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Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
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E-mail: office@ddtjournal.com

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Mini-Review

DOI: 10.5582/ddt.2024.01014

# Need for a consensus definition of chronic dehydration: A scoping review

**Yoko Hasegawa[1,§], Katsunori Kato[2,§], Kazuhiro Ogai[1], Chizuko Konya[3], Takeo Minematsu[3,*]**

[1] Department of Bio-engineering Nursing, Graduate School of Nursing, Ishikawa Prefectural Nursing University, Ishikawa, Japan;
[2] Department of Adult Nursing, Graduate School of Nursing, Ishikawa Prefectural Nursing University, Ishikawa, Japan;
[3] Department of Adult Nursing, Faculty of Nursing, Ishikawa Prefectural Nursing University, Ishikawa, Japan.

**SUMMARY** Dehydration is common in older adults and impacts their clinical outcomes. Chronic dehydration is especially important as it has been under-recognized. This scoping review aimed to summarize the available definitions of chronic dehydration to identify gaps between each definition and discuss future research needs. Four databases (Pubmed, CINAHL, Cochrane Library, Science Direct) were systematically searched for peer-reviewed articles that clearly described the definition of chronic dehydration published from inception to June 8th, 2023. Two researchers reviewed the articles independently, and any disagreement was solved upon discussion. We identified five articles with a wide range of subjects from children to older adults. Chronic dehydration was defined as a state of persistently elevated blood urea levels; weight loss ≥ 1% as a result of fluid loss; a ratio of blood urea nitrogen to creatinine > 20; serum osmolarity ≥ 295 mOsm/kg; and a dehydrated state lasting 72 hours or longer. The definition varied among studies, indicating the need to establish an international consensus on the definition of chronic dehydration.

***Keywords*** water, dehydration, review, aging, diagnosis

## 1. Introduction

Water is an essential nutrient for human life, comprising 60% of the human body and playing a key role in numerous physiological processes such as digestion, absorption and use of nutrients, detoxication and excreting waste products, and whole-body thermoregulation (*1*). Total body water is closely regulated and distributed throughout the body in the intra- and extra-cellular compartments (*2*).

Dehydration is a condition with a depletion of total body water content primarily due to excessive fluid loss, lack of intake, or a combination of both (*3,4*). Older adults are especially at higher risk of dehydration. The prevalence of dehydration in older adults is approximately 20-38% in long-term care (*5-7*) and 19-20% in the community (*7,8*). Dehydration is associated not only with frailty (*2,9*), sarcopenia (*10*), and diminished physical and cognitive performance (*1,11*) but also with morbidity and mortality (*12,13*). Dehydration also causes higher medical care costs (*13,14*). Thus, appropriate assessment and intervention are necessary for preventing dehydration (*15*).

Dehydration can be either acute or chronic (*1*). Acute dehydration is relatively easily noticed and subject to treatment because it can be severe and caused by acute medical events that require medical intervention, such as infection, vomiting and diarrhea (*16*). In contrast, chronic dehydration is implicit because its severity is often mild and asymptomatic (*16,17*). Chronic dehydration is common, being found in approximately 17% of older adults living in nursing homes, and is related to dementia and higher body mass index (*18*). Since chronic dehydration is asymptomatic, in contrast to the notable symptoms of acute dehydration, its pathophysiology may differ from acute dehydration.

Therefore, there is an urgent need for further study regarding the prevalence, clinical impacts, and pathophysiology of chronic dehydration. Defining chronic dehydration is the first step to initiate research on chronic dehydration. However, researchers have applied various operational definitions of chronic dehydration as there is no international consensus definition yet. Therefore, this scoping review aimed to summarize the available definitions of chronic dehydration to identify gaps between each definition and discuss future research needs.

## 2. Materials and Methods

### 2.1. Research design and methods

This scoping review was conducted per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses for Scoping Review (PRISMA-ScR) guideline (*19*). The study protocol was developed by YH and KK and reviewed by a senior researcher (TM) prior to conducting the study.

### 2.2. Literature search

Four databases (Pubmed, CINAHL, Cochrane Library, and Science Direct) were searched on June 8th, 2023 for all previous records for all previous records with no date limits. There was a limited number of records when searching the terms ("chronic dehydration" AND defin* AND English[la]). Therefore, we used the search terms ("chronic dehydration" AND English[la]) to maximize our literature search and reviewed the definition of chronic dehydration.

### 2.3. Inclusion and exclusion criteria

Studies were eligible for inclusion if they were peer-reviewed research articles, the subjects were either human, mammals or laboratory animals, written in English, and included a clear statement of the definition of chronic dehydration. Both quantitative and qualitative research and research with experimental approaches were included. We included studies with human subjects and experimental animals to comprehensively capture the definition of chronic dehydration. The articles for inclusion were original articles, reviews, clinical reports, brief reports, comments, letters, editorials, short communications, and conference abstracts. Articles were excluded if they were written in languages other than English and not peer-reviewed. For review articles, we assessed the reference lists for additional references manually.

### 2.4. Data extraction

All retrieved citations were imported into Rayyan (Qatar Computing Research Institute, Doha, Qatar), and any duplicates were removed. Two reviewers (YH and KK) independently screened the titles and abstracts. Disagreements were resolved by consensus or discussed with a third reviewer if needed. Potentially eligible literature was subjected to a full-text review by the same reviewers. Reference lists were also reviewed for potentially relevant studies. After the full-text review, data on the author, year of publication, study design, subjects and a definition of chronic dehydration were collected by two authors (YK and KK) and validated by each other. No quality assessment was performed as this is a scoping review.

## 3. Results

The literature search identified 776 articles, and an additional 7 were included manually (Figure 1). After duplicates were removed, 730 articles were screened for title and abstract. Of these, 134 articles were eligible for full-text screening, and 129 articles were excluded because the definition of chronic dehydration was not

