule + Diane-35 + Metformin is better than Guizhi Fuling Capsule in reducing FSH, and it is also better in increasing E2. There was no significant difference between the two in LH and T hormones. There was no significant difference between Kuntai Capsules + Diane-35 + Metformin and Guizhi Fuling Capsules + Diane-35 + Metformin. As for the effect in lessen insulin resistance, Kuntai Capsule+Diane-35+Metformin was significantly better than Guizhi Fuling Capsules+Diane-35+Metformin.

**Keywords:** PCOS; Infertility; Guizhi Fuling Capsule; Kuntai Capsule; Diane-35; Metformin

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## 1.Introduction

Polycystic ovary syndrome (PCOS) is one of the most common reproductive endocrine metabolic diseases, with a range of clinical manifestations such as oligo-anovulation, hyperandrogenism, metabolic abnormalities (insulin resistance and impaired fasting glucose levels), infertility and obesity<sup>[1-6]</sup>, affecting up to 20% of women of all ages and 5% to 10% of women of reproductive age<sup>[5]</sup>. Although PCOS don't cause sudden deaths, it will greatly reduce the quality of life of women with the disease, for it can hardly be cured but to improve symptoms.

According to some studies, insulin resistance (IR) occurs in 70% of the patients with PCOS<sup>[7-9]</sup>. International guidelines for the treatment of polycystic ovary system recommend metformin as the medicine for PCOS patients with IR. Some studies have shown that combined use of metformin and Diane-35 has better curative effect in serum hormone level including Luteinizing hormone (LH), Follicle Stimulating Hormone (FSH), Testosterone (T), Estradiol (E2) than using metformin or Diane-35 alone<sup>[10-12]</sup>.

In recent years, many studies have concentrated on the treatment of PCOS with traditional Chinese medicine (TCM), for instance, Kuntai capsule and Guizhi Fuling capsule have been confirmed effective for reducing the serum FSH and TSH level and ameliorating insulin resistance situation with little toxic side effects<sup>[13-14]</sup>. Moreover, the RCT studies of Ming Luo<sup>[15]</sup>, Haiyan wang et al<sup>[16]</sup> and Xiu Li state that the combine use of Chinese and western medicine shows a better improvement of the PCOS patients in serum hormone level (LH, FSH, T or androgen) or conditions of ovulation and pregnancy than using the western medicine alone.

However, there are few systemic reviews and meta-analysis on the effect of combine use of TCM and western medicine on the treatment of PCOS. Qian-wen Ma et al<sup>[17]</sup> conducted a meta-analysis to conclude co-treatment with TCM and letrozole was more effective than letrozole monotherapy in the treatment of PCOS, but they didn't concentrate on certain kinds of TCM, so the conclusion can be difficult to put into clinical application, for as is well known, the prescription of some TCM can be variable and individual specific<sup>[18]</sup>.

Chinese patent medicine such as Kuntai capsule<sup>[19]</sup> and Guizhi Fuling capsule<sup>[14]</sup> have fixed formulations and recommended dosage, that is to say, the extensive clinical application and promotion of them can have realistic significance. However, up to date, no study has compared the effects of combining use of Kuntai capsule or Guizhi Fuling capsule and western medicine with using western medicine alone.

## 2.Objectives

The primary aim of this study was therefore to undertake a comprehensive systematic review and meta-analysis comparing the effect of Guizhi Fuling capsule/ Kuntai capsule + metformin + Diane-35 with metformin + Diane-35 in PCOS on a range of indexes including serum FSH, LH, E2, T levels and reproductive parameters including ovulation rate and pregnancy rate. We also conduct subgroup analyses to provide evidences for the choice of Guizhi Fuling capsule and Kuntai capsule as the TCM in the combine medication.

## 3.Materials and Methods

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement to conduct our systematic review. The full review protocol was registered with PROSPERO under registration number CRD 42021281360.

### 3.1 Study eligibility criteria

The PICO (population, intervention, comparison, outcome) framework was used to establish a priori selection criteria and included all RCTs comparing (i) Guizhi Fuling capsule + metformin + Diane-35 versus metformin + Diane-35, (ii) Kuntai capsule + metformin + Diane-35 versus metformin + Diane-35 for women of any age with PCOS.

Exclusion criteria: (i) the participants of the study were presence of other etiologies for hyperandrogenism or infertility, such as hypothyroidism, congenital adrenal hyperplasia and Cushing's syndrome, or concurrent medication use (e.g. OCPs, orlistat, clomiphene citrate, etc.) even if the same between groups; (ii) the study did not include the outcome measures we will

be studying; (iii) abstract data from conference and scarce literature; (iv) the quality evaluation of the study shows very poor quality.

Primary outcomes included metabolic parameters including serum hormone levels: Luteinizing hormone (LH), Follicle Stimulating Hormone (FSH), Testosterone (T), Estradiol (E2); and Secondary outcomes included reproductive parameters including ovulation rate and pregnancy rate.

### 3.2 Literature search methodology

English literature was searched in PubMed, web of science and Medline, and the literature in Chinese was searched in VIP, Wan Fang and China HowNet (CNKI) databases. There were no limits on year of publication. Language limit is Chinese and English.

The retrieval condition is as follows: “Chinese medicine”, “Chinese herbal medicine” “traditional Chinese and western medicine”, “Fuling Guizhi capsule/pill”, “Guizhi Fuling capsule/pill”, “Kuntai capsule/pill”, “metformin”, “Diane-35”, “Ethinylestradiol cyproterone”, and “Polycystic ovary syndrome”, “PCOS” were searched in title, key words or abstract.

### 3.3 Study selection

In compliance with the inclusion criteria, we primarily screened the titles and abstracts, then retrieved the full texts of all potentially eligible studies. Two review authors independently examined and selected the eligible articles according to the inclusion criteria for the current review. Disagreement with respect to study eligibility was resolved via discussion with the third reviewer author.

### 3.4 Data extraction

Two review authors independently extracted the data from each eligible study. Any disagreements were resolved by discussion with a third review author. Data retrieved the basic characteristics of included study including sample size, mean age, interventions and duration time, as well as outcome measures. Authors were contacted for additional or missing information as required.

### 3.5 Risk of bias

The risk of bias of the included studies was assessed independently by two authors using the Cochrane Collaboration’s tool for assessing risk of bias with respect to the following aspects: random sequence generation, allocation concealment, blinding of participants or personnel, blinding of outcome assessment, reporting bias, loss to follow up, other sources of bias. Any discrepancies were resolved via discussion with the third review author to reach a consensus.

### 3.6 Statistical analysis

Statistical processing was done using the statistical software package R (<http://www.R-project.org/>). The results were reported as mean difference (MD) with 95% CI for continuous outcomes of serum FSH, LT, E2, T levels and odds ratios (OR) with 95% confidence interval (95% CI) for dichotomous outcomes of ovulation rate and pregnancy rate. Subgroup analyses were performed based on the kind of TCM used in the integrated Chinese and Western medicine group. Publication bias was estimated by Egger’s test<sup>[20]</sup> in addition to funnel plot.

Heterogeneity between studies was examined using the I-square ( $I^2$ ) index to quantify the degree of heterogeneity. If the test results present high heterogeneity ( $I^2 \geq 50\%$ ), meta-analysis is performed using a random effect model; if the experimental results present low-moderate heterogeneity ( $I^2 < 50\%$ ), meta-analysis is performed using a fixed effect model<sup>[21]</sup>.

## 4. Results

### 4.1 Study Selection and Characteristics

In the presence of heterogeneity, the two researchers checked the data entered and explored the variation by conducting sensitivity analysis. In the initial search, 370 relevant articles were identified, of which 130 articles were from CNKI, 92 articles from VIP, 139 articles from Wan Fang, 2 articles were from pubmed, 2 articles from medline, and 5 articles from web of science. After the exclusion of 197 duplicate articles using EndNote X9 software, 173 articles underwent a title and abstract review. A total of 156 studies were excluded in this process, of which 12 articles are review studies, 6 articles are animal research, the intervention methods of 138 articles did not meet the inclusion criteria. For the remaining 17 articles, the full text was reviewed and 8 of them were excluded for following reasons: the dosage form is decoction but not pill or

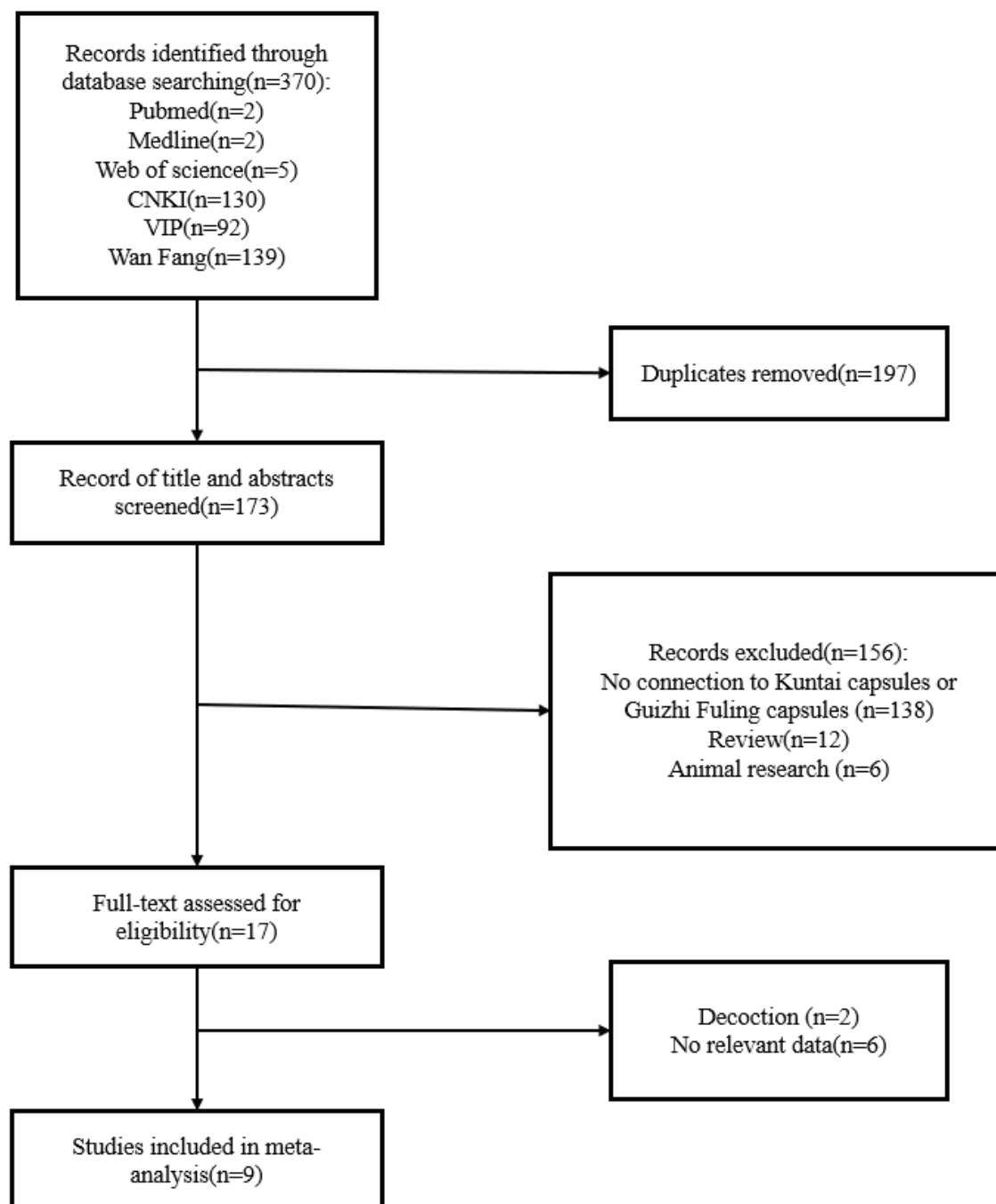
capsule( $n=2$ ), which means that the other herbs may be added to the potion; article does not report the data required for this study ( $n=6$ ). The nine remaining studies fulfilled the eligibility criteria and were included in the meta-analysis<sup>[13,15-16,22-27]</sup>. The literature review and identification process are shown in Figure 1. The meta-analysis evaluated the efficacy of two Chinese medicine preparations combined with Diane-35 and Metformin versus Diane-35 and Metformin for a total of 1035 patients with PCOS across nine clinical studies<sup>[13,15-16,22-27]</sup>. Baseline clinical characteristics of these patients are summarized in Table 1.

Table 1 The characteristics from the eligible studies

Study	Sample size		Mean age(years)		Therapeutic method		Outcome	Duration
	Experi-mental	Con-trol	Experimen-tal	Control	Experimental	Control		
Xiu Li, 2020	40	40	31.17±6.65	30.29±5.31	Diane-35+Met-formin+Cassia twig tuckahoe capsule	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T); adverse reaction(trans-aminase,myocardial enzyme, creatinine, bone density); recrudescence	63 days
Liyun ZHANG, 2016	55	55	30.8±6.9	29.8±6.6	Diane-35+Met-formin+Cassia twig tuckahoe pill	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T, Fins, FBG, TC, TG, HDL-C, LDL-C,); HOMA-IR; ISI; BMI; WHR; acne	Three menstrual cycles
Ying TIAN, 2017	54	53	29.8±4.5	29.3±4.1	Diane-35+Met-formin+Cassia twig tuckahoe capsule	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T), HOMA-IR; HOMA-β, BMI; WHR; polytrichia; Ovarian volume; acne	Three menstrual cycles
Ming LUO, 2019	53	53	26.87±1.69	27.04±1.59	Diane-35+Met-formin+Kuntai capsule	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T); HOMA-IR; HOMA-β	4 months
Haiyan WANG, 2020	80	80	26.50±1.73	26.19±1.54	Diane-35+Met-formin+Kuntai capsule	Di-ane-39+Met-formin	serum sex hormone levels(LH, FSH, E2, T); Ovarian volume; menstrual cycle	63 days
Qionglin LIN, 2016	49	49	26.53±1.46	26.61±1.47	Diane-35+Met-formin+Kuntai capsule	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T); HOMA-IR; HOMA-β; Dizziness; headache; nausea; vomiting; skin irritation; breast tender	84 days
Hongmei Xv, 2020	52	52	26.96±1.49	26.80±1.45	Diane-35+Met-formin+Kuntai capsule	Di-ane-35+Met-formin	serum sex hormone levels(LH, FSH, E2, T); HOMA-IR; HOMA-β; polytrichia; acne;	Three menstrual cycles
Tong-shan HU, 2017	30	30	33.15±5.21	33.19±5.10	Diane-35+Met-formin+Kuntai capsule	Di-ane-35+Met-formin	HOMA-β; serum sex hormone(FSH); ovulation rate; pregnancy rate	100 days

Study	Sample size		Mean age(years)		Therapeutic method		Outcome	Duration
	Experimental	Control	Experimental	Control	Experimental	Control		
Yan LIU, 2017	105	105	29.45±9.41	29.23±9.09	Diane-35+Metformin+Kuntai capsule	Diane-35+Metformin	serum sex hormone levels(LH, FSH, E2, T)	4 months

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram of the search process.