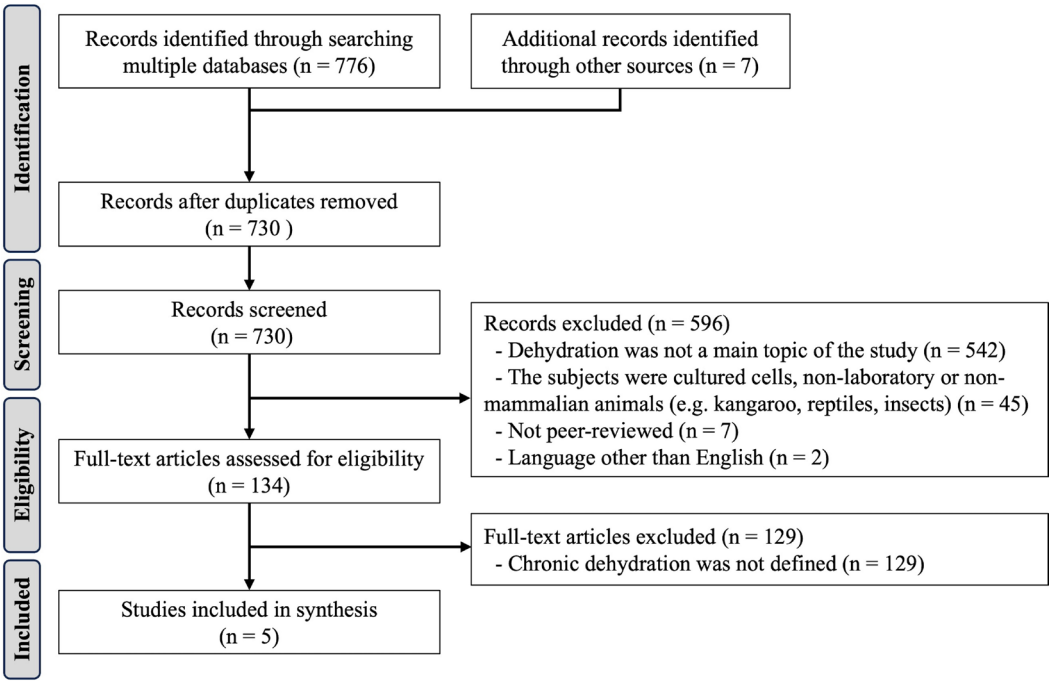


**Figure 1. Flow diagram of scoping review.**

**Table 1. The definitions of chronic dehydration**

| No. | Article | Study design | Subjects | Definition of chronic dehydration |
|---|---|---|---|---|
| 1 | Udassin R. *et al.* (1992) | Prospective cohort study | Children with familial dysautonomia underwent Nissen fundoplication and gastrostomy | Persistently elevated blood urea levels No specific numerical values were mentioned |
| 2 | Kleiner SM. (1999) | Review | Not specified | 1% or greater loss of body weight as a result of fluid loss |
| 3 | Bennett JA. *et al.* (2004) | Prospective cohort study | Patients age 75 or older who were admitted to the hospital or sent home from the emergency department of a hospital | A ratio of blood urea nitrogen to creatinine > 20 |
| 4 | Nagae M. *et al.* (2020) | Prospective cohort study | Residents aged ≥65 years living in nursing homes for ≥1 week without requiring urgent medical care for an acute illness | Serum osmolality ≥295 mOsm/kg |
| 5 | Katz B. *et al.* (2020) | Systematic review | Healthy or diseased adults aged older than 18 years | Chronic hydration status was defined as dehydrated states lasting 72 hours or longer |

described. Finally, 5 articles met the inclusion criteria for this review.

3.1. Study characteristics

Table 1 presents the characteristics of the included studies. All included studies were human-based research articles, including two with older adults, one with adults, and another with children. The study designs were three prospective cohort studies and a systematic review. No experimental studies with animal subjects defined chronic dehydration.

3.2. Definition of chronic dehydration

Udassin *et al*. defined chronic dehydration as persistently elevated blood urea levels in children with familial dysautonomia who underwent Nissen fundoplication and gastrostomy, but no specific numerical values were defined (*20*). Kleiner *et al.* defined chronic dehydration as 1% or greater loss of body weight as a result of fluid loss (*1*). Bennett *et al*. used the definition of a blood urea nitrogen to creatinine ratio (BUN/Cr) > 20 in the patients aged 75 or older who were admitted to the hospital or sent home from the emergency department (*21*). Nagae *et al.* defined chronic dehydration as serum osmolality ≥ 295 mOsm/kg in older adults living in nursing homes (*18*). They included only the residents in the nursing homes for ≥ 1 week without requiring urgent medical care for an acute illness. Katz *et al*. defined chronic dehydration in a systematic review to examine studies investigating the spectrum of hydration status and executive function in healthy or diseased adults as the dehydrated states lasting 72 hours or longer (*22*).

## 4. Discussion

This scoping review included five studies that stated the definition of chronic dehydration. All studies were human-based, with a wide range of subjects from children to older adults. The definitions employed in the included articles were (*i*) a state of persistently elevated blood urea levels, (*ii*) weight loss ≥ 1% as a result of fluid loss, (*iii*) BUN/Cr >20, (*iv*) serum osmolarity ≥ 295 mOsm/kg, and (*v*) a dehydrated state lasting 72 hours or longer.

The definition of chronic dehydration varied among studies. Udassin *et al*. (20) and Bennett *et al.* (*21*) define chronic dehydration using blood urea levels and BUN/Cr ratio, respectively. These parameters are elevated with dehydration but also in cases of kidney failure, gastrointestinal bleeding, and heart failure, while parameters are lower in cases of malnutrition (*23*). These conditions are common in older adults; therefore, the definition including these parameters might not be best for defining chronic dehydration. Kleiner defined chronic dehydration as weight loss ≥ 1% due to fluid loss (*1*); however, this definition cannot distinguish acute and chronic dehydration. Similarly, serum osmolality is difficult to differentiate acute and chronic dehydration, although it is considered the most reliable biomarker to determine a state of overall dehydration (*15*). Katz *et al.* (*22*) defined chronic dehydration as the dehydrated states lasting 72 hours or longer, but it is uncertain whether 72 hours is long enough to define chronicity. Considering Nagae *et al.* (*18*) exclusively studied older adults institutionalized for ≥ 1 week without requiring urgent medical care for an acute illness to differentiate from acute dehydration, chronic dehydration may be developed over a longer period than Katz *et al*. examined. Taken together, there has been no definitive definition of chronic dehydration yet.

This scoping review identified the need to establish an international consensus on the definition of chronic dehydration that can differentiate acute and chronic

states to support future research. Concurrently, we identified the need to apply an operational definition to research chronic dehydration at this stage. Because none of the indicators included in this review could discriminate acute and chronic dehydration by themselves, it would be useful to define chronic dehydration by narrowing down the subjects to exclude those with possible acute dehydration. Nagae *et al*. included only the residents living in the nursing homes for ≥ 1 week who did not require urgent medical care for an acute illness; this eliminated subjects with possible acute dehydration and specifically included those with chronic dehydration. Although the duration of absence of acute illness and urgent medical care is controversial, it is reasonable to define chronic dehydration by such a methodology. More detailed pathophysiology of chronic dehydration needs to be elucidated in order to reach a consensus definition.

The limitation of this study is that we included articles published only in English, which might omit some important studies defining chronic dehydration. However, we searched multiple databases for peer-reviewed articles, including reviews, conference abstracts, letters and editorials to maximize our literature search. This is the first scoping review that specifically summarizes the definition of chronic dehydration.

## 5. Conclusion

This scoping review identified the existing definition of chronic dehydration in five human subject studies. Chronic dehydration was defined as a state of persistently elevated blood urea levels; weight loss ≥ 1% as a result of fluid loss; BUN/Cr > 20; serum osmolarity ≥ 295 mOsm/kg, and a dehydrated state lasting 72 hours or longer. The definition varied among studies, indicating the need to establish an international consensus on the definition of chronic dehydration.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Takeo Minematsu, Department of Adult Nursing, Faculty of Nursing, Ishikawa Prefectural Nursing University, Gakuendai 1-1, Kahoku City, Ishikawa 929-1210, Japan.
E-mail: takeom@ishikawa-nu.ac.jp

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Review

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# Tuberculosis in pregnancy and assisted reproductive technology

**Wenli Cao[1,§], Xiayan Fu[1,§], Haiyang Li[1], Jialu Bei[1], Lisha Li[2,3,4,*], Ling Wang[2,3,4,*]**

[1] Reproductive Medicine Center, Zhoushan Maternal and Child Health Care Hospital, Zhoushan, Zhejiang, China;
[2] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[3] The Academy of Integrative Medicine, Fudan University, Shanghai, China;
[4] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China.

**SUMMARY** Tuberculosis is a chronic infectious disease caused by mycobacterium tuberculosis infection. In the world, tuberculosis is an important factor affecting women's reproductive health, which can cause reproductive tract anatomy abnormalities, embryo implantation obstacles, ovarian reserve and ovulation dysfunction, leading to female infertility. This group of women usually need to seek assisted reproductive technology to conceive. Latent tuberculosis infection during pregnancy has no clinical manifestation, but may develop into active tuberculosis, leading to adverse pregnancy outcomes. Most pregnant women do not need to be treated for latent tuberculosis infection, unless they are combined with high-risk factors for tuberculosis progress, but they need close follow-up. Early diagnosis and treatment of active tuberculosis in pregnancy can reduce the incidence rate and mortality of pregnant women and newborns, and treatment needs multidisciplinary cooperation.

***Keywords*** tuberculosis, pregnancy, anti tuberculosis treatment, assisted reproductive technology

## 1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by mycobacterium tuberculosis (MTB) infection, which is the 13th leading cause of death in the world. According to the summary of the global tuberculosis report in 2023 (*1*), a total of 7.5 million people in the world will be diagnosed with tuberculosis in 2022, which is the highest number since WHO began global tuberculosis surveillance in 1995. Despite an 18% decrease in TB notifications during the COVID-19 pandemic, rates of severe TB and related mortality have continued to rise (*2*). Pregnancy with tuberculosis (PWT) is a collection of diseases caused by MTB infections that occur either during pregnancy or when TB remains untreated during that time. Latent tuberculous infection (LTBI) is a special state in which the host has not yet developed the disease after being infected with MTB. It is characterized by a strong positive purified protein derivative test (PPD) test or a recent transition from negative to positive, without the clinical manifestations and imaging signs of active tuberculosis. LTBI patients have a 5%-10% risk of developing active TB, and most of the rest have no obvious clinical symptoms. The global incidence rate of PWT is between 5-7% according to statistics (*3,4*). Notably, developing countries have a relatively higher incidence rate of tuberculosis compared to developed ones due to differences in economy and medical technology (*1*).

Due to various reasons, the incidence rate of infertility has increased year by year. According to statistics in China, the infertility rate of couples of childbearing age has increased from 2.5%-3% in 1993 to approximately 18% in 2020 (*5*). With the rapid development of assisted reproductive technology (ART), especially *in vitro* fertilization embryo transfer (IVF-ET), more and more infertility patients have achieved their desire to conceive. However, whether IVF increases the risk of TB in infertile patients and its impact on pregnancy outcomes has attracted widespread clinical attention. PWT is not rare in clinical practice. In China, with the relaxation of the three child policy and the increase of IVF, the number of cases of this disease has significantly increased, but its treatment level has mostly stagnated in the 1980s to 1990s (*6,7*).

Worldwide, TB is the leading infectious disease that causes women's deaths, which can cause more than 1 million women's deaths every year. Active TB is also the main disease that causes maternal deaths (*8*). The delayed diagnosis of PWT is the main cause of adverse pregnancy outcomes. To prevent significant severe TB and related mortality, standardized whole process management of PWT is essential. This article will review the pathophysiology, diagnosis, and treatment of PWT after IVF-ET, to provide basis and suggestions for clinical diagnosis and treatment of patients.

## 2. The relationship among TB, pregnancy and ART

### 2.1. The effects of TB on female fertility

After MTB infects the lungs, it can infect the internal genitalia within approximately one year, first invading the fallopian tubes (90-100%), then the endometrium (50-80%), ovaries (20-30%), cervix (10 -20%), as shown in Figure 1. This may lead to anatomical abnormalities in the reproductive tract, embryo implantation disorders, reduced ovarian reserve, and ovulation dysfunction, leading to infertility (*9*).

The fallopian tube is the most frequently invaded pelvic organ by MTB. When MTB further infects, causing the fallopian tube to stiffen and thicken, presenting typical bead like changes, and forming cheese like substances in the lumen. When it reaches the level where tuberculosis can be detected, the function of the fallopian tube has basically lost. There are many reports of infertility caused by MTB infection in clinical practice (*10*). Pontifex *et al.* reported the HSG results of 3,773 patients, and found that 37% of the patients with primary infertility had tubal obstruction, and almost half of tubal obstruction originated from tuberculosis (*11*).

Uterine tuberculosis often spreads from fallopian tube tuberculosis through both uterine horns. The endometrium of patients with genital tuberculosis (GTB) exhibits different abnormal manifestations depending on the extent and severity of the lesion, and can affect embryo implantation. Mild endometrial lesions manifest as chronic endometritis and tuberculous granuloma. Moderate manifestation is cheesification or adhesion. Severe manifestations include adhesion of the entire uterine cavity, adhesion of the fallopian tubes, and complete damage to the endometrium, leading to Asherman syndrome. A retrospective study found that the positive rate of acid fast bacilli test on the endometrium of 60 infertile patients was 46.7% (14/60), compared to 13.3% (4/60) in the control group (*12*). Sharma *et al.* conducted a retrospective study on the results of HSG in 70 patients with GTB, and the results showed that, 57.1% of women have normal uterine cavities, 18.5% have irregular cavities, 2.8% have shrunken cavities, and 18.5% have irregular filling defects (*13*). The study by Pan *et al.* showed that, compared with the control group, patients with endometrial latent tuberculosis had fewer retrieved embryos, available embryos, and high-quality embryos (*14*). However, regular anti tuberculosis treatment before assisted reproductive assistance resulted in a similar fresh cycle live birth rate as the control group. A study on assisted reproductive technology results in Indian women with genital tuberculosis showed that after anti tuberculosis treatment, the number of oocytes and grade I embryos obtained from fresh cycles, endometrial thickness, and endometrial blood flow in patients were significantly increased; the thickness of the endometrium and endometrium during the frozen embryo transfer cycle also significantly increased (*15*). Therefore, early diagnosis of FGT and anti-tuberculosis treatment before IVF treatment are the primary measures to increase the probability of pregnancy (*16*).

Tubercul-associated immune abnormalities have an adverse effect on pregnancy. The most significant immune response of the host to MTB is the T cell-mediated cellular immune process, where T lymphocyte subsets and cytokines shift towards the Thl direction, which may affect embryo implantation (*17*). MTB can change the immune response, accompanied by fibrosis and adhesion, thus activating antiphospholipid antibodies and microthrombosis, ultimately leading to embryo implantation failure (*18*).

In addition, MTB infection also affects ovarian reserve and ovulation function. As early as 1985, relevant literature pointed out that tuberculosis infection can lead to a decrease in ovarian reserve function, which is characterized by high basal follicle stimulating hormone, fewer sinus follicles, low inhibin B, slow peak velocity of ovarian blood flow during systole, low pulsatile index, and ultimately low number of retrieved oocytes (*19,20*). Dam *et al.* found that LTBI can lead to ovarian reserve dysfunction, reducing the number of follicles and high-quality embryos (*15*). Although ovarian tuberculosis is relatively rare, in severe cases of tuberculosis infection, tuberculosis nodules, caseous necrosis, and abscesses appear on the surface and inside the ovaries, affecting the blood supply to the ovaries, leading to a decrease in the number and quality of follicles.