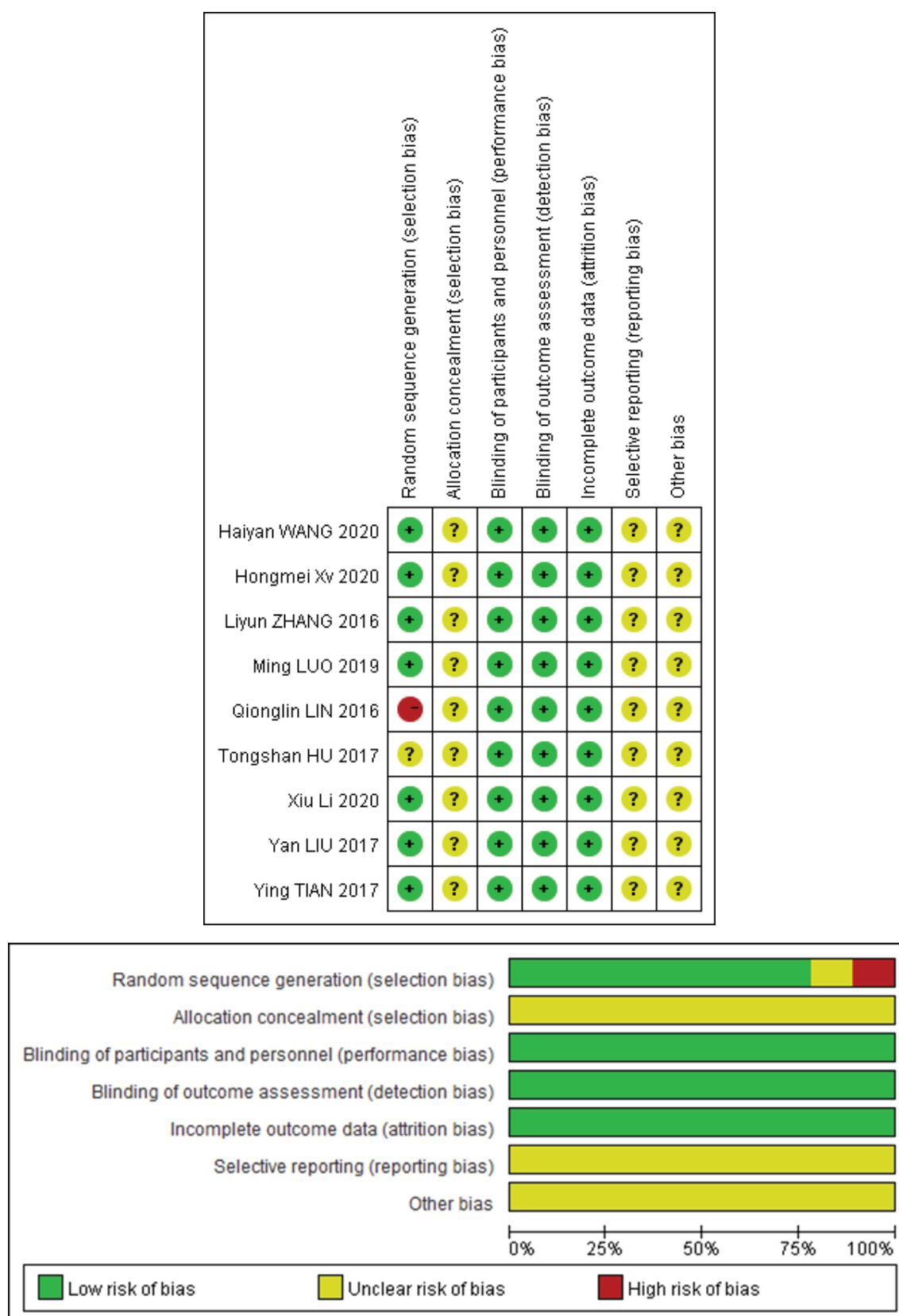


## 4.2 Quality Assessment

Trial quality was assessed using Cochrane risk of bias tool. In the nine studies, only one study were assessed as high risk of bias and no study were assessed as low risk of bias (Figure 2).



Figure 2. Assessment of risk of bias of included RCTs

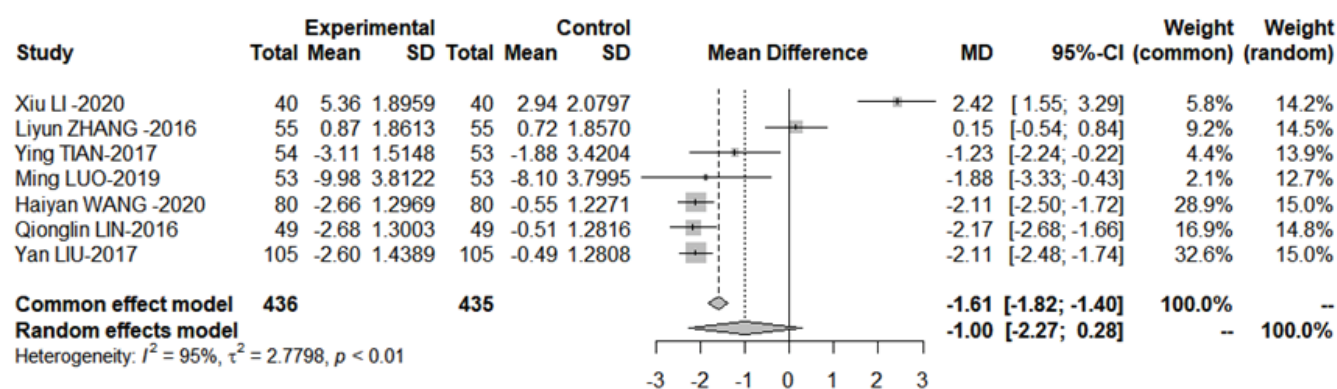


### 4.3 Anthropometric parameters

#### 4.3.1 Follicle Stimulating Hormone(FSH)

Seven studies reported the change in FSH involving 871 patients. There is no significant difference between the two therapies in FSH changes (MD:-1.00, 95%CI -2.27, 0.28;Unit:U/L;  $P < 0.01$ ;  $I^2=95\%$ )( Figure 3) .

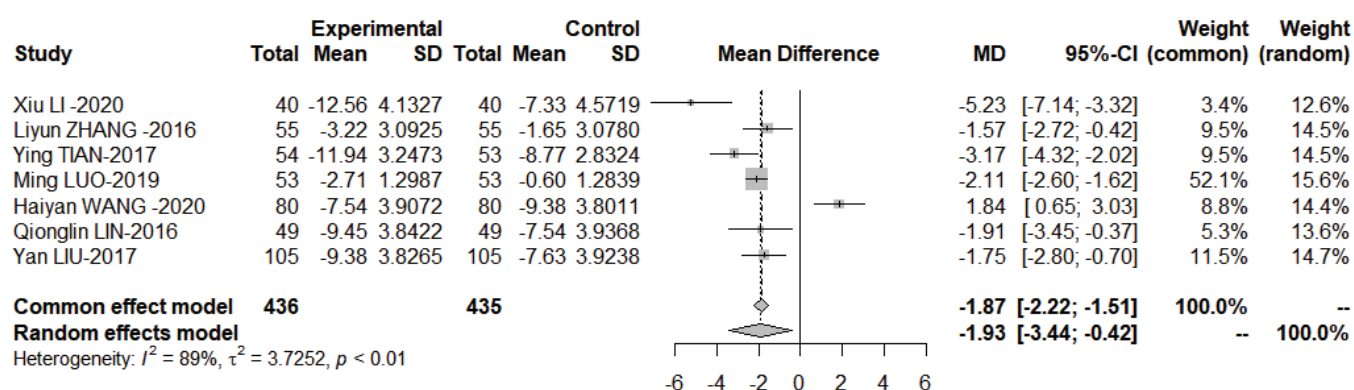
Figure 3 Meta-analysis of TCM +D+M versus D+M for FSH(U/L). CI, confidence interval.



### 4.3.2 Luteinizing hormone(LH)

Seven studies reported the change in LH involving 871 patients. Comparing to D+M, TCM +D+M significantly reduced LH(MD: -1.93, 95%CI -3.44, -0.42;Unit:U/L;P < 0.01;  $I^2=89\%$ )( Figure 4) .

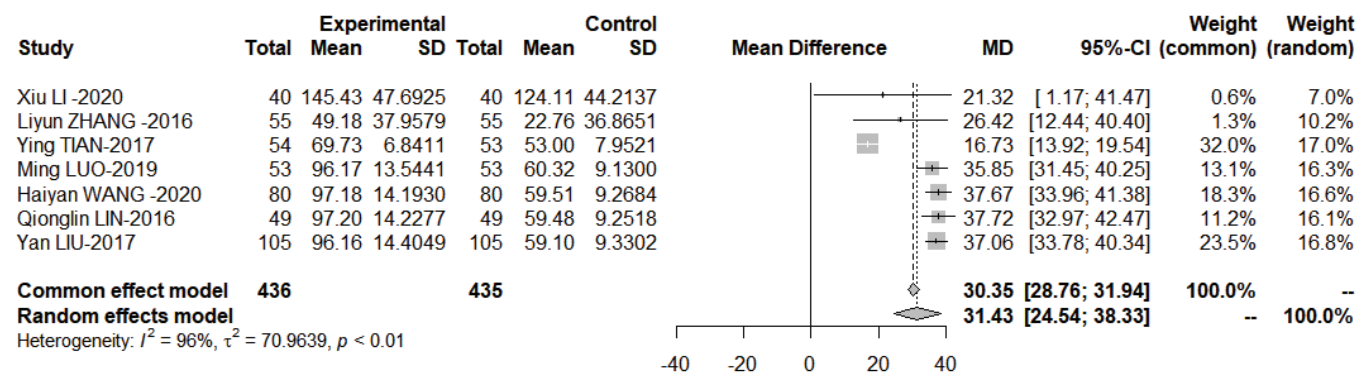
Figure 4 Meta-analysis of TCM+D+M versus D+M for LH(U/L). CI, confidence interval.



### 4.3.3 Estradiol(E2)

Seven studies reported the change in LH involving 871 patients. Comparing to D+M, TCM+D+M significantly increased E2(MD: 31.43, 95%CI 24.54, 38.33;Unit:pmol/L;P < 0.01;  $I^2=96\%$ )( Figure 5) .

Figure 5 Meta-analysis of TCM +D+M versus D+M for E2(U/L). CI, confidence interval.

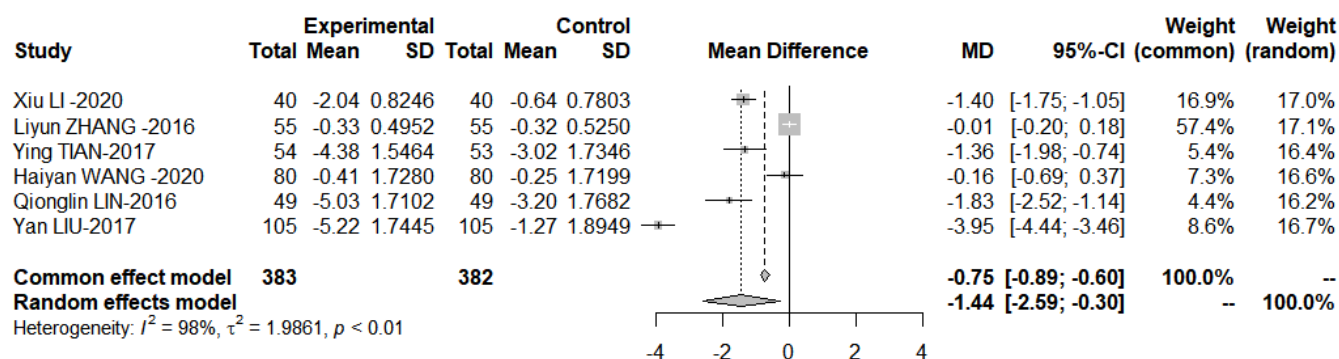


### 4.3.4 Testosterone(T)

Six studies reported the change in T involving 765 patients. Comparing to D+M, TCM +D+M significantly reduced T(MD: -1.44, 95%CI -2.59, -0.30;Unit:nmol/L ;P < 0.01;  $I^2=98\%$ )( Figure 6) .



Figure 6 Meta-analysis of TCM +D+M versus D+M for T(nmol/L). CI, confidence interval.

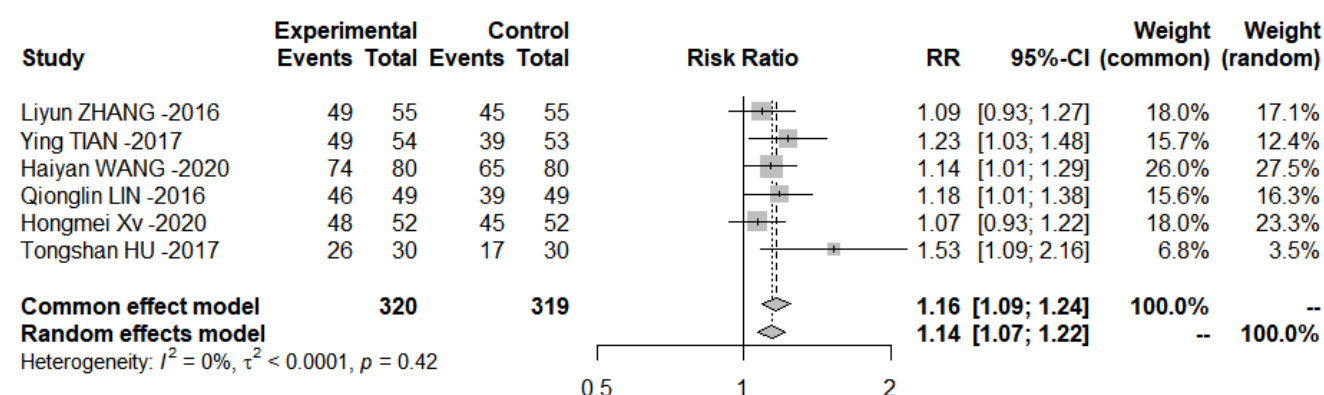


## 4.4 Clinical outcomes

### 4.4.1 Ovulation rate

Six studies were included for the meta-analysis of ovulation rate involving 639 PCOS patients. Comparing to D+M, TCM +D+M group has higher ovulation rate(RR:1.14 95%CI 1.07,1.22; P=0.42;  $I^2=0\%$ )( Figure 7).