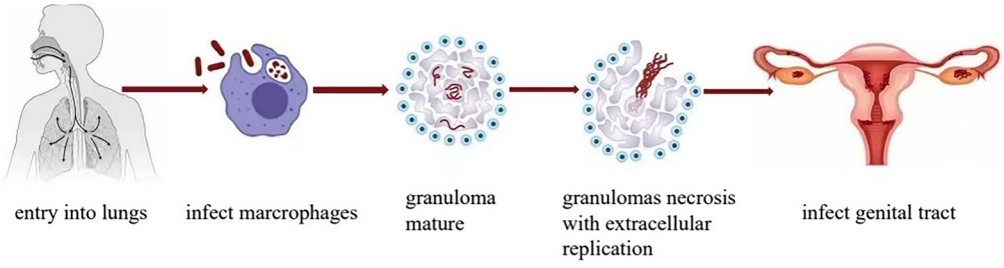


**Figure 1. The process of female reproductive tract tuberculosis infection.** Tuberculosis infection first infects the lungs and is engulfed by macrophages, gradually forming granulomas. When granulomatous necrosis accompanied by extracellular replication occurs, tuberculosis bacteria can spread to the female reproductive tract, such as the fallopian tubes, endometrium, ovaries, and cervix.

2.2. The interaction between pregnancy and TB

Pregnant women are generally susceptible to MTB. During pregnancy, due to neuroendocrine regulation, the body exhibits a high metabolic state, with mild congestion and edema of the respiratory mucosa, which is conducive to the invasion and reproduction of pathogens. The increase of circulating blood volume of women during pregnancy can reactivate stable tuberculosis foci in the lungs, leading to the recurrence of tuberculosis in the incubation period, and may induce cavity bleeding, leading to secondary infection in the lungs, increasing the difficulty of tuberculosis treatment (*21,22*).

In order to prevent fetal allograft rejection during pregnancy, the maternal immune system has undergone significant changes. The existence of specific immune tolerance and non-specific immunosuppression between mother and fetus may be the reason for the rise of tuberculosis incidence rate during pregnancy (*23*). At the same time, the regulation of estrogen and progesterone during pregnancy maintains maternal fetal immune tolerance, which is one of the reasons for the increased susceptibility to MTB and the possibility of activation of latent tuberculosis. Research suggests an increase in the level and activity of phagocytes and plasma like dendritic cells in pregnant women, downregulation of natural killer (NK) cell toxicity by progesterone induced blocking factors and interleukin (IL-10), and the production of interferon γ (γ-IFN) is also reduced, indicating that cellular responses are generally inhibited, the function of immune cells decreases, and the opportunity for immune escape of Mycobacterium tuberculosis is increased (*24*).

In addition to the increase in susceptibility to tuberculosis due to physiological changes unique to pregnancy, the risk factors of pregnant women infected with tuberculosis are shown in Table 1. PWT seriously threatens the health of mothers and infants. The delay in diagnosis of TB in pregnancy, the progress of the disease, the lack of formal treatment and the combination of extrapulmonary tuberculosis are all important factors leading to the adverse outcome of pregnancy. An American study on obstetric outcomes pointed out that compared with pregnant women without tuberculosis infection, PWT patients were more likely to have chorioamnionitis, premature delivery, postpartum anemia, bleeding, blood transfusion, pneumonia, acute respiratory distress syndrome and mechanical ventilation, and the maternal mortality rate of tuberculosis was significantly increased (*25*). Therefore, standardized whole process management of PWT is essential to prevent major maternal and perinatal complications.

**Table 1. High risk factors of tuberculosis**

| Disease state | High risk factors |
|---|---|
| TB infection | Contacts with active TB patients<br>People from a country where TB is common, including Africa, Asia, the Caribbean, Eastern Europe, Latin America, Russia<br>Living or working in high-risk environments |
| LTBI progression | Human immunodeficiency virus infection<br>Tuberculosis infection within the past 2 years<br>Intravenous drug user<br>Immunocompromise<br>No standardized treatment for tuberculosis in the past |

2.3. ART increases the risk of PWT

Female pelvic tuberculosis is highly susceptible to tubal infertility, and the recovery of reproductive function is very difficult. It is often necessary to seek assisted reproductive assistance for pregnancy. Some studies report that the changes of hormone levels in the body of infertile women undergoing ART during ovulation induction, the interference of ovum retrieval operation on old pelvic lesions, and the reduction of immune function after pregnancy can all lead to the recurrence of latent tuberculosis foci, and even a large number of MTB multiply into the blood, resulting in acute hematogenous disseminated pulmonary tuberculosis (*26*). IVF superovulation leads to extremely high levels of estrogen in the body, while progesterone luteal support can cause high levels of progesterone. Estrogen and progesterone have a direct inhibitory effect on T lymphocytes in a dose-dependent manner, inducing lymphocyte apoptosis, inhibiting cellular immunity, and leading to transform old and latent tuberculosis into active tuberculosis (*27*).

Addis *et al.* first reported cases of pregnancy complicated with bilateral miliary tuberculosis after IVF-ET (*28*). The patient was 33 years old and underwent IVF-ET for 10 years due to unexplained primary infertility, resulting in a single pregnancy. Intermittent vaginal bleeding occurred during early pregnancy, accompanied by influenza like symptoms and bilateral miliary tuberculosis, and spontaneous abortion occurred at 14 weeks of pregnancy. It is suggested that the IVF-ET treatment process and pregnancy may activate the potential tuberculosis focus, leading to active TB and spontaneous abortion. The research results of Gull *et al.* also showed that both IVF-ET treatment and pregnancy can exacerbate LTBI and even cause life-threatening miliary tuberculosis, leading to spontaneous abortion (*29*). Therefore, it is necessary for infertile patients to screen for tuberculosis before ART treatment to avoid adverse pregnancy outcomes.

## 3. Diagnosis of PWT

The clinical manifestations of PWT are diverse and lack of specificity. Early diagnosis is very difficult. In addition, clinicians are lack of vigilance, and are prone to misdiagnosis, missed diagnosis and delayed treatment. The key to avoid missed diagnosis and misdiagnosis

**Table 2. Diagnostic tools of tuberculosis**

| Tuberculosis test classification | Investigation |
|---|---|
| Immunological examination | Purified protein derivative test<br>γ-IFN release test<br>ESAT6-CFPl0 assay |
| Bacteriology examination | Smear method<br>Culture method |
| Imaging examination | Chest X-ray, CT, MRI examination<br>Hysterosalpingography |
| Molecular biology diagnosis | Polymerase chain reaction<br>Gene sequencing<br>Xpert MTB/RIF test |
| Others | Routine bronchoscopy examination<br>Percutaneous transbronchial biopsy<br>Immunohistochemical diagnosis technology Traditional pathological diagnosis technology |

is to strengthen the understanding of tuberculosis in pregnancy and screen suspected patients for tuberculosis in time before IVF-ET. Screening of PWT includes immunology, bacteriology, imaging, molecular biology and other means, which can be selected according to different clinical manifestations and disease progression stages of patients. There are various tools to improve the accuracy of PWT diagnosis, as shown in Table 2.

### 3.1. Immunological examination

Immunological examination of TB is a rapid examination technique to determine the antibodies and antigens of human TB bacteria. Tuberculin skin test (TST) is an important screening test for TB, especially for high-risk groups. The currently recommended tuberculin for use by World Health Organization (WHO) is PPD. TST results are not affected by pregnancy, so it is recommended for screening PWT (*30*). The advantage of TST testing lies in its simple operation and low cost, but its specificity is relatively poor, and its sensitivity is low for patients with immune deficiency.