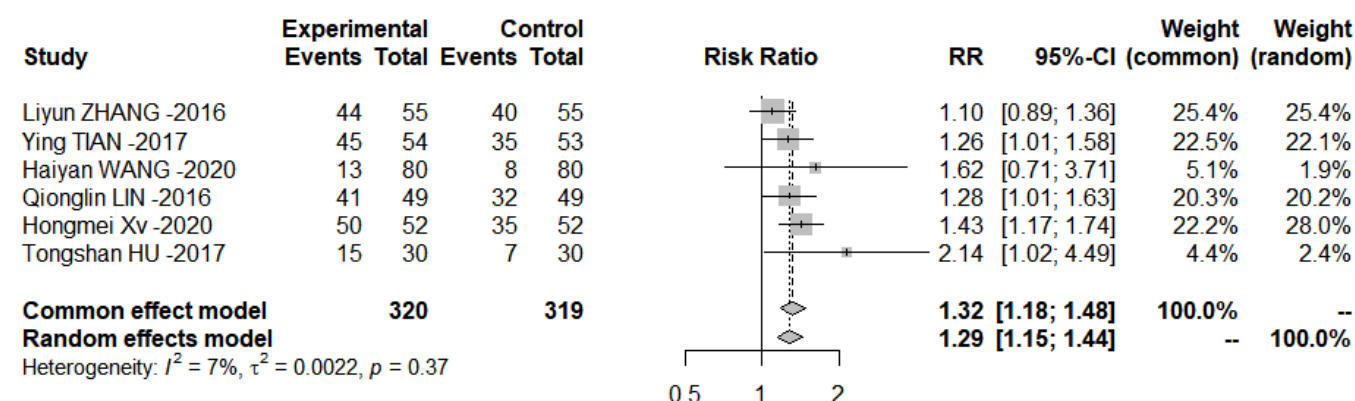
Figure 7 Meta-analysis of TCM+D+M versus D+M for ovulation rate(%). CI, confidence interval.



### 4.4.2 Pregnancy rate

Six studies were included for the meta-analysis of ovulation rate involving 639 PCOS patients. Comparing to D+M, TCM +D+M group has higher pregnancy rate(RR : 1.29 95%CI 1.15,1.44;P=0.37;  $I^2=7\%$ )( Figure 8).

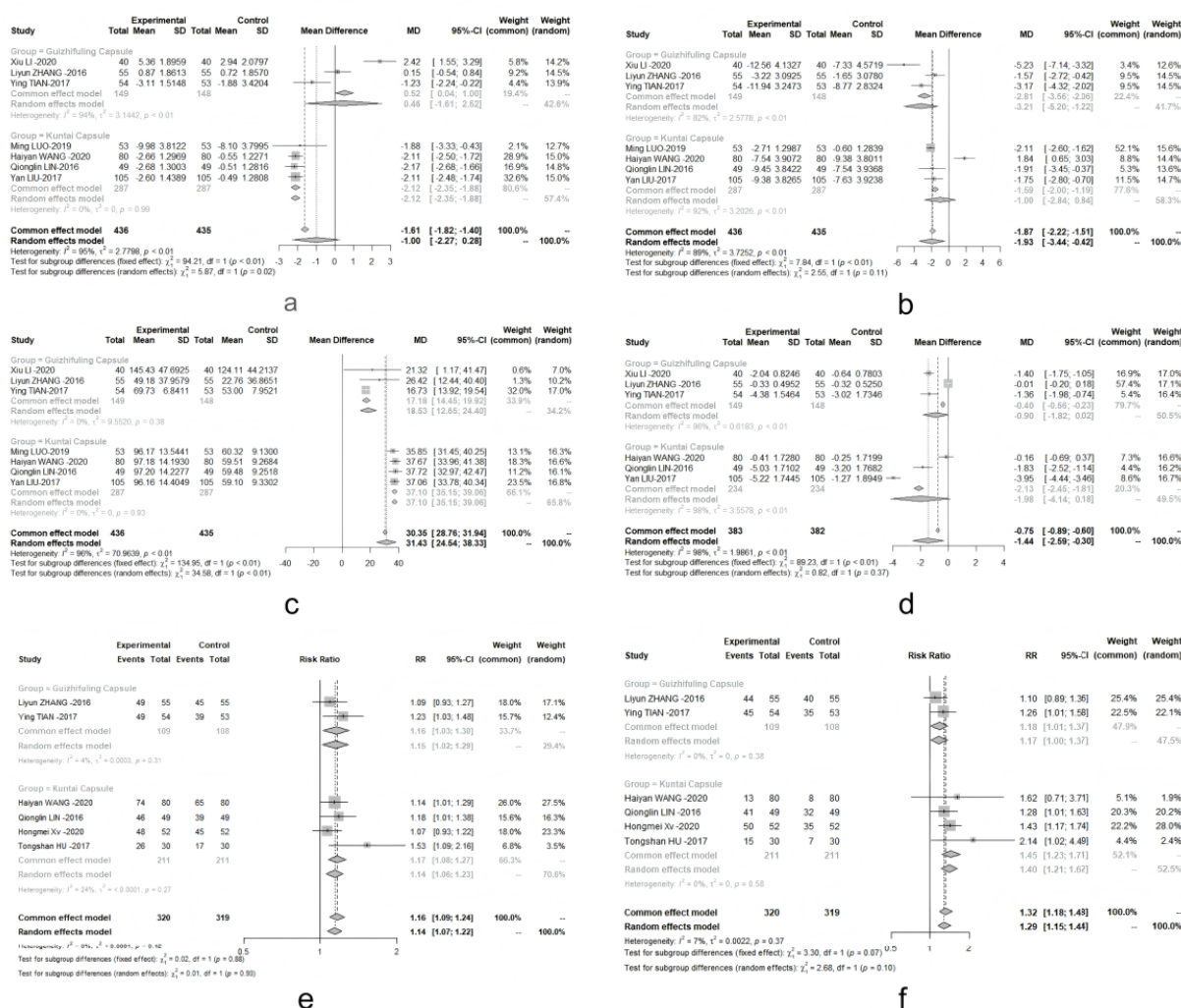
Figure 8 Meta-analysis of TCM+D+M versus D+M for pregnancy rate(%). CI, confidence interval.



## 4.5 Subgroup analysis

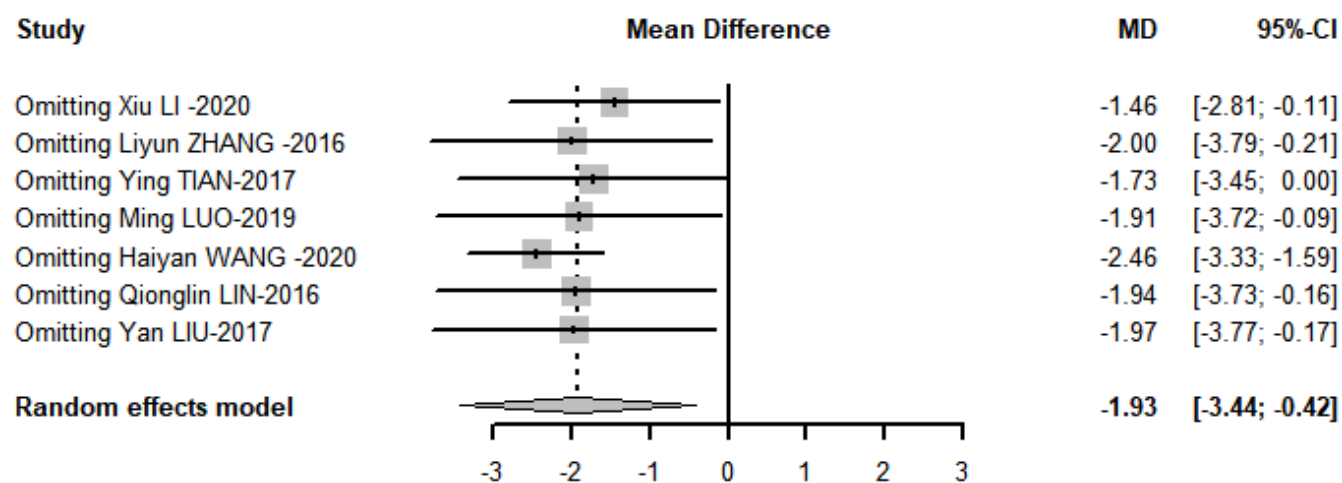
To analyze the difference in efficacy of the two kinds of traditional Chinese medicine (Guizhifuling Capsule and Kuntai Capsule), we conducted subgroup analysis of all outcome indicators(Figure 9). Compared with the Guizhi Fuling capsule group, the Kuntai capsule group had more FSH reduction and E2 increase more, suggesting that Kuntai capsule may have better efficacy than Guizhi Fuling capsule. In other outcome indicators, the two groups did not show significant differences.

Figure 9 Subgroup analysis of G+D+M versus K+D+M for LSH(a), LH(b), E2(c), T(d), ovulation rate(e), and pregnancy rate(f). CI, confidence interval



## 4.6 Sensitivity Analysis

Figure 11 Sensitivity analysis of LH



#### 4.7 Publication Bias

The publication bias of the pooled LH (Egger's test:  $P = 0.9776$ ), pooled pregnancy rate (Egger's test:  $P = 0.2543$ ) were examined with funnel plot and Egger's regression tests (Figure 12-15). The results did not reveal evident publication bias in LH and pregnancy rate.

Figure 12 Funnel plot of LH

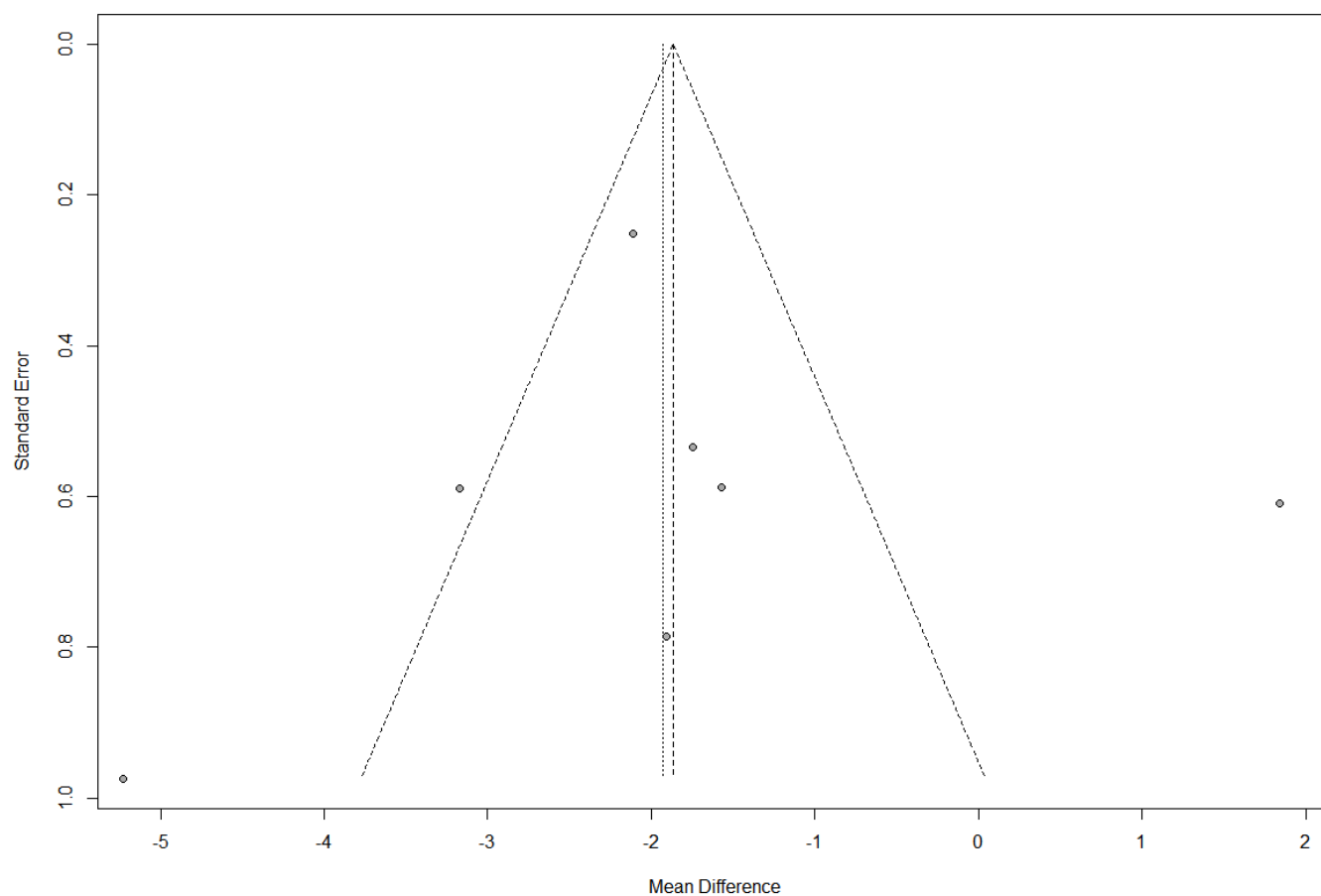


Figure 13 Egger's test of LH

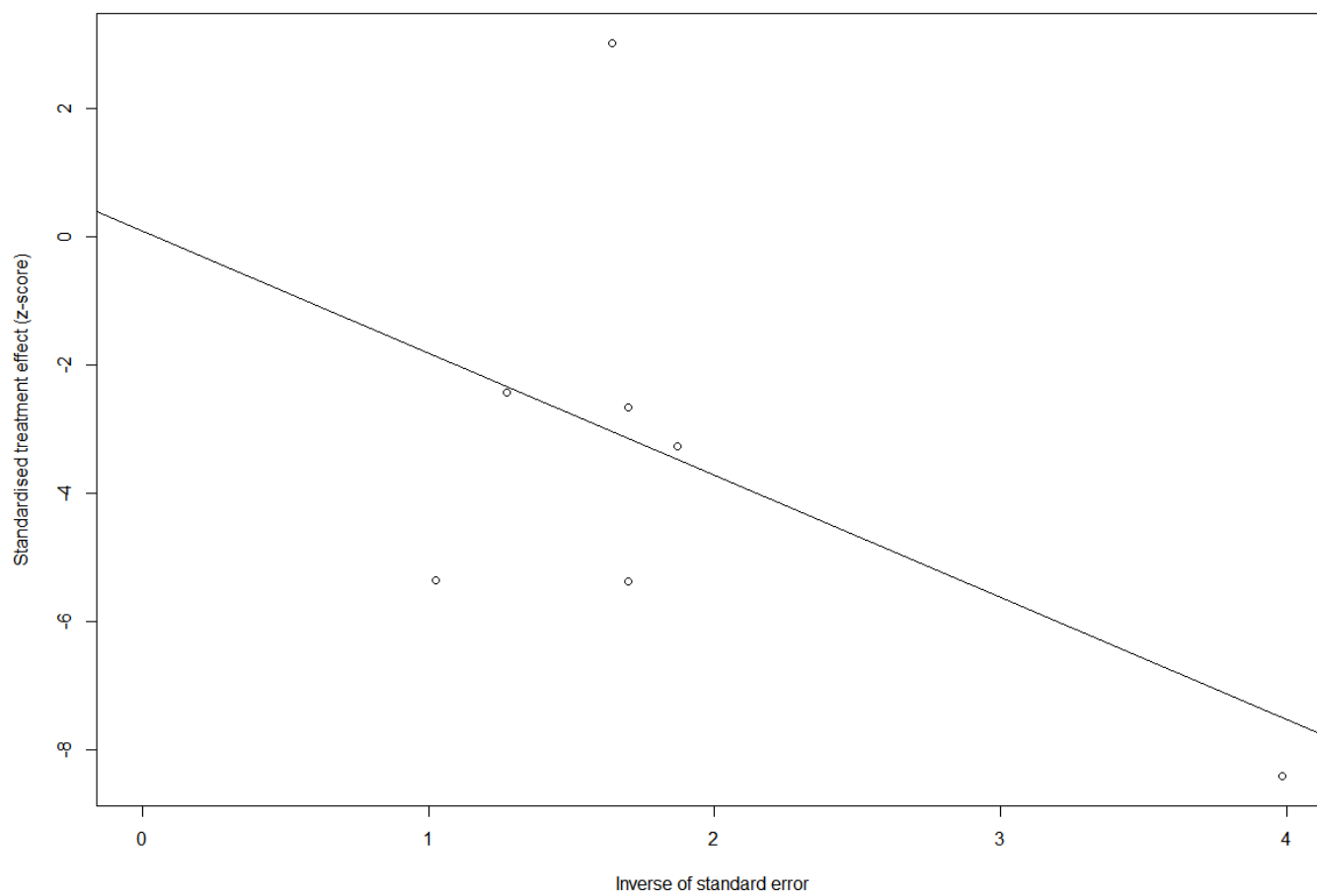


Figure 14 Funnel plot of pregnancy rate

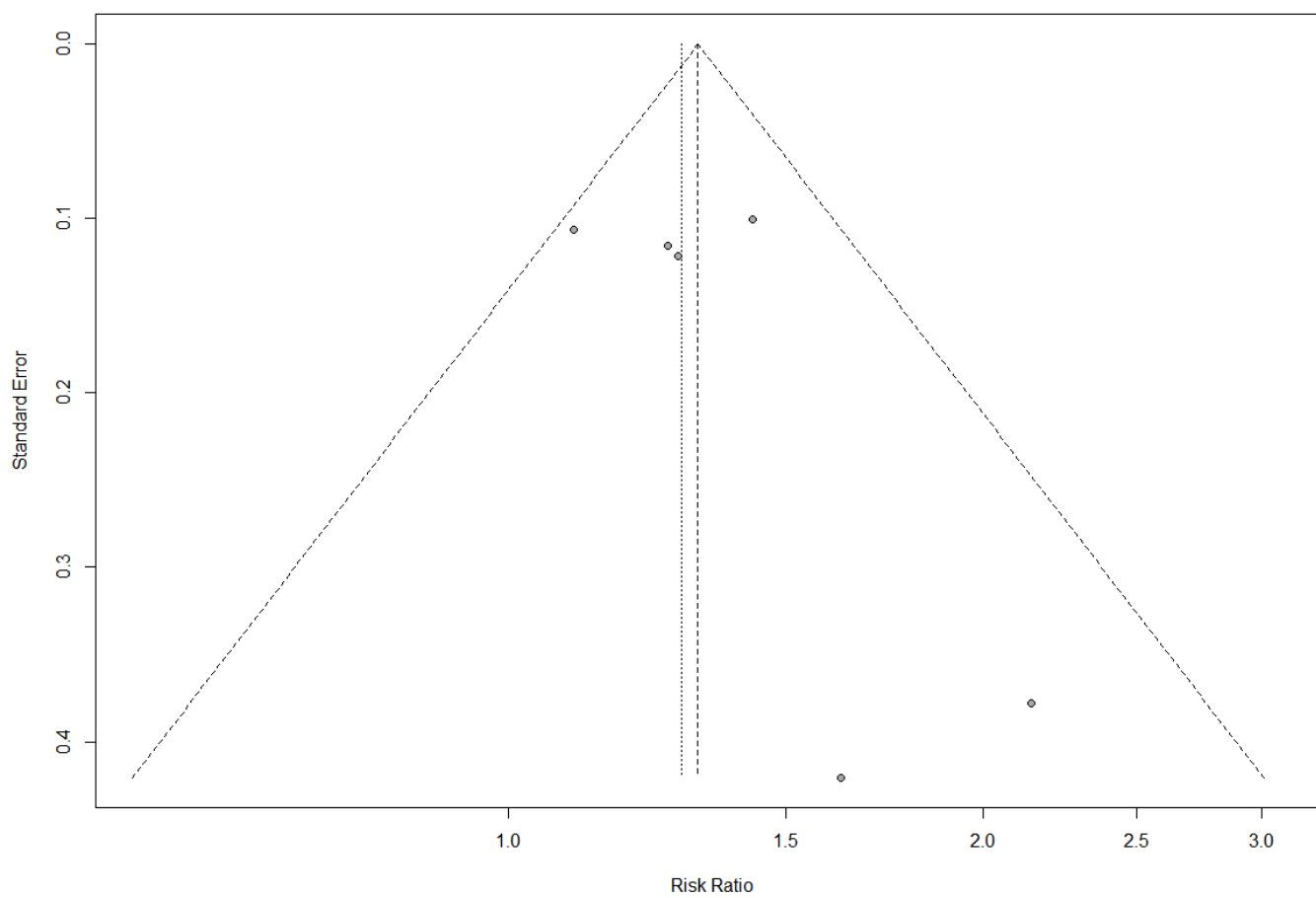
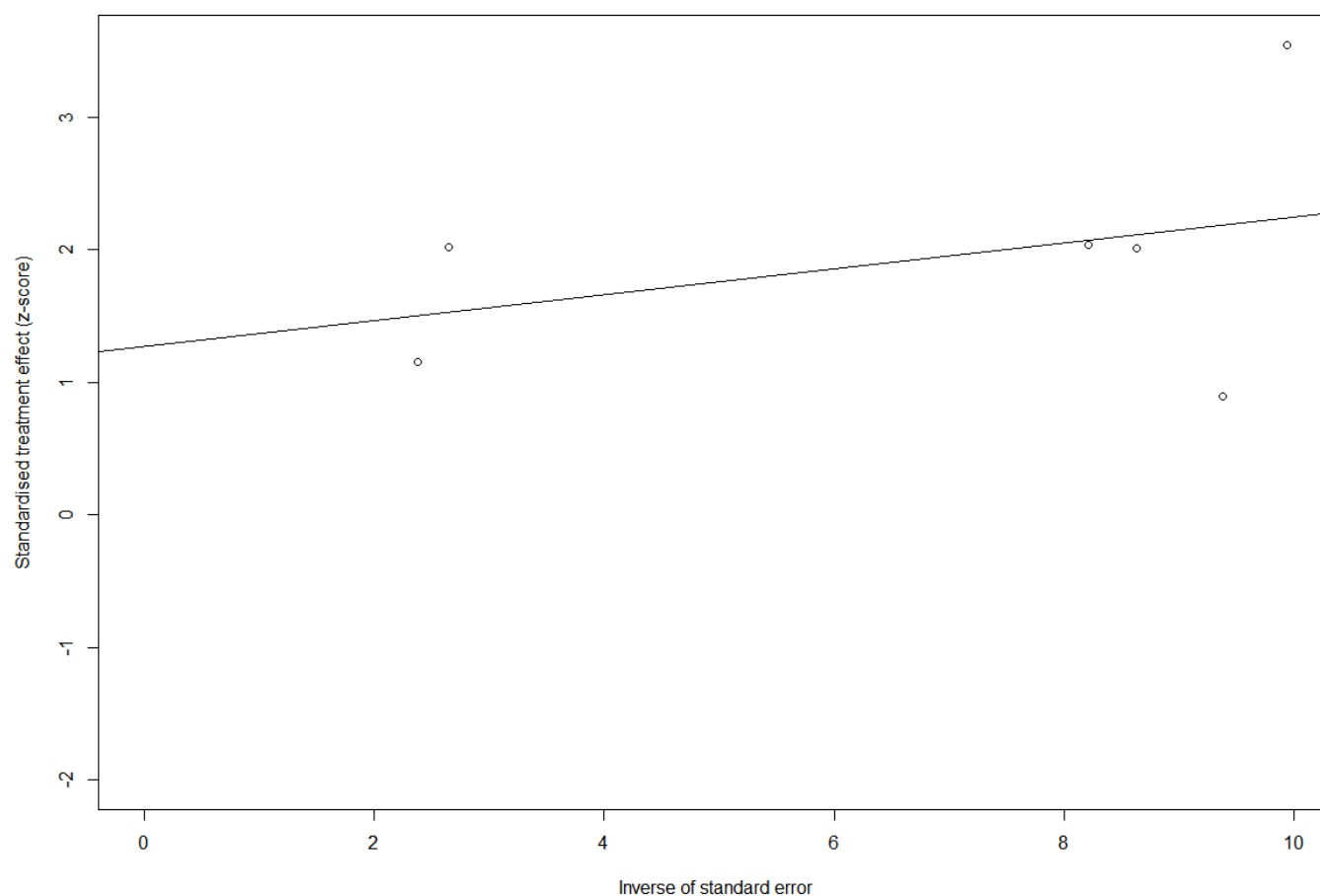


Figure 15 Egger's test of pregnancy rate



## 5. Discussion

Polycystic ovary syndrome (PCOS) is the most common kind of endocrine disorder characterized by chronic anovulation, hyperandrogenism (HA) and insulin resistance (IR) and adversely affects the normal life of 6% to 20% of women in reproductive age globally<sup>[28-29]</sup>.

Some studies found that the excessive androgen lead to the inhibition of hypothalamic feedback, in other words, the elevated level of LH in serum cannot inhibit the further secretion of gonadotropin-releasing hormone (GnRH), which subsequently lead to the growing amount of LH<sup>[30-31]</sup>. Excessive LH and low-dose FSH continuously stimulate the ovary, which contributing to the arrest of the growing of the dominant follicle.

However, the exact pathogenesis of PCOS still remains unclear, which result in the treatment strategy of this disease is dominated by symptomatic treatment, including weight control, life-style modification for the mild case and the regulation of menstrual cycle, anti-hyperandrogenemia (HA) therapy, management of insulin resistance (IR) and metabolic disorders for the severe one<sup>[32-34]</sup>. One of the first line medication, Oral contraceptive pills (OCPs), have long been used to reduce HA level in serum and to regulate menstrual cycles in women with PCOS<sup>[35-36]</sup>. OCPs not only possess the ability to increase the production of sex hormone-binding globulin (SHBG) to reduce serum free testosterone (T) hormone, the precursor of androgen, but also can reduce LH and androgen secretion by inhibiting hypothalamic gonadotropin<sup>[37]</sup>. Diane-35, ethinyl estradiol (EE) plus cyproterone acetate (CPA), is the representative of OCPs and widely being used in China, Australia and Europe<sup>[38]</sup>.

As a biguanide used for the standard first-line treatment of type-2 diabetes mellitus, Metformin is applied in clinics to handle IR of patients with PCOS, it can enhance insulin sensitivity both in the liver and in the peripheral tissue through inhibiting hepatic glucose production and promoting uptake and utilization, respectively<sup>[39-41]</sup>. A meta-analysis including 12 RCTs compared the effect of metformin +lifestyle change with placebo +lifestyle change, the result of which indicated that the former demonstrated more admirable effect in lowering BMI (MD: 20.73 kg/m<sup>2</sup>, 95% CI: 21.14, 20.32) and decreasing

subcutaneous adipose tissue (MD :292.49 cm<sup>2</sup> , 95% CI: 2164.14, 220.84) and increasing the number of menstrual cycles (MD :1.06, 95% CI :0.30, 1.82) than the latter after 6 months intervention<sup>[42]</sup>. Another meta-analysis focused on the Effect of metformin in overweight women also come to the similar conclusion that metformin reduces BMI, FSH, LH, low density lipoprotein (LDL), cholesterol and testosterone level in individuals with PCOS<sup>[43]</sup>.

An systematic review published in 2008 compared the efficacy of Diane-35 and metformin with Diane-35 alone, the result of which indicated that Diane-35 and metformin combination showed better result than Diane-35 monotherapy in lowering BMI(WMD 1.05; 95% CI 0.09, 2.01) in patients with PCOS<sup>[38]</sup>.