γ-IFN release test (IGRAs) plays an important role in the diagnosis of bacterial negative pulmonary tuberculosis and extrapulmonary tuberculosis by stimulating lymphocytes with specific antigens to detect cytokines. Currently, it still cannot distinguish between latent and active tuberculosis infections. It is known as one of the most promising diagnostic techniques for tuberculosis because of its advantages of rapidity, high sensitivity and specificity (*31*). However, its high cost makes it difficult to be widely used in developing countries with high prevalence of tuberculosis.

ESAT6-CFPl0 assay is a newly developed skin test protocol for detecting mycobacterium TB infection. It has high sensitivity and specificity, low cost, simple operation, and is not affected by Bacille Calmette-Guérin (BCG) vaccination (*32*). Due to its comparable performance to TST, it can be used as an alternative testing method for TST in areas with high HIV prevalence and widespread BCG vaccination (*33*).

### 3.2. Bacteriology examination

Bacteriology examination of mycobacterium TB includes smear method and culture method. The advantages of sputum smear examination are simplicity, speed, and ease of implementation. However, its sensitivity during pregnancy is relatively low, it is recommended to conduct more than three sputum tests to avoid missed diagnosis. Although the culture of mycobacterium TB is considered as the gold standard for the diagnosis of TB, its clinical application is limited by the long culture time.

### 3.3. Imaging examination

A retrospective study showed that 50% (14/28) of PWT patients did not undergo chest X-ray or CT examination at their first visit, which resulted in delayed diagnosis (*34*). According to the latest guidelines, X-ray and CT examinations are safe during pregnancy and lactation, with radiation levels much lower than those that have adverse effects on the fetus (*35*). Multi slice spiral CT can find small lesions that are difficult to be found by X-ray, which is of great significance for early diagnosis of TB. Among them, High resolution CT has irreplaceable advantages in diagnosing tuberculosis negative and active TB (*36*). With the development of MRI technology, the impact of respiration and heartbeat on imaging quality has been effectively eliminated, making it more sensitive in detecting caseous necrosis, liquefaction, active cavities, lymph node abnormalities, and pleural abnormalities (*37*). It is suitable for pulmonary tuberculosis patients who require long-term routine follow-up, especially children, women, and pregnant women. Compared to CT, due to the clearer display of anatomy and lesions in the brain, spine, and spinal cord, MRI is more suitable for the diagnosis of extrapulmonary tuberculosis (*38*).

Pelvic genital tuberculosis is an important cause of infertility in developing countries, and hysterosalpingography (HSG) is the initial evaluation method. HSG before IVF-ET can not only understand the morphology of the uterine cavity and the patency of the fallopian tubes, but may also detect granulomatous calcification, irregular or extensive scar formation of the endometrium, and even uterine cavity collapse and adhesions. Chavhan *et al.* reviewed 492 cases of HSG due to infertility and found 37 cases of pelvic genital tuberculosis (*39*). The research results of Eng *et al.* are similar, and found that the incidence of contrast medium reflux is high in patients with endometrial tuberculosis undergoing HSG (*40*). Although the diagnosis of pelvic genital tuberculosis depends on the presence of clear reproductive tract MTB, HSG is also a reliable diagnostic indicator.

3.4. Molecular biology diagnosis

The molecular biology examination of PWT mainly includes polymerase chain reaction (PCR), gene sequencing, Xpert MTB/RIF test, *etc.*, as it is not affected by drug resistance and cell phenotype, it has high specificity. With the rapid development and application of molecular biology technology, various PCR based techniques and gene roll up techniques can also be used for the diagnosis of tuberculosis, such as the restriction fragment length polymorphism genotyping method of the insertion sequence IS6110 of tuberculosis strains, to understand the drug resistance and molecular mechanisms of drug resistance of tuberculosis bacteria (*41*). Gene chip technology can quickly and specifically detect mutations in the rpoB, katG, and inhA genes of most Mycobacterium TB isolates, analyze drug-resistant genotypes of Mycobacterium TB, and guide clinical medication (*42*).

It is suggested that Xpert MTB/RIF test should be used as a routine method to screen whether pregnant women with infectious fever after IVF-ET are complicated with TB (*43*). The method is relatively fast and has higher sensitivity and specificity, which can provide reference for clinical diagnosis and selection of treatment plans for drug-resistant TB (*44*).

3.5. Other detection technologies

On the basis of routine bronchoscopy examination, transbronchial ultrasound guided percutaneous transbronchial biopsy can more accurately confirm the location of the lesion and improve the diagnostic accuracy of TB, but whether it can be applied in pregnancy still lacks authoritative clinical guidelines.

Immunohistochemical diagnosis technology and traditional pathological diagnosis technology can effectively improve the accuracy of pathological diagnosis of tuberculosis. However it is more difficult to perform lesion extraction in pregnancy.

## 4. Pregnancy assistance strategies for infertile women infected with TB

Early treatment of GTB can improve the reproductive prognosis of patients. Jindal *et al.* found that in women with asymptomatic endometrial TB, administering anti-tuberculosis treatment can increase the chance of natural conception (*45*). If the damage caused by TB is too great, usually only ART can be sought for conception. With the continuous improvement of the success rate of IVF-ET, the pregnancy rate of IVF-ET also increases for infertility caused by GTB. However, the overall embryo implantation rate and pregnancy rate are lower than those of non GTB, and the abortion rate is higher than that of non GTB (*46*). A study in India on repeated failure of IVF-ET caused by LTBI also shows that, the pregnancy rate of LTBI patients is significantly lower than that of non tuberculous infertility patients, and the natural abortion rate is higher (*15*). GTB can lead to abnormal energy metabolism, increased amino acid synthesis, and significantly reduced glucose production in the endometrium, which can lead to embryo implantation failure (*47*). In addition, changes in the immune response of tuberculosis patients can activate antiphospholipid antibodies, leading to the activation of antiphospholipid antibodies and the formation of microthrombosis, leading to embryo implantation failure (*15*).

The low success rate of IVF-ET is believed to be related to untreated GTB. A large study involving over 10000 infertile patients receiving IVF-ET treatment showed that the clinical pregnancy rates (31.7% *vs.* 38.1%) and live birth rates (23.8% *vs.* 30.6%) after IVF-ET in untreated past PWT patients were significantly lower than those receiving treatment (*48*). Combined anti-tuberculosis treatment can significantly improve ovarian reserve function and endometrial receptivity, and the number of retrieved oocytes is also increased (*15*).

The process of assisted pregnancy treatment may trigger tuberculosis lesions or complicated with non genital active tuberculosis. The systemic blood transmission of maternal tuberculosis infection after vaginal oocyte recovery has been reported (*49*). In addition, reports of transplacental fetal transmission have once again raised concerns, with consequences including late abortion, premature delivery and congenital tuberculosis (*50*). Given the increasing adoption of IVF-ET as the gold standard treatment for tubal infertility, it is expected that a large proportion of women with GTB will receive IVF-ET under tuberculosis pretreatment. The coexistence of primary tubal infertility and untreated tuberculosis does exist the risk of tuberculosis transmission, which may cause fatal consequences for mothers and children. Therefore, infertility experts must be vigilant about the harm of GTB before undergoing ART, and carry out appropriate pre-treatment to achieve ideal pregnancy outcomes.