Some studies hypothesized that obesity might be more responsible than the elevated androgen level for the development of anovulation in PCOS and obesity induced metabolic abnormalities are closely related to insulin resistance(IR)<sup>[44- 47]</sup>. When IR occurs in individuals, the function of insulin cannot be exerted which results in the synthesis and secretion of insulin from pancreatic cell being increased in a compensatory way<sup>[48]</sup>. Hyperinsulinemia further deteriorates androgen-dependent anovulation via two main pathways<sup>[48]</sup>. One is that insulin indirectly accelerates the androgen production through enhancing the effects of LH on ovarian theca cells<sup>[49-50]</sup>. Another is that insulin inhibits the release of sex hormone-binding globulin (SHBG) from the liver, therefore the amount of bio-active androgen (unbound with SHBG) in blood circulation will increase<sup>[51-52]</sup>. The excess androgen lead to menstrual irregularity, which in turn inhibit follicular maturation<sup>[53]</sup>. Thus, obesity and IR will deteriorate hyperandrogenism in this feed-back circle, in addition to directly control the level of androgen, the active management of IR induced by obesity is of vital importance in the treatment of PCOS.

Western medicine treatment is mainly to adjust the menstrual cycle, improve ovulation, but none of them can permanently relieve the symptoms of patients, and even lead to ovarian overstimulation and low pregnancy rate<sup>[54-56]</sup>. Moreover, metformin frequently induces gastrointestinal discomfort in patients, and in some cases even causes hypoglycemia, as dangerous as hyperglycemia, when uptake overdose or in fasting circumstances<sup>[57-58]</sup>.

Therefore, we intended to investigate whether the addition of Traditional Chinese Medicine(TCM) to metformin and Diane-35 would improve the clinical outcome of patients with PCOS.

TCM possess two major characteristic distinct from western medication, holistic therapy and multisystem regulation<sup>[59]</sup>.

In viewpoint of traditional Chinese medicine (TCM), the disorders of the kidneys, liver and spleen might result in the occurrence of PCOS, the deficiency in kidney in particular, since kidney is believed to govern reproductive function of humans<sup>[60-62]</sup>. When the function of the liver is impaired, the blood circulation and the menstrual cycle of an individual will be adversely affected, while the condition of spleen will influence body type, obesity might occur if it doesn't perform well<sup>[63]</sup>.

Guizhi fuling capsule (GF) is one of the most frequently prescribed Chinese herbal formulas for treating uterine fibroids and widely accepted for the management of gynaecological conditions<sup>[64]</sup>. The ingredients of guizhi fuling capsule consist of the following 5 herbs: Cinnamomum cassia (L.) J. Presl (Guizhi), Poria cocos (Schw.) Wolf (Fuling), Paeonia lactiflora Pall. (Chishao), Juglans regia L. (Taoren), and Paeonia suffruticosa Andr. (Mudanpi)<sup>[65]</sup>.

Some studies indicated that GF might ameliorate PCOS-IR through regulating related target proteins and pathways in hormone generation and secretion<sup>[66-68]</sup>. The function of GF is not limited to alleviating IR resistance , reducing fasting blood glucose together with insulin and correcting abnormal lipid metabolism, can also shorten the menstrual cycle, restore ovulation, and improve the pregnancy rate<sup>[69-71]</sup>.

Another kind of the traditional Chinese medicine, Kuntai capsule (KT), consists of prepared Dihuang (Rehmannia glutinosa (Gaertn.) DC.), Huanglian (Rhizoma Coptidis), Baishao (Radix Paeoniae Alba), Huangqin (Radix Scutellariae Baicalensis), Ejiao (Colla Corii Asini), and Fuling (Poria)<sup>[72]</sup>. The result of a meta-analysis demonstrated that Kuntai capsule has better performance in regulating serum sex hormone levels including LH, LH/FSH and T than placebo in patients with PCOS<sup>[72]</sup>.

In the mechanism analysis of the modern pharmacology, the component dang gui and baishao may reduce the release of insulin and androgen through phosphatidylinositol 3-hydroxy kinase(PI3K)/protein kinase B (AKT)/glucose transporter 4 (GLUT4) signal pathway and oxidative stress<sup>[73-75]</sup>.

Studies have shown that traditional Chinese medicine can inhibit the apoptosis and excessive autophagy of ovarian granulosa cells, reduce follicular atresia, improve ovarian reserve function, regulate serum endocrine hormone levels, restore the



number of ovarian granulosa cells, so as to maintain ovarian function and promote follicle development<sup>[76-77]</sup>.

The pooled results indicated that the efficacy of TCM+ Diane-35+ metformin was significantly superior to that of Diane-35+ metformin in lowering LH level (MD: -1.93, 95%CI: -3.44, -0.42; Unit: U/L;  $P < 0.01$ ;  $I^2=89\%$ ) and T level (MD: -1.44, 95%CI: -2.59, -0.30; Unit: nmol/L;  $P < 0.01$ ;  $I^2=98\%$ ) and increasing Estradiol (E2) (MD: 31.43, 95%CI: 24.5, 38.3; Unit: pmol/L;  $P < 0.01$ ;  $I^2=96\%$ ), Ovulation rate (RR 1.14 95%CI: 1.1, 1.2;  $P=0.42$ ;  $I^2=0\%$ ) and Pregnancy rate (RR 1.29 95%CI: 1.2, 1.4;  $P=0.37$ ;  $I^2=7\%$ ).

A similar result can be found in a systematic review, which concludes that traditional Chinese medicine (TCM) has the competence to significantly increase the pregnancy rate and the ovulation rate in participants struggling with infertility due to anovulation<sup>[78]</sup>.

In attempt to find out whether there was some difference towards the type of TCM, we conducted subgroup analysis stratified studies in according to the type of TCM. KT achieved better performance in increasing Estradiol (E2) (pooled MD: 31.4, 95%CI: 24.5, 38.3, Unit: pmol/L,  $P<0.01$ ;  $I^2 = 95.6\%$ ) and lowering FSH (pooled MD: -1.00, 95%CI: -2.27, 0.28; Unit: pmol/L,  $P=0.02$ ;  $I^2 = 95\%$ ) in comparison with GF.

Only one study reported the incidence of drug related adverse events<sup>[23]</sup>, as mentioned by which, there were no severe adverse events in both groups and occasionally some patients developed slight increase in transaminase, myocardial enzyme and creatinine, which return to normal after two weeks. As a consequence, the pooled analysis is only conducted to the efficacy, while toxicity hasn't undergone pooled analysis.

## 6. Limitation

Limitation of our studies include: first, the included studies have not reported the diagnostic criteria and important outcome indicator like the live birth rate and adverse events, which result in the limited information was obtained in this meta-analysis; Second, although we conducted comprehensive search, included studies were all carried out in China, the result of the current meta-analysis might cannot be applied to the other part of the world<sup>[79]</sup>; last, we suppose there might exist methodological flaws since neither of them reported the calculation of sample size in the initiation and the drop-out rate in the end.

## 7. Conclusion

The results of our systematic review and meta-analysis validated the efficacy of TCM including GF and KT in the treatment of patients with PCOS. TCM containing therapy might be a potential option to enhance the efficacy of metformin and Diane-35. However, since the number of eligible studies is limited and most of them are relatively moderate quality, the results should be interpreted with caution. To provide stronger evidence for the application of GF or HYKT-containing TCM in the treatment of PCOS, more high quality randomized controlled trials are needed.

## Funding

no

## Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# Research Progress on Detection Technologies for *Pseudomonas Aeruginosa*

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogen widely distributed in the natural environment, which can cause a variety of infections, especially in people with low immunity and high pathogenicity. In recent years, significant progress has been made in the detection technology of *Pseudomonas aeruginosa*, covering traditional methods, molecular biology techniques, immunological methods and automated detection systems. Traditional methods such as the national standard method and the filter membrane method are easy to operate, but have the problems of long time consuming and limited sensitivity. Molecular biological techniques (such as PCR, gene cloning) and immunological methods (such as ELISA, colloidal gold immunochromatography) have significantly improved the sensitivity and specificity of detection, but they require high equipment and technology, and are expensive. Automated detection systems, such as VITEK 2 Compact and AutoMS 1000 mass spectrometry identification system, are excellent in improving detection efficiency and accuracy, but their high cost and complex operation process limit their wide application. In addition, the resistance of *Pseudomonas aeruginosa* to bacteriostatic agents further increases the difficulty of detection. In this paper, the development and application of immunological detection technology, molecular biological technology and immunological technology of *Pseudomonas aeruginosa* were reviewed, and the principles, advantages, disadvantages and research progress of various detection technologies of *Pseudomonas aeruginosa* were described, and the future development trend was prospected, in order to provide reference for the optimization and development of detection methods of *Pseudomonas aeruginosa*.

**Keywords:** *Pseudomonas Aeruginosa*; Detection Technology; Molecular Biology; Immunology; Automated Detection

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*Pseudomonas aeruginosa* is a Gram-negative bacterium widely distributed in nature. It belongs to the family *Pseudomonas*, and is commonly found in soil, water, plant surface, and hospital environment. The cell is rod-shaped and its width is about 0.5-1.0um and its length is about 1.5-5.0um. Unlike *Salmonella*, *Pseudomonas aeruginosa* does not usually have a capsule, but is able to produce a polysaccharide substance called alginate that forms a biofilm that helps the bacteria survive in harsh environments. *Pseudomonas aeruginosa* has unipolar flagella, which are motile, and has pili that help it attach to the host cell surface. *Pseudomonas aeruginosa* is not highly nutrient demanding, can grow under simple carbon and nitrogen source conditions, and can grow in the temperature range of 4 °C to 42 °C and the pH range of 5.5 to 8.5.

*Pseudomonas aeruginosa* is an important opportunistic pathogen that mainly infects people with low immunity. It can

cause a variety of infections, including respiratory tract infection, urinary tract infection, wound infection, sepsis, etc. Its pathogenicity is related to A variety of virulence factors produced by it, such as exotoxin A, elastase, phospholipase C, etc., which can damage host cells and tissues and lead to the spread of infection.

## 1. Detection method

### 1.1 National Standard Law

GB/T 8538-2008 "Inspection Methods for Drinking Natural mineral Water" stipulates the detection process of *Pseudomonas aeruginosa*, mainly including sample filtration, selective culture (such as Cetrimide AGAR medium), colony morphology observation and biochemical identification (such as oxidase test, pigment production test, etc.). The results were confirmed by selective medium inhibition of other bacterial growth combined with specific biochemical reactions of *Pseudomonas aeruginosa*. The determination of the test results was based on whether the bacteria were detected in accordance with the characteristics, and *Pseudomonas aeruginosa* in drinking natural mineral water should not be detected. In the detection process, it is necessary to strictly aseptic operation, ensure the quality of medium and culture conditions to ensure the accuracy of results<sup>[1]</sup>.

According to the provisions of "National Standard for Food Safety Packaged Drinking Water" (GB 19298-2014), *Pseudomonas aeruginosa* could not be detected in any of the five packaged samples. *Pseudomonas aeruginosa* shows a certain tolerance to disinfection methods commonly used in the production of packaged drinking water (such as ozone, ultraviolet light and chlorine dioxide)<sup>[2-3]</sup>, which makes it difficult to completely eliminate possible *Pseudomonas aeruginosa* in the water, resulting in products that do not meet the standards. Although the content of *Pseudomonas aeruginosa* detected by enterprises in the early stage of production may be extremely low, due to the long shelf life of packaged drinking water, the bacteria may multiply in large numbers during transportation and sales, and the concentration may even reach  $10^4$  CFU/mL, which will also lead to unqualified products<sup>[4]</sup>.

In the National standard for Food Safety - Quality Requirements for Food microbiological Test Media and Reagents (GB 4789.28-2013), the detection content of *Pseudomonas aeruginosa* mainly includes the following aspects: The medium and reagents used for detection should meet the quality requirements in the standard to ensure their applicability and reliability. The medium should have good selectivity and discrimination, which can effectively inhibit the growth of other microorganisms and promote the growth and characteristics of *Pseudomonas aeruginosa*. The standard specifies the detection steps, including sample processing, inoculation, culture and identification, etc. The commonly used medium is Cetrimide Agar (cetyl trimethylammonium bromide AGAR), which is selective for *Pseudomonas aeruginosa*<sup>[5]</sup>. The culture conditions are usually 30-35°C for 24-48 hours. After cultivation, it is necessary to observe the characteristics such as colony morphology and color. *Pseudomonas aeruginosa* usually shows green or blue-green colonies on Cetrimide Agar, and may produce fluorochrome. After the initial identification, it needs to be further confirmed by biochemical tests (such as oxidase test, pigment production test, etc.), and molecular biological methods (such as PCR) can be used if necessary. According to the test results, whether *Pseudomonas aeruginosa* was detected in the sample was reported, and the colony count and identification results were recorded. The specific operation should strictly refer to the detailed provisions of GB 4789.28-2013 to ensure the accuracy and reliability of the test.

### 1.2 Filter membrane method

The detection method adopted the national standard filter membrane method. The water sample was filtered by aseptic operation, so that bacteria were trapped on the filter membrane, and then the filter membrane was attached to *Pseudomonas* AGAR medium for culture, and the number of typical colonies was counted. The method is simple and sensitive, and is suitable for the detection of *Pseudomonas aeruginosa* in drinking water.

Mai Miao proposed that sample collection is the key step of detection<sup>[6]</sup>. When sampling bottled water, it is necessary to disinfect the barrel wall and sampling port with disinfectant water to ensure that the sampling container and apparatus are sterile and avoid cross contamination. The sampling personnel need to sterilize their hands, and shake the water sample sufficiently before sampling to make the bacteria evenly distributed and ensure the uniformity of sampling. The quality control of medium is the core to ensure the accuracy of detection. The selection, preparation and use of media should be

strictly in accordance with the “National standard for Food Safety: Quality Requirements for Culture Media and Reagents for Microbiological Examination of Food” (GB 4789.28-2013) to ensure that the proportion of nutrients and pH value meet the standards. Laboratories should formulate quality control standards and quality control instructions for medium, and strengthen the quality control of compounding water and verification reagents. The preparation method, sterilization method and storage conditions of the medium should be standardized to ensure the stability of its quality.

Special attention should be paid to aseptic operation during inoculation and culture. The filter membrane should be tightly attached to the medium to avoid air bubbles, so as to ensure that *Pseudomonas aeruginosa* can fully contact the medium and grow. The temperature and humidity of the incubator should be reasonably controlled to provide a suitable growth environment for *Pseudomonas aeruginosa* and improve the accuracy of strain counting within a suitable environmental range<sup>[7]</sup>. The accuracy and reliability of *Pseudomonas aeruginosa* detection can be effectively improved by strictly regulating sample collection, medium quality control and inoculation and culture operations<sup>[5]</sup>.