## 5. Treatment of tuberculosis during pregnancy

The key to improving the prognosis of pregnant women with tuberculosis lies in early diagnosis and treatment. The treatment of tuberculosis in pregnancy is a multi-disciplinary problem, which requires the joint participation of obstetricians, infectious physicians, neonatal pediatrics and public health units. The treatment of pregnant women with tuberculosis should generally refer to the treatment principles of non pregnant tuberculosis. The American College of Obstetricians and Gynecologists (ACOG), the American Academy of Pediatrics (AAP), and Centers for Disease Control and Prevention (CDC) recommend that all women at high risk of TB should be screened for symptoms and physical examination at the beginning of prenatal care, including

cough, expectoration, fever, night sweats, weight loss, *etc*. If the patient has signs or symptoms related to TB, and TB infection develops into active tuberculosis risk factors screening is positive, TB testing should be carried out as soon as possible. The diagnostic process for pregnant patients is shown in Figure 2. Once diagnosed as PWT, treatment should be carried out immediately to control the progress of TB and treatment should be targeted and personalized.

Isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) are four first-line drugs for the treatment of TB, which are classified as Class C drugs in pregnancy by the Federal Drug Administration (FDA). INH can pass through the placenta, but even if taken in the early stages of pregnancy, it will not cause deformities. RIF may have a small risk of teratogenesis (*51,52*). A study of 204 pregnancy cases showed that 4.4% of pregnant women found abnormalities related to the use of RIF, including hydrocephalus, anencephaly, and limb defects, higher than the 1.8% reported in other studies (*53*). However, in a large-scale latent tuberculosis infection trial using INH + RIF or INH, 125 pregnant women did not show any unexpected fetal loss rate or congenital abnormalities (*54*). Given decades of experience using rifampicin and limited data on potential teratogenicity, most experts believe that using rifampicin during pregnancy is appropriate.

### 5.1. Treatment of active TB

Although tuberculosis drugs are all defined as Class C drugs by FDA, considering the adverse maternal and infant outcomes of untreated active tuberculosis, the benefits of treatment outweigh the potential risks of drugs. Compared with the second and third trimesters of pregnancy, if active tuberculosis is treated in the first trimester of pregnancy, it can almost eliminate the increased risk of premature delivery, low birth weight and perinatal death, and maternal complications are also reduced (*55*). Reasonable anti tuberculosis treatment has a relatively small impact on the growth and development of infants and young children, which is beneficial for the clinical cure of patients in the middle and late stages of pregnancy (*56*). A prospective cohort study showed that the treatment success rate of PWT without HIV infection was comparable to that of non pregnant women (*57*). Therefore, it is recommended that active TB during pregnancy should be actively treated.

WHO recommends that INH, RIF, EMB and PZA should be used together every day for 2 months, and then INH, RIF and EMB should be used every day for 4 months (2HREZ+4HRE). This plan can cure 90% of cases, have good drug compliance, and improve maternal and perinatal outcomes (*4,58*). However, CDC recommends that pregnant women with active TB should first choose INH, RIF and EMB for 2 months, and then INH and RIF for 7 months (HREx2+HRx7), because the impact of PZA on the fetus is still unclear (*59*). During treatment, pyridoxine and vitamin K should be supplemented as INH may increase maternal liver toxicity, and regular liver function testing should be conducted. Obstetricians and gynaecologists can cooperate with disease experts, including infectious disease experts, tuberculosis medical consultants and health departments, to ensure timely and accurate diagnosis and treatment, while strengthening the link with nursing to strengthen patients' treatment compliance.

In addition to medication treatment, PWT patients should also be advised to have a balanced diet. Medical personnel should pay attention to their mental health, regularly follow up, and timely implement psychological intervention measures for mentally unhealthy individuals to alleviate their tension, anxiety, and depression, which is beneficial for patients' recovery and return to normal

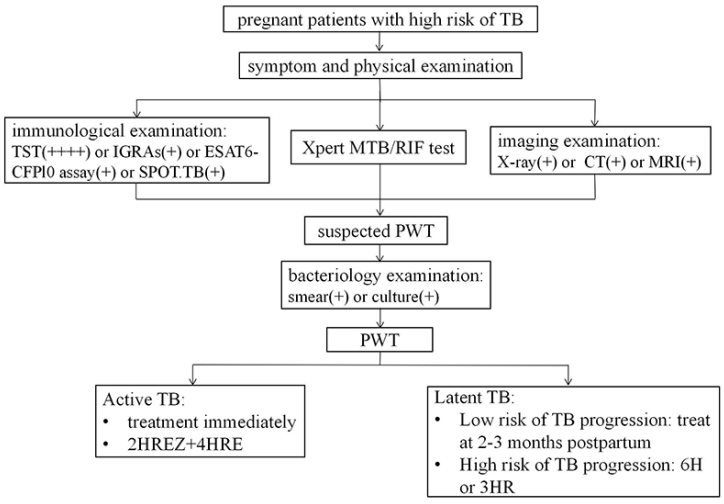


**Figure 2. Diagnosis process of PWT.** First, evaluate the symptom and physical of pregnant patients with high risk of PWT. Second, if there are abnormal signs, perform immunological, Xpert MTB/RIF test or imaging examination. If any one is positive, it is a suspected PWT patient, and further bacteriology examination is required. Once diagnosed with PWT, individualized treatment should be carried out accordingly.

life. And infection control management is also crucial, and screening of staff and family members is necessary.

5.2. Treatment of LTBI

The treatment of pregnant women with latent tuberculosis infection during pregnancy is still controversial. To avoid using unnecessary medications during pregnancy, CDC recommends delaying treatment until 2-3 months postpartum unless there is a clear risk of disease progression (*59*). When the risk of LTBI progression is high, as shown in Table 1, it is recommended to use INH treatment for 6 months or RIF+INH treatment for 3 months (*60*). A study evaluated the results of 125 cases of LTBI during pregnancy who received RIF + INH or RIF monotherapy for prevention and treatment. The abortion rates were 13% (4/31) and 14% (8/56), respectively, and the incidence of congenital abnormalities was 0% (0/20) and 5% (2/41), respectively. This indicates that the RIF+INH regimen can also be used for LTBI prevention and treatment (*54*).

Before the standardized treatment of tuberculosis in pregnancy, the basic diseases such as liver disease should be excluded first, and the liver function and other related indicators should be monitored during the treatment period to avoid liver injury (*61*). At the same time, plasma endotoxin, C-reactive protein and procalcitonin are regularly tested to identify whether tuberculosis patients are complicated with pulmonary bacterial infection, guide clinical rational drug use and timely adjust treatment (*62*). The recently released CDC guidelines review data on the preferred and alternative treatment options for LTBI during pregnancy, and evidence suggests that a 6-9 month course of INH treatment remains the recommended option for pregnant women, as there is currently no supporting data on the use of rifampicin during pregnancy (*59*). There is limited research data on LTBI treatment for pregnant women, and there is currently a lack of sufficient evidence on the safety, tolerability, and long-term treatment outcomes of LTBI treatment during pregnancy.

5.3. Timing of Termination of PWT

TB is not an indication for terminating pregnancy, but it is recommended to terminate pregnancy in the following situations (*63*). *i*) Severe pulmonary TB is accompanied by decreased lung function and cannot tolerate continued pregnancy and childbirth. *ii*) Active pulmonary tuberculosis requires timely anti-tuberculosis treatment, considering the adverse effects of drugs on the fetus that are difficult to avoid. *iii*) Those who cannot continue pregnancy due to other systemic diseases. *iv*) Pregnancy of AIDS patients complicated with tuberculosis. *v*) Indications for obstetric termination of pregnancy. The termination of pregnancy is generally within 3 months of pregnancy. If the pregnancy has exceeded 3 months, with the informed consent of family members and pregnant women, appropriate anti-tuberculosis drug treatment can be chosen to maintain pregnancy.

## 6. Conclusion and prospects

PWT is not uncommon in clinical practice. In view of its poor reproductive prognosis, it is recommended that infertile women receive screening for tuberculosis before IVF-ET. Once tuberculosis is suspected after pregnancy, relevant examinations should be conducted to confirm the diagnosis and avoid delaying treatment due to symptoms similar to those of early pregnancy. Once pregnancy is diagnosed with active tuberculosis, start anti-tuberculosis treatment as soon as possible, and form a safe and effective anti-tuberculosis treatment plan during pregnancy to reduce the abortion rate and death rate. In addition to drug therapy, medical personnel should pay more attention to the mental health of patients. The treatment of PWT requires multidisciplinary joint management, joint development of anti-tuberculosis treatment plans, monitoring of treatment effectiveness and pregnancy outcomes.

The training and management of medical staff should be strengthened to make use of more advanced molecular biological diagnostic technology for early diagnosis and early treatment of PWT. At the same time, the informed understanding of tuberculosis of patients in the first and second trimesters should be strengthened, and the whole process management should be standardized jointly by doctors and patients, so as to achieve good outcomes for mothers and children of patients with gestational tuberculosis during the whole pregnancy.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai, China 200011.
E-mail: dr.wangling@fudan.edu.cn

Lisha Li, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai, China 200011.
E-mail: lishasmv@163.com

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# Evaluation of appropriateness of alerts overrides and physicians' responses of the medication-related clinical decision support system in China, a hospital-based study

**Li Jin[1,§], Huan Fang[2,§], Jie Shen[1], Zhigao He[1,*], Yi Li[3,*], Liang Dong[4], Jiali Feng[5], Tetsuya Asakawa[6,*]**

[1] Department of Pharmacy, Longhua Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China;
[2] Department of Pharmacy, Jinshan Hospital of Fudan University, Shanghai China;
[3] Department of Nephrology, Longhua Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China;
[4] Department of Information Technology, Longhua Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China;
[5] Department of Oncology, Longhua Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China;
[6] Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, Shenzhen, China.

**SUMMARY** This study was designed to investigate the *state quo* of the appropriateness of alerts overrides of the medication-related clinical decision support system (MRCDSS) in China. The medication-related alerts in one hospital from Jan 2022 to Dec 2022 were acquired and sampled. Rates of alert overrides, appropriateness of alert generation and physicians' responses were observed. Total 14,612 medication-related alerts (≤ level 3) were recorded, of those, 12,659 (86.6%) alerts were overridden. The top 3 alert types were: drug and diagnosis contraindications (23.8%), drug and test value contraindications (23.3%), and compatibility issues (17.7%). Of all sampled 1,501 alerts, 80.2% of them were appropriately overridden by the physicians. The appropriate rate of alert generation was 57.9% and the inappropriate rate was 42.1%. The inappropriate rate of physicians' responses was 17.8%, and 2.0% physicians' responses were undetermined. A few medications accounted for over 10% of overrides, 88.3% of "overridden reasons" inputted by the physicians were meaningless characters or values, indicating an obvious "alert fatigue" in these physicians. Our results indicated that the overridden rate of MRCDSS in China was still high, and appropriateness of generation of alert was quite low. These data indicated that the MRCDSS currently using in China still needs constantly optimization and timely maintenance. Proper sensitivity to reduce triggering of useless alerts and generation of alert fatigue might play a vital role. We believed that these findings are helpful for better understanding the *state quo* of MRCDSS in China and providing useful insights for future developing and improving MRCDSS.

***Keywords*** medication-related clinical decision support system (MRCDSS), alerts, alert fatigue, hospital information system (HIS)

## 1. Introduction

Medication-related clinical decision support system (MRCDSS) has become an indispensable component of hospital information system (HIS) (*1*). It's algorithms commonly include data of clinical characteristics of patients (demographic data, results of laboratory examinations, severity, *etc.*), drug instructions based on pharmacopoeia, and clinical guidelines (*2*). Once the medical orders are determined as "risky", it may generate "alerts", which are reportedly more specific than alerts from basic medication surveillance (*2*). Most of MRCDSS alerts are interruptive, and require users to choose "accept" or "override" the alerts before continuing. Alerts from MRCDSS cannot be ignored during the processes of making a medical order, which are greatly helpful for the physicians to prevent potential errors during the prescription. However, due to the limitations of software and algorithms (*2*), alerts generated by the MRCDSS might be "appropriate" or "inappropriate", of those, many alerts might be overridden by the physicians in light of the clinal pathophysiological state of a certain patients. Meanwhile, the responses of physicians are also not always appropriate. Thus, evaluation of the override rate is useful for better understanding the *state quo* of the performance of the MRCDSS in use

and the knowledge, attitude and practice (KAP) of the physicians concerning the MRCDSS which is important to improve the performance of MRCDSS, fix the bugs of software, and carry out education/training to improve the operation skill of the physicians. Poly *et al.* reviewed that approximately 46.2%-96.2% of alerts were overridden, of those, 29.4%-100% of the overrides alerts were identified as "appropriate" (*3*). Another study in Australia reported that approximately 90% physicians complained that too many alerts were triggered in the electronic medical records (EMRs). They therefore suggested that most of alerts should be removed and the alerts should be triggered with a more specific and less sensitive manner (*4*). A study in Korea found that the override rate of alerts of MRCDSS was 92.9%. Conversely, only 7.3% of the alerts were clinically appropriate. Too many unnecessary alerts were generated (*1*). These published findings indicate that the MRCDSS so far is far from satisfactory. Main problem lies in oversensitivity of the MRCDSS induces too many unnecessary alerts are generalized, which may make the medical staff fatigue and begin to ignore the alert information. Such negative attitude to the alerts of MRCDSS might lead an inappropriate override, which are reportedly associated with enhanced risk of generation of adverse events during the clinical practice (*5,6*). Hence, development of more optimized algorithms for MRCDSS has been an ultimately task for global researchers, which first step is grasping the *state quo* of alert generation and override rate of the MRCDSS in use in a certain country. However, these data are unreported and unavailable in China, which has limited development of better algorithms for MRCDSS in China.

On the basis of the aforementioned backgrounds, we designed this single-center, observational, retrospective study to investigate the *state quo* of MRCDSS performance in China. We attempt to provide useful information regarding the type of overridden alert, appropriate rate of alert display, and physicians' responses. We believe that the findings of the present study are helpful for development of optimized algorithms for MRCDSS in China.