### 1.3 Construction and expression of OprF prokaryotic expression vector

The experimental method is based on PCR amplification and gene cloning technology. First, genomic DNA is extracted from *Pseudomonas aeruginosa*, the target gene fragment is amplified by specific primers, and the amplified products are separated and purified by agarose gel electrophoresis. The purified gene fragment was ligated into the cloning vector and transformed into host cells, such as *E. coli* DH5 $\alpha$  competent cells. Positive clones containing target gene fragments were obtained by antibiotic screening and identification by PCR or enzyme digestion<sup>[8]</sup>. The positive clones were screened and the recombinant expression plasmid was constructed. The target gene fragment was digested from the cloning vector and ligated into the expression vector (e.g., pET28b). The recombinant plasmids were transformed into competent cells, and positive clones were selected by medium containing antibiotics and further verified by PCR and enzyme digestion. Positive clones were inoculated into the medium containing the inducer to induce expression of the target protein. The expressed cells were separated by ultrasonic fragmentation and centrifugation to obtain the supernatant and precipitate, and then the target protein was purified by affinity chromatography (such as Ni-NTA). Finally, SDS-PAGE was used to analyze the protein expression, and Western blot was used to verify the expression and molecular weight of the target protein.

Wu Qing<sup>[9]</sup> et al. ‘s method successfully achieved the cloning, expression, protein purification and identification of OprF gene by PCR, enzyme digestion, SDS-PAGE and Western blot, which provided reliable technical support for *Pseudomonas aeruginosa* related research.

### 1.4 Rapid detection based on sugar functionalized magnetic nanoparticle material enrichment and fluorescence method

This assay is based on glycosylfunctionalized magnetic nanoparticles (GNPS) and loaded GNP@FITC functionalized nanofiber membranes for the detection of *Pseudomonas aeruginosa* in packaged drinking water. 5 mg of sugar-functionalized magnetic nanoparticles was added to 250 mL of packaged drinking water and shaken at 37°C and 160 r/min for 30 min. The nanoparticles were adsorbed by magnet and the upper bacterial solution was removed (magnetic field separation). Then 20 mL of liquid medium was added and incubated for 6 hours. The membrane was loaded with GNP@FITC functionalized nanofiber membrane, and the fluorescence intensity was measured by fluorescence spectrophotometer after 30 minutes. By comparing the fluorescence intensity ratio ( $I/I_0$ ) between the initial ( $I_0$ ) and the ( $I_0$ ) after 30 minutes of culture ( $I/I_0$ ) less than 0.7, the *Pseudomonas aeruginosa* was identified.

In the experiments conducted by Huang Hui et al.<sup>[10]</sup>, the optimal experimental conditions were determined by optimizing the addition amount of glycofunctionalized magnetic nanoparticles, the co-culture time with *Pseudomonas aeruginosa*, and the co-culture time of the membrane loaded with GNP@FITC functionalized nanofibers and adsorbed bacteria. This method can quickly and sensitively detect *Pseudomonas aeruginosa* by changing the fluorescence intensity, which provides an efficient technical means for microbial detection of packaged drinking water.

### 1.5 PCR amplification

Single PCR amplification : Liu Huan et al. used a single PCR amplification technique to construct a PCR reaction system by using the mixed bacterial solution as a template, adding specific primers, DNA polymerase, buffer, and pure water. The

amplification program includes the steps of predenaturation, denaturation, annealing, and extension, and the amplification is completed after multiple rounds of cycling. The amplified products were separated by agarose gel electrophoresis and the amplified bands were analyzed in a gel imaging system to verify the presence of the target gene fragment<sup>[11]</sup>.

**Multiplex PCR amplification :** Based on the single PCR, the primer volume in the multiplex PCR system was adjusted to 0.125 to 1  $\mu$ L, and the other reaction components and conditions were consistent with the single PCR. By optimizing the primer ratio and reaction conditions, the specific amplification of multiple target bacteria was achieved at the same time. The amplified products were similarly analyzed by 2% agarose gel electrophoresis and gel imaging system to verify the specificity and sensitivity of the multiplex PCR. Primer specificity was verified by single PCR using the mixed bacterial solution as a template. The concentration of five control bacteria was detected by dilution plate smear counting method, and the concentration of each bacteria in the mixed bacterial solution was calculated. The detection limit of multiplex PCR was analyzed by agarose gel electrophoresis and gel imaging using mixed bacterial solutions with different concentration gradients as templates. The reproducibility of the method was evaluated by repeating 10 times of multiplex PCR using  $1 \times 10^5$  diluted bacterial mixture as template.

Liu Huan et al. proposed the need for the preparation of simulated samples and the detection of control bacteria<sup>[11]</sup>. Six sterilized medical protective masks were used to evenly smear 1 ml of  $1 \times 10^7$  CFU/ml mixed bacterial solution, and placed at room temperature until no visible droplets were observed to prepare simulated samples for controlling bacterial contamination. Test sample solution was prepared according to GB 15979-2002 "Hygienic Standard for Disposable Sanitary products" Appendix B method<sup>[12]</sup> : weighing ( $10 \pm 1$ ) g sample, cutting into pieces, adding 200 ml sterile physiological saline, and thoroughly mixing. Five  $\mu$ l of the sample solution was used as a multiplex PCR template to detect the five control bacteria in the simulated samples.

## 1.6 Preparation of monoclonal antibodies and ELISA

In the study of detection methods for *Pseudomonas aeruginosa*, the preparation of monoclonal antibodies is one of the key steps. BALB/c mice were immunized with the recombinant protein Pa-OprF mixed with Freund's complete adjuvant at a volume ratio of 1:1. When the serum antibody titer of the mice reached 1:64 000 and the OD value was greater than 0.4, the recombinant protein Pa-OprF was injected intraperitoneally to enhance the immune effect<sup>[13]</sup>. Three days after immunization, splenocytes from immunized mice were harvested for cell fusion with SP2/0 cells at a ratio of 5:1. The recombinant protein Pa-OprF and inactivated *Pseudomonas aeruginosa* were used as coating antigens, and the positive hybridoma cell lines that could specifically recognize *Pseudomonas aeruginosa* were obtained after two subcloning and indirect enzyme-linked immunosorbent assay. The selected positive hybridoma cell lines were injected intraperitoneally into BALB/c mice, and serum was collected and purified to obtain monoclonal antibodies. Using the recombinant protein Pa-OprF as the coating antigen, indirect ELISA was used to detect the titer of monoclonal antibody.  $S/N > 2.1$  was judged as positive, and the highest dilution of antibody corresponding to  $S/N > 2.1$  was taken as the titer of the antibody<sup>[14]</sup>. This step provides antibody tools with high specificity and sensitivity for the development of subsequent detection methods.

In this method, in order to evaluate the binding characteristics and specificity of the monoclonal antibody to the antigen, the monoclonal antibody biofilm interference technique and Western Blot were used for detection. Octet Red instrument and Mouse immunoglobulin quantification sensor (AMC) were used to detect the antigenantibody affinity of the purified monoclonal antibodies. Origin 8.5 software was used to process the experimental data and generate the binding dissociation curve to quantitatively analyze the binding kinetic parameters of monoclonal antibodies and antigens. It provides an important basis for the optimization of antibody performance and the improvement of detection methods<sup>[15]</sup>. To further verify the specificity of the monoclonal antibodies, the method was identified by Western Blot. *Pseudomonas aeruginosa* was sonicated and the supernatant was collected by centrifugation. After denaturation, the supernatant and the recombinant protein Pa-OprF were separated by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membrane, blocked with blocking solution and incubated with prepared Pa-2\* and Pa-3\* monoclonal antibodies as primary antibodies and sheep anti-mouse IgG-HRP as secondary antibodies. Chemiluminescence reagents were used to develop the monoclonal antibody, and the specific recognition ability of the monoclonal antibody to *Pseudomonas aeruginosa* was verified by the development results.

This step ensures the reliability and accuracy of the antibody in the detection method and provides a solid experimental basis for subsequent applications.

By adopting these methods, Cao Jiamin et al.<sup>[16]</sup> provided highly specific monoclonal antibodies for the detection of *Pseudomonas aeruginosa*, and verified the titer, affinity and specificity of the antibodies by ELISA, biofilm interference technology, Western Blot and other methods, laying a solid foundation for the development of efficient and sensitive detection methods for *Pseudomonas aeruginosa*.

### 1.7 Colloidal gold immunochromatography

In order to evaluate the sensitivity of colloidal gold immunochromatographic test strips in the detection of *Pseudomonas aeruginosa*, the test strips were prepared and tested according to the method in literature<sup>[17]</sup>. *Pseudomonas aeruginosa* concentrations were diluted to a series of six standard turbidity gradients, and PBS was used as a negative control. The minimum detection limit was determined by observing the positive results of the test strip. This test provides an important basis for the sensitivity of the dipstick in practical applications, ensuring that it can detect low concentrations of *Pseudomonas aeruginosa*. To verify the specificity of the test strips, *Pseudomonas aeruginosa* and 10 other common respiratory pathogens, including *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus salivans* and *Streptococcus pyogenes*, were detected at a concentration of 1.0 McManus unit. PBS was used as a negative control, and its specific recognition ability to *Pseudomonas aeruginosa* was evaluated by the reaction results of the test strip. This test ensures that the dipstick can accurately distinguish *Pseudomonas aeruginosa* from other pathogens and avoid cross-reactions in practice. To evaluate the stability and repeatability of the strips, an accelerated aging test was performed. The test strips were placed in 45°C environment, and random samples were taken at a certain time interval. *Pseudomonas aeruginosa* at 1.0 McConkey unit concentration was used as positive sample, and PBS was used as negative control. According to the test results, the storage time of the test strip at room temperature was deduced by formula. This test provides reliable data support for the long-term storage and use of the test strip, and ensures its stable detection performance within the validity period<sup>[16]</sup>.

In this study, sensitivity, specificity and stability tests were performed to comprehensively evaluate the potential application of GICA strips in the detection of *Pseudomonas aeruginosa*. Combined with relevant studies in the literature, this method is rapid, sensitive, specific and stable, which provides strong technical support for the field detection of *Pseudomonas aeruginosa*.

### 1.8 VITEK 2 Compact automatic bacterial identification

VITEK 2 Compact is a fully automated microbial identification system that is widely used for rapid identification of bacteria in clinical and environmental Settings. Based on biochemical reactions and microbial metabolic properties, this system can efficiently and accurately identify a variety of bacteria, including *Pseudomonas aeruginosa*. In this study, VITEK 2 Compact was used to identify the isolated and purified strain 47 and the standard strain ATCC15442.

In the identification process, Shen Peiyao et al.<sup>[18]</sup> performed Gram staining on strain 47 and standard strain ATCC15442 to confirm that it was Gram-negative bacteria. According to the staining results and colony morphology, the corresponding identification cards were selected for subsequent identification. Single colonies of the strains to be tested were selected and prepared into bacterial suspension with appropriate concentration to ensure the accuracy of identification results. The prepared bacterial suspension was injected into the identification card of VITEK 2 Compact system, and the system automatically performed biochemical reaction detection and data analysis. Identification results were systematically generated by comparing metabolic features in the database. Strain 47 and standard strain ATCC15442 were *Pseudomonas aeruginosa*, and the identification probability was 97% and 93%, respectively, by VITEK 2 Compact. This result was consistent with the characteristics of the positive strain in the GB 8538-2016 identification criteria, and verified the identity of strain 47 as *Pseudomonas aeruginosa*.

With its high efficiency, accuracy and high automation, VITEK 2 Compact system significantly shortens the identification time of bacteria. Its rich database covers the metabolic characteristics of a variety of bacteria and can provide high confidence identification results. In this study, the system confirmed the *Pseudomonas aeruginosa* characteristics of strain 47 with the



results of LAMP assay and culture medium experiments. Combined with LAMP and traditional culture medium methods, VITEK 2 Compact optimized the detection process, improved the detection efficiency, and provided a reliable identification tool for microbiology laboratories.

### 1.9 Identification by AutoMS 1000 mass spectrometry

AutoMS 1000 mass spectrometry identification system is an efficient microbial identification tool based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technology, which is widely used for rapid identification of bacteria in clinical and environmental samples. In this study, AutoMS 1000 was used for the identification of *Pseudomonas aeruginosa* and carbapenemase detection, which was performed according to the 2022 CLSI operating standards to improve its accuracy and efficiency in microbial detection to a certain extent<sup>[19]</sup>.

In the study of Lu Dan et al.<sup>[20]</sup>, they collected *Pseudomonas aeruginosa* strains isolated and preserved between January 2022 and October 2023, including 50 carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) and 30 carbapenem-sensitive *Pseudomonas aeruginosa* (CSPA). All strains were identified by AutoMS 1000 mass spectrometry identification system and compared with the results obtained by Vitek 2 Compact automatic microbial analysis system. AutoMS 1000 could quickly and accurately identify *Pseudomonas aeruginosa* by analyzing the protein profile of bacteria. The identification results were consistent with the original stored strains, which verified the high reliability of AUTOMS 1000. AutoMS 1000 can also be used to detect whether *Pseudomonas aeruginosa* produces carbapenemases. In the study, imipenem solution was prepared with bacterial suspension, and after reacting the strains with imipenem, mass spectrometry analysis was performed using AutoMS 1000. The complex adduct peak (489 m/z) of imipenem and matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) disappeared when carbapenemase was produced. This method can quickly detect the carbapenemase activity of *Pseudomonas aeruginosa*, and provide an important basis for clinical treatment. In this study, the AutoMS 1000 mass spectrometry identification system has the characteristics of simple operation, fast detection speed and high accuracy, which can complete bacterial identification and drug resistance detection in a short time. The detection method based on mass spectrometry technology does not require complicated sample pretreatment and significantly shortens the detection cycle. In this study, the identification results of AutoMS 1000 were consistent with the results of K-B method drug susceptibility test, which further verified its reliability in the detection of *Pseudomonas aeruginosa*.

The AutoMS 1000 mass spectrometry identification system showed significant technical advantages in the identification and carbapenemase detection of *Pseudomonas aeruginosa*, providing an efficient and reliable tool for the rapid detection of *Pseudomonas aeruginosa* in clinical and environmental samples. Combined with the relevant studies in the literature, AutoMS 1000 has a broad application prospect in the field of microbial detection, which can significantly improve the detection efficiency and provide strong support for clinical diagnosis and treatment.

### 1.10 Vitek 2 Compact automatic microbial identification drug sensitivity analyzer

Vitek 2 Compact automatic microbial identification and drug sensitivity analyzer is a highly efficient microbial identification tool based on colorimetric turbidimetry, which is widely used in rapid identification and drug sensitivity testing of bacteria in clinical samples. By analyzing the metabolites and biochemical characteristics of bacteria, the instrument can complete bacterial identification and drug sensitivity analysis in a short time. It has the characteristics of simple operation, high accuracy and good repeatability. In this study, Vitek 2 Compact was used to identify *Pseudomonas aeruginosa* and other Gram-negative bacteria. By colorimetric turbidimetry, the instrument was used to detect the identification card at three wavelengths every 15 minutes, a total of 16 orientations, and each position was detected three times. Gram-negative bacteria (GN) identification cards can be completed within 3 to 8 hours, depending on the species. The results showed that 73 out of 75 strains of Gram-negative bacteria were correctly identified, and the correct rate was 97.33%, indicating that Vitek 2 Compact has a high accuracy in the identification of Gram-negative bacteria such as *Pseudomonas aeruginosa*. Vitek 2 Compact was used to read the transmittance values of the reaction Wells of the drug susceptibility test plate, and the sensitivity of bacteria to drugs was determined according to the standard curve.

In the present study by Wu Shu et al.<sup>[21]</sup>, the coincidence rate of drug sensitivity test results of Gram-negative bacteria was 93.33%, indicating that the instrument had high reliability in drug sensitivity test. The results of drug susceptibility test were



divided into coincidence, serious error, major error and general error, and the coincidence rate was as high as 93.33%, which further verified the accuracy of Vitek 2 Compact in the drug susceptibility test of *Pseudomonas aeruginosa*.

Vitek 2 Compact automatic microbial identification and drug sensitivity analyzer is characterized by its high efficiency, accuracy and high automation, which can complete bacterial identification and drug sensitivity test in a short time. Based on the detection principle of colorimetric turbidimetry, it can quickly analyze the metabolic characteristics of bacteria and significantly shorten the detection time of traditional methods. In this study, Vitek 2 Compact identification and drug susceptibility test results showed high accuracy and reliability, which provides an important basis for clinical diagnosis and treatment. The analyzer showed significant technical advantages in the identification and drug sensitivity test of *Pseudomonas aeruginosa*, providing an efficient and reliable tool for the rapid detection of clinical samples. Combined with the relevant literature, Vitek 2 Compact has a broad application prospect in the field of microbial detection, which can significantly improve the detection efficiency and provide strong support for clinical diagnosis and treatment.

## 2. Discussion

The detection technology of *Pseudomonas aeruginosa* has made significant progress in recent years, covering a variety of methods from traditional culture methods to modern molecular biology and immunological techniques. Although these techniques have their own advantages in sensitivity, specificity, and detection speed, they also face some common challenges and limitations. Although the traditional national standard method and the filter membrane method are simple to operate and low cost, their detection cycle is long, usually takes several days, and the operation skills of the experimental personnel are high, which may have the problem of insufficient sensitivity when detecting low concentrations of bacteria. Despite their widespread use in laboratory Settings, these methods are less efficient in field applications where rapid detection is required.

Detection methods based on molecular biology, such as PCR and LAMP technology, have high sensitivity and specificity, and can be completed in a short time. However, these methods require high equipment and reagents, are relatively expensive, and require high operational skills of the experimental personnel. When PCR and LAMP techniques detect complex samples, they may be interfered by inhibitors in the samples, which may affect the accuracy of the detection results. Immunological methods, such as ELISA and colloidal gold immunochromatography, are fast, sensitive and specific, and are suitable for rapid detection in the field. However, the stability and reproducibility of these methods still need to be further optimized, especially in the process of long-term storage and use, false positive or false negative results may occur. However, the high cost of development and production of immunological methods limits their promotion in large-scale applications.

Automated detection systems, such as VITEK 2 Compact and AutoMS 1000, can rapidly and accurately identify *Pseudomonas aeruginosa* and assess its drug susceptibility by combining biochemical reaction and mass spectrometry. These systems have demonstrated significant advantages in clinical diagnostics, but their high equipment costs and [] maintenance costs limit their use in resource-limited Settings. In conclusion, although the detection technology of *Pseudomonas aeruginosa* is improving, there is still a need for further research and development of low-cost, high-throughput, and suitable for rapid detection in the field. Future research should focus on improving the sensitivity, specificity and stability of detection methods while reducing the cost of detection to meet the needs of different application scenarios. Through multidisciplinary cooperation and technological innovation, the detection technology of *P. aeruginosa* is expected to play a greater role in the fields of clinical diagnosis, food safety and environmental monitoring.

Although the detection technology of *Pseudomonas aeruginosa* has made significant progress, there are still some problems to be solved. Although traditional detection methods such as national standard method and filter membrane method are simple to operate, they are time-consuming and limited in sensitivity, which are difficult to meet the needs of rapid detection. Although molecular biological techniques (such as PCR, gene cloning, etc.) and immunological methods (such as ELISA, colloidal gold immunochromatography, etc.) have improved the sensitivity and specificity of detection, these methods require high equipment and technology, and are expensive, limiting their application in resource-limited Settings. The tolerance of *Pseudomonas aeruginosa* to disinfectants and its potential ability to reproduce in packaged drinking water make it difficult to completely eliminate the risk of contamination with existing detection methods. Although automated detection systems, such as VITEK 2 Compact and AutoMS 1000 mass spectrometry identification system, have significantly improved the detection

efficiency and accuracy, their wide application is still limited by the high equipment cost and complex operation process. In the future, the development of *Pseudomonas aeruginosa* detection technology should focus on the following aspects: the development of more rapid, sensitive and low-cost detection methods, such as fluorescence detection technology based on nanomaterials and portable detection equipment, to meet the needs of rapid on-site detection; Further optimization of molecular biology and immunology techniques to improve their specificity and stability, while reducing the cost of detection; To strengthen the research on the drug resistance mechanism of *Pseudomonas aeruginosa* and develop new detection methods for drug-resistant strains. Promote the popularization of automated testing systems, and make them available in more laboratories and medical institutions through technical improvement and cost control. Interdisciplinary cooperation and technology integration will also provide new ideas and solutions for the development of *Pseudomonas aeruginosa* detection technology, and ultimately achieve the goal of more efficient and accurate detection.

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## Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# Feasibility Study on the Use of Wireless Optogenetic Regulation of PD-L1 Expression to Remodel the Immune Microenvironment of Glioblastoma

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**Abstract:** This study is based on wireless optogenetic technology, utilizing the CRY2/CIB1 photosensitive system to achieve spatiotemporal control of PD-L1 expression. In vitro experiments showed that the surface PD-L1 positivity rate of cells increased from  $28.6 \pm 3.1\%$  to  $67.3 \pm 5.4\%$  ( $P < 0.001$ ). In animal experiments, the terminal tumor volume in the light exposure group was  $450 \pm 90$  mm<sup>3</sup>, with a tumor inhibition rate of approximately 49.4% ( $P < 0.001$ ), and the median survival was extended to 32 days (compared to 24 days in the control group,  $P = 0.004$ ). Immunological tests revealed a significant increase in CD8<sup>+</sup> T cell infiltration ( $112 \pm 18$  vs  $52 \pm 10$  cells/HPF,  $P < 0.01$ ), a 30% decrease in the proportion of Tregs ( $P < 0.05$ ), and an increase in the M1/M2 macrophage ratio to 1.8. The results suggest that the wireless optogenetic system can not only precisely regulate PD-L1 but also remodel the tumor immune microenvironment, providing a new approach for precise immunotherapy of GBM.

**Keywords:** Wireless Optogenetics; Photosensitive System; PD-L1 Expression; Spatiotemporal Control; Tumor Suppression

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## Introduction

Glioblastoma (GBM) is one of the most common malignant gliomas in the central nervous system, characterized by rapid proliferation, infiltrative growth, and high resistance to conventional radiochemotherapy<sup>[1]</sup>. At present, the standard treatment regimen is surgical resection combined with radiochemotherapy. However, due to the diffuse infiltration of tumor cells at the microscopic level, residual tumor cells are easily left behind. The significant heterogeneity and redundant molecular pathways of GBM lead to a low median survival and survival rate in patients<sup>[2]</sup>. One of the key factors in the immune suppression mechanisms of GBM is the high expression of programmed death ligand 1 (PD-L1). PD-L1 can bind to the PD-1 receptor on the surface of tumor-infiltrating lymphocytes, triggering an immune inhibitory signaling pathway that leads to the exhaustion of effector T cells, allowing tumor cells to evade immune system clearance<sup>[3]</sup>. In addition, optogenetics is a cutting-edge technology that uses light-sensitive proteins to achieve spatiotemporal-specific control of cellular activity or gene expression. It has been widely applied in neuroscience, developmental biology, and gene therapy<sup>[4]</sup>. By introducing specific light-sensitive elements, the expression of target genes can be activated or inhibited under specific wavelengths of light, enabling highly precise regulation of cellular functions.

Based on the above background, this paper employs a wireless optogenetic method, using wireless energy transfer and

a micro light source to remotely and controllably inhibit the expression of PD-L1 in brain tumor sites. Compared with traditional optogenetic methods, wireless optogenetics avoids the trauma and physical restrictions caused by wires, making it more suitable for deep brain tissues and long-term in vivo experiments. This approach is not only technically feasible but also has the potential to break through the current bottleneck in glioblastoma immunotherapy, providing a new direction for theoretical and technical research in creating high-precision immune intervention measures.

## 1. Materials and Methods

### 1.1 Cell and Animal Models

#### 1.1.1 Cells

Two human glioblastoma (GBM) cell lines, U87 and LN229 (American Type Culture Collection, ATCC, Manassas, VA, USA) [5], were selected for this study.

The cells were cultured in high-glucose DMEM (Dulbecco's Modified Eagle Medium, Gibco, Thermo Fisher Scientific, Cat# 11965-092, USA) supplemented with 0% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Cat# 10099-141, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Cat# 15140-122, USA). The cultures were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> (Thermo Scientific™ Forma™ Series II Water Jacket CO<sub>2</sub> Incubator, Model 3110, USA).

When the cell confluence reached approximately 80%, the cells were digested with 0.25% trypsin-EDTA (Gibco, Thermo Fisher Scientific, Cat# 25200-056, USA). After rinsing with PBS (Phosphate Buffered Saline, HyClone, Cytiva, Cat# SH30256.01, USA), the cells were passaged.

Based on the optogenetic system and light exposure conditions, the cells were divided into the following groups: control group (no light/no vector), empty vector group (transfected with the optogenetic system vector but no light exposure), light exposure group (optogenetic system vector + blue light exposure), and negative control group (empty vector + blue light exposure).

#### 1.1.2 Animal Models

Immunodeficient mouse models were established using 6–8-week-old female BALB/c nude mice (SPF grade), which were employed for xenograft glioblastoma (GBM) tumor models. Humanized immune system mouse models were generated using NOD-scid IL2R<sup>γ</sup> null (NSG) mice through hematopoietic stem cell transplantation to reconstitute a humanized immune system (hu-PBMCs), enabling evaluation of immune microenvironment modulation. U87 or LN229 cells were resuspended in PBS at a concentration of  $1 \times 10^7$  cells/mL, and 100 μL of suspension was subcutaneously inoculated into the right axilla of the mice. Each model included four groups: control, empty vector, light stimulation, and negative control (n = 8 per group). Specifically, immunodeficient models were established with 6–8-week-old female BALB/c nude mice (SPF grade, Beijing Vital River Laboratory Animal Technology Co., Ltd., China). Humanized immune system models were constructed with 6–8-week-old female NSG mice (SPF grade, The Jackson Laboratory, Cat# 005557, USA). Human peripheral blood mononuclear cells (PBMCs) for transplantation were obtained from Stemcell Technologies, Vancouver, Canada. U87 or LN229 cells were resuspended in PBS (HyClone, Cytiva, Cat# SH30256.01, USA) at  $1 \times 10^7$  cells/mL, and 100 μL of the suspension was subcutaneously injected into the right axilla using a microsyringe (Hamilton, 25 μL/100 μL, Model 701/702, USA).

Mice were maintained in an IVC (individually ventilated cage) system (Tecniplast, Italy, Model GM500) under controlled temperature ( $22 \pm 2$  °C), humidity ( $50 \pm 10\%$  RH), and a 12-h light/dark cycle, with free access to standard laboratory chow and water.

### 1.2 Optogenetic System Design

#### 1.2.1 Light-Controlled Genetic Elements

To achieve reversible and spatially specific control of PD-L1 expression, this study constructed a light-controlled transcriptional regulation element based on the CRY2/CIB1 light-induced dimerization system. Under blue light irradiation ( $\lambda \approx 470$  nm), CRY2 dimerizes with CIB1, recruiting the bound transcriptional activator (VP64) to the PD-L1 promoter region to enhance the transcription of the PD-L1 gene. Lentiviral vectors pLenti-EF1α (Addgene, USA) were used to package the CRY2-transcription factor fusion protein and the CIB1-DNA binding domain fusion protein, respectively, and a light-sensitive



regulatory element was introduced upstream of the PD-L1 promoter. A blue light LED light source (470 nm, M470L4, Thorlabs, USA) was used in conjunction with a fiber optic coupling system (Doric Lenses, Canada) to achieve localized light exposure both in vitro and in vivo. The light response characteristics and PD-L1 expression changes were verified by transient transfection in HEK-293T cells (ATCC, USA).

### 1.2.2 Gene Delivery

Transfection was performed using Lipofectamine™ 3000 (Thermo Fisher Scientific, USA), and PD-L1 baseline levels were assessed 48 h post-transfection. For in vivo delivery, lentiviruses carrying the optogenetic elements ( $1 \times 10^8$  TU/mL, Hanbio Biotechnology, China) were intratumorally injected at a volume of 50  $\mu$ L every other day for a total of three injections.

## 1.3 Wireless Light Delivery Device

### 1.3.1 Wireless Light Delivery Device Construction

The main structure of the device is composed of a miniaturized wearable LED module, a wireless battery, and a Bluetooth control unit. The total weight is 1.5 g, which is designed to be fixed on the back of a mouse without affecting its free movement; the size is 10×8×5 mm. A lightweight silicone sleeve and medical tape are used for fixation to ensure that the light is accurately targeted at the tumor site.

### 1.3.2 Optical Parameters

Wavelength ( $\lambda$ ):  $470 \pm 5$  nm (blue light); power density: 5–10 mW/cm<sup>2</sup>; adjustable emission modes: continuous or pulsed light (10 Hz, 50% duty cycle); the heat dissipation system consisted of an aluminum substrate combined with miniature cooling fins to prevent the skin temperature of the animals from rising above 2 °C.