## 2. Materials and Methods

### 2.1. The rational medication smart prescription review system currently used in China

All data of the present study were acquired from the medical records of the HIS (HIS, Winning Health Technology Group Co., Ltd., China) in a 1200-bed comprehensive hospital. All medication orders were inputted to the system, then the medication-related clinical decisions were firstly made by a commercial Rational Medication Smart Prescription Review software (Visense Technology Co., Ltd., China, *http://www.ivisense.com/*), which is a representative and widely used MRCDSS software in hospitals in China (*7-9*). The knowledge base of this software includes enormous quantity of drug instructions, the Chinese Pharmacopoeia and Chinese Pharmacopoeia Clinical Medication Instruction (*10*). This system can conduct risk review of all inputted medication orders based on the 6-category build-in drug use risk review rules including dose range, drug interactions, drug duplicate, compatibility issues, administration route, contraindications. Once the system detected a medical order was "risky", it would general the alerts. All alerts are displayed in one window. A single drug may generate multiple alerts, which are individually counted in the data table. This system can provide four levels of alerts. Level 1 to level 4 are presented from strength to weak. Level 1 alerts are mandatory. Accordingly, involved medical orders were directly intercepted and physicians must the modify prescriptions. Level 2 alerts are determinable, physicians are allowed to choose accept or override the alerts. If they choose "accept", they need to modify the prescribed medications; if they choose "override", they need to state the reasons for the override. Level 3 alerts are reminder only, the physicians need not revise the prescriptions or enter the reasons for override. Level 4 alerts is not prompted which are only recorded in the back end.

### 2.2. Experimental design

This is a single-center, observational, retrospective study based on the medication-related alerts with levels 2 and 3 generated by the HIS from January 2022 to December 2022. All the alerts were processed with stratified sampling in random using the combination of "department + drug name + alert type" with the 1% sampling rate and at least one case per combination. The appropriateness of each alert override was independently analyzed by 2 clinical pharmacists and their consistency was tested as well. In case of any disagreement, discussion would be held by 1 clinical physician to reach mutual agreement.

This study used the evaluation framework described in a previous study (*11*). Alerts are classified by the results of appropriateness evaluation. Due to the fact that Level 3 alerts do not have reasons of override, there are situations that their appropriateness could not be determined, which were defined as "non-decidable" here. In brief, the appropriateness of alert overrides includes both appropriateness of alert generation and appropriateness of physicians' responses. The appropriateness of alert generation refers to the situation whether the alert is appropriately generated when the conditions reach the criteria of the rule of "risky" including the administrative, laboratory, and treatment-related data and trigger the alerts. Alerts might be incorrectly triggered due to the system deficiencies (such as lack of data and maintenance errors, or bugs of software) are considered "inappropriate". Appropriate physicians' responses refer to the situations that the

physicians have to override the alerts based on the patients' conditions, or evaluation of the benefits/risks of the medications in light of guidelines, expert consensus, and literature data, although the alerts are relevantly and accurately generated. We provided the evaluation criteria of overridden criteria in Table S1 in various situations (Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=198*), which were developed by senior pharmacists. In addition, the demographic data in the patients of the involved recorders, including gender, age, severity evaluated by Charlson comorbidity index, and the number of alert overrides were acquired from the EMRs.

This study was designed and conducted as per the guidelines of the Declaration of Helsinki of the World Medical Association (2000), and was approved by the Ethics Committee of Longhua Hospital of Shanghai University of Traditional Chinese Medicine (approval number: 2024LCSY013). Informed consent was signed by the tested clinical pharmacists.

### 2.3. Analysis

A SPSS software (V 25.0, IBM, USA) was used for statistical analysis. The alert override rate and appropriateness of alert overrides of different alert types were presented in percentage. The consistency of evaluation in two clinical pharmacists was verified using a Cohen's Kappa approach (*12*), where a score above 0.8 was considered as good consistency. $P < 0.05$ was considered as the significant difference.

## 3. Results

A total of 303,600 medication orders of 31,625 admitted patients involving 1,513 types of drugs were involved in this study. Of those, 60,497 alerts were generated, with 14,612 alerts ≤ level 3, of which 12,659 (86.6%) alerts were overridden. Finally, a total of 1,501 alerts involved 1,387 patients were randomly sampled according to combination of "department + drug name + alert type" (Figure 1).

The baseline data were presented in Table 1. Of all involved patients, 52.42% were male and 47.58% were female, 72.23% patients were aged over 60 years. The severity of comorbidities assessed by Charlson Comorbidity Index shows that 40.88% of patients had a score of 6 or above. In terms of the department-wise, general surgery, oncology-hematology, critical care medicine, and nephrology were the main departments covered by the alerts, contributing 51.3% alert. Most of the patients had a single overridden alert (Table 1).

Table 2 shows the appropriateness of alert generation and physicians' responses. The total appropriate rate of

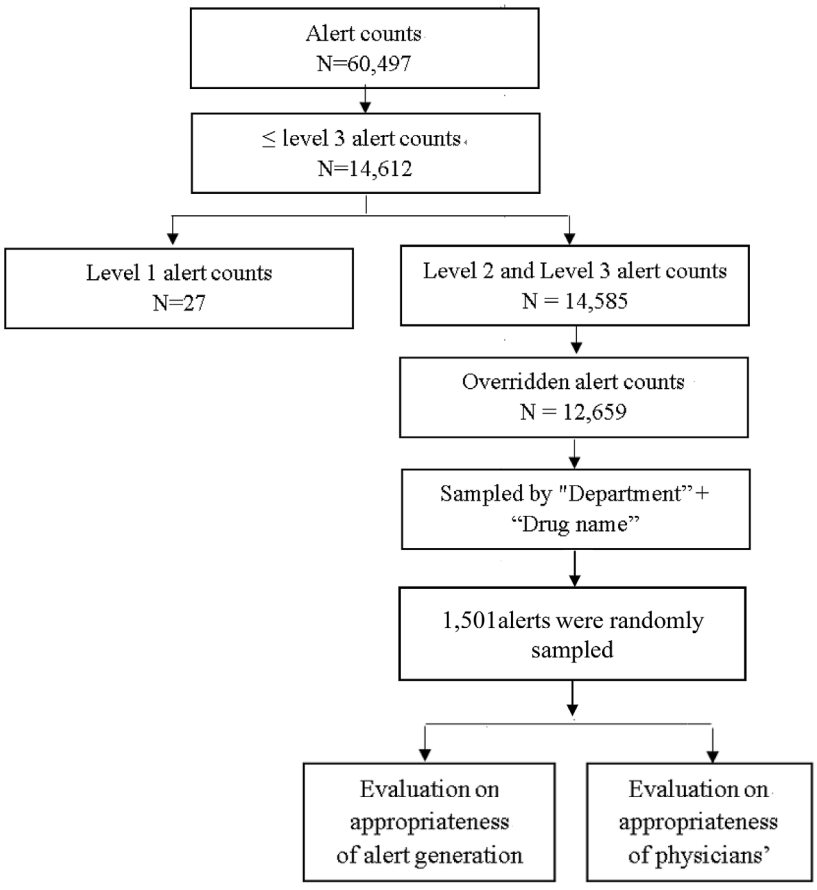


**Figure 1. The flow chart of the present study.**

physician's responses regarding alert display was 80.2% whereas the inappropriate rate was 17.8%. Meanwhile, the total appropriate rate of alert display was 57.9% whereas the inappropriate rate was 42.1%. In the appropriate generated alert display, 38.1% of physician's responses were correct, whereas 17.8% were incorrect. In addition, 2.0% of the physician's responses