### 1.3.3 Control Methods

The lighting duration, power, and mode were remotely controlled via Bluetooth 5.0. The light was set for 30 minutes daily for 14 consecutive days. In control mice without the carrier, 7 consecutive days of illumination caused no skin burns or restricted activity.

## 1.4 Statistical Analysis

Western blot: Total protein was extracted using RIPA lysis buffer, separated by SDS-PAGE, and transferred onto PVDF membranes. The membranes were incubated with anti-PD-L1 (1:1000, CST) and anti-GAPDH (1:5000, Abcam) antibodies, followed by ECL detection. Band intensity was quantified using ImageJ software.

qPCR: Total RNA was extracted with TRIzol reagent, reverse-transcribed into cDNA using the PrimeScript RT kit (Takara), and subjected to quantitative PCR with SYBR Green for detection of PD-L1 mRNA expression, with  $\beta$ -actin as the internal control.

Flow cytometry: Cells were labeled with PD-L1–FITC antibody, and surface PD-L1 positivity was analyzed on a FACSCalibur flow cytometer. Immunohistochemistry (IHC): Paraffin-embedded tumor sections were incubated with antibodies against CD8, CD4, F4/80, and Foxp3, followed by DAB chromogenic staining. Images were acquired under a light microscope, and infiltrating immune cells were quantified.

Single-cell suspensions were stained with immune markers (CD45, CD8, CD4, CD11b, etc.) and analyzed to determine the proportions of different immune cell subsets.

Tumor volumes were measured every 3 days using a caliper, and calculated according to the formula:

$$V = \frac{1}{2} \times \text{length} \times (\text{width})^2 \quad (1)$$

Tumor Growth Inhibition (TGI):

$$V = \frac{1}{2} \times \text{length} \times 100 \quad (2)$$

Kaplan-Meier survival curves were used, and differences between groups were assessed using the log-rank test. All experiments were repeated at least three times. Data are presented as mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparisons between groups.  $P < 0.05$  was considered statistically significant.



## 2.Results

### 2.1 Regulation of PD-L1 Expression

HEK-293T, U87, and LN229 cells were transfected with the optogenetic vector and subjected to blue light illumination (470 nm, 5 mW/cm<sup>2</sup>, 30 min). PD-L1 protein and mRNA levels were measured by Western blot and qPCR, respectively, as shown in Table 1. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. The results indicated that PD-L1 expression remained at baseline levels in the control and empty vector groups. In the illuminated group, PD-L1 protein expression was significantly upregulated (U87: 2.1 ± 0.3-fold; LN229: 1.8 ± 0.2-fold, P<0.01), with mRNA levels showing a corresponding increase. No significant changes were observed in the negative control group. Flow cytometry further confirmed differences in cell surface PD-L1 positivity: the illuminated group exhibited a positive rate of 67.3 ± 5.4%, significantly higher than the control group (28.6 ± 3.1%, P<0.001), suggesting that the optogenetic system can enhance PD-L1 expression under spatially specific conditions.

*Tab.1 Results of Light-Controlled Regulation of PD-L1 Expression in Different Cell Lines*

Cell lines	Group	PD-L1 protein expression	PD-L1 mRNA expression	PD-L1 positivity rate
HEK-293T	Control Group	1.0±0.1	1.0±0.1	15.2±2.4
	Empty vector group	1.1±0.1	1.0±0.1	16.7±2.1
	Light group	1.5±0.2*	1.6±0.2*	32.8±4.3*
	Negative control group	1.0±0.1	1.1±0.1	14.9±2.7
U87	Control Group	1.0±0.1	1.0±0.1	28.6±3.1
	Empty vector group	1.0±0.1	1.1±0.1	29.4±3.0
	Light group	2.1±0.3**	2.0±0.3**	67.3±5.4***
	Negative control group	1.1±0.1	1.1±0.2	27.8±2.9
LN229	Control Group	1.0±0.1	1.0±0.1	22.5±2.6
	Empty vector group	1.0±0.1	1.0±0.1	23.1±2.4
	Light group	1.8±0.2**	1.7±0.2**	54.7±4.6***
	Negative control group	1.1±0.1	1.0±0.2	22.0±2.5

### 2.2 Applicability and Safety

In the BALB/c nude mouse GBM xenograft model, animals wearing the wireless light delivery device exhibited normal free movement, and their weight curves showed no significant differences compared to the group without the device. During light exposure, the skin temperature on the back of the mice increased by no more than 1.8°C, which is within the safety standard. The results in Table 2 show that in the safety pre-experiment (n=5), continuous light exposure for 7 days (30 min/d) did not result in skin damage or restricted movement. Histological sections revealed no significant inflammatory response, confirming the feasibility of long-term application of the device.

*Tab.2 Safety Evaluation of the Wireless Light Delivery Device in BALB/c Nude Mouse GBM Model*

Indicator	Control Group (No Device, n=5)	Device Group (With Device, n=5)	Light Safety Group (With Device, n=5, Continuous 7 days, 30 min/d)	Statistical Difference
Weight Change (g)	Initial: 18.4 ± 1.2 7 days: 19.1 ± 1.3	Initial: 18.6±1.1 7 days: 19.0±1.4	Initial: 18.7±1.0 7 days: 19.2±1.2	NS(P>0.05)
Weight Change Rate (%)	+3.8±1.2	+2.7±1.5	+2.9±1.3	NS
Back Skin Temperature Increase (°C)	0.2±0.1	0.3±0.1	1.8±0.2 (During Light Exposure)	< Safety Threshold 2°C
Skin Damage	None observed	None observed	None observed	-

Indicator	Control Group (No Device, n=5)	Device Group (With Device, n=5)	Light Safety Group (With Device, n=5, Continuous 7 days, 30 min/d)	Statistical Difference
Activity Status	Normal, free movement	Normal, free movement	Normal, free movement	-
Histological Examination (HE Staining)	Normal skin and subcutaneous tissue structure	Normal	No significant inflammation or necrosis	-

## 2.3 Effect of Optogenetic Modulation on Tumor Growth

In the U87 and LN229 xenograft tumor models, tumor volume was monitored every 3 days. Table 3 shows that tumors in the control and empty vector groups grew rapidly, reaching volumes of  $890 \pm 120 \text{ mm}^3$  and  $865 \pm 105 \text{ mm}^3$  on day 14, respectively. Tumor growth in the illumination group slowed significantly, reaching a terminal volume of only  $450 \pm 90 \text{ mm}^3$ , with a tumor inhibition rate (TGI) of approximately 49.4% ( $P < 0.001$ ). There was no significant difference between the negative and control groups ( $P > 0.05$ ). Survival analysis showed that the median survival in the illumination group was approximately 35% longer than in the control group (24 days in the control group vs. 32 days in the illumination group,  $P = 0.004$ ), suggesting that optogenetic modulation has an anti-tumor effect in vivo.

Tab.3 Anti-tumor effects of optogenetic regulation on U87 and LN229 transplanted tumor models

Indicator	Control Group	Empty vector group	Light group	Negative control group	P value
End-stage tumor volume U87( $\text{mm}^3$ )	$890 \pm 120$	$865 \pm 105$	$450 \pm 90$	$880 \pm 115$	$P < 0.001$
End-stage tumor volume LN229( $\text{mm}^3$ )	$910 \pm 130$	$885 \pm 125$	$470 \pm 85$	$900 \pm 120$	$P < 0.001$
Tumor inhibition rate (TGI,%)	0%	2.8%	49.4%	1.1%	$P > 0.05$
Median survival time (d)	24	25	32	23	$P = 0.004$
Extended survival rate (%)	0%	4.2%	35%	-4.2%	$P = 0.004$

## 2.4 Changes in the Tumor Immune Microenvironment

As shown in Table 4, the light exposure group exhibited a significant increase in CD8<sup>+</sup> T cell infiltration in the tumor tissue, with  $112 \pm 18$  cells/HPF compared to  $52 \pm 10$  cells/HPF in the control group, representing more than a twofold increase ( $P < 0.01$ ). The number of CD4<sup>+</sup> T cells also increased ( $P < 0.05$ ). The proportion of Foxp3<sup>+</sup> Treg cells decreased ( $P < 0.05$ ), indicating a partial reversal of the immunosuppressive state. F4/80<sup>+</sup> macrophage infiltration in the light exposure group showed an increase in the M1 phenotype and a decrease in the M2 phenotype (immunostaining ratio M1/M2 $\approx$ 1.8), suggesting that optogenetic regulation may promote antitumor immune polarization.

Flow cytometry analysis of single-cell suspensions revealed that the proportion of CD8<sup>+</sup> T cells in the light exposure group increased to  $28.6 \pm 3.2\%$ , significantly higher than the control group ( $15.2 \pm 2.1\%$ ) ( $P < 0.001$ ). The proportion of CD4<sup>+</sup> T cells also increased ( $P < 0.05$ ). The proportion of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) decreased by 30% ( $P < 0.05$ ). Among myeloid cells (CD11b<sup>+</sup>), the proportion of M1 phenotype increased, indicating a shift towards an immunologically activated tumor microenvironment.

Tab.4 Effects of Optogenetic Regulation on the Tumor Immune Microenvironment in GBM Xenograft Models

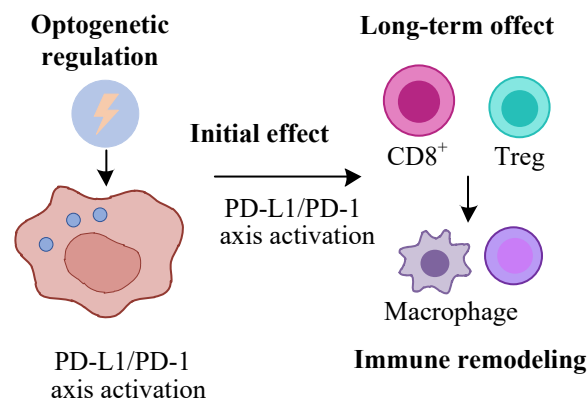
Indicator	Control Group	Light Exposure Group	P-value
CD8 <sup>+</sup> T cell infiltration (cells/HPF, IHC)	$52 \pm 10$	$112 \pm 18$	$P < 0.01$
CD4 <sup>+</sup> T cell infiltration (cells/HPF, IHC)	$68 \pm 12$	$92 \pm 15$	$P < 0.05$
Foxp3 <sup>+</sup> Treg infiltration (cells/HPF, IHC)	$41 \pm 9$	$28 \pm 7$	$P < 0.05$
F4/80 <sup>+</sup> Macrophages (M1/M2 ratio, IHC)	$0.9 \pm 0.2$	$1.8 \pm 0.3$	$P < 0.01$
CD8 <sup>+</sup> T cell proportion (% of CD45 <sup>+</sup> , Flow Cytometry)	$15.2 \pm 2.1\%$	$28.6 \pm 3.2\%$	$P < 0.001$

Indicator	Control Group	Light Exposure Group	P-value
CD4+ T cell proportion (% of CD45+, Flow Cytometry)	22.5±3.0%	28.1±3.5%	P<0.05
Treg proportion (CD4+CD25+Foxp3+, % of CD4+)	12.0±1.8%	8.4±1.5%	P<0.05
Myeloid cells (CD11b+, % of CD45+, Flow Cytometry)	18.5±2.6%	19.2±2.8%	NS
M1 macrophage proportion (% of CD11b+, Flow Cytometry)	34.2±4.1%	52.5±5.0%	P<0.01
M2 macrophage proportion (% of CD11b+, Flow Cytometry)	38.5±4.3%	29.1±3.6%	P<0.05

## 2.5 Mechanistic Hypotheses

The dual mechanisms of optogenetic regulation of the tumor immune microenvironment are shown in Figure 1. The optogenetic system upregulates PD-L1 expression on the surface of tumor cells, which initially may promote the activation of the PD-L1/PD-1 axis. However, long-term local blue light stimulation may counteract immune evasion through immune remodeling effects (such as enhanced CD8<sup>+</sup> T cell infiltration, Treg suppression, and macrophage polarization), thereby leading to an overall antitumor effect. This phenomenon suggests that optogenetic regulation can not only be used for studying PD-L1 function but may also serve as an experimental basis for precise spatiotemporal immunotherapy strategies.

*Fig.1 Optogenetic regulation of PD-L1 and immune remodeling*



## 3. Conclusion

This study has confirmed that the wireless optogenetic system can effectively regulate PD-L1 expression and improve the immune environment in GBM both in vitro and in vivo. In vitro experiments showed that blue light stimulation significantly enhanced PD-L1 levels on the surface of tumor cells. In the xenograft tumor model, the system achieved marked tumor suppression (terminal volume 450±90 mm<sup>3</sup>, TGI=49.4%) and significantly extended the median survival of mice to 32 days. Immunological analysis revealed that in the light exposure group, CD8<sup>+</sup> T cell infiltration doubled (112±18 vs 52±10 cells/HPF), the proportion of Foxp3<sup>+</sup> Tregs decreased (8.4±1.5% vs 12.0±1.8%), and the proportion of M1 macrophages increased (52.5±5.0% vs 34.2±4.1%). These results indicate that optogenetic regulation not only elucidates the function of PD-L1 but also has the potential to reverse immune suppression and promote immune activation, thereby exerting antitumor effects. Wireless optogenetics provides a feasible experimental basis for precise, spatiotemporally controlled immunotherapy strategies for GBM and holds potential for clinical translation.

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no

## Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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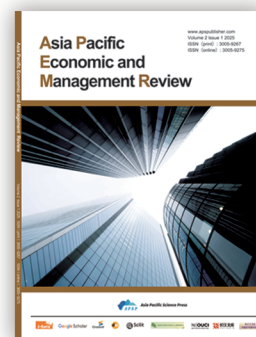
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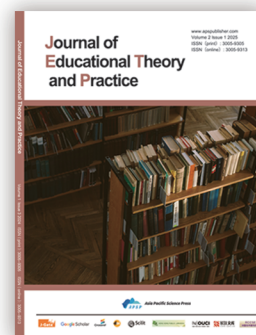
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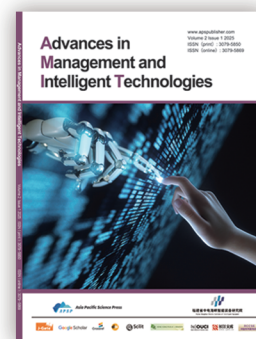
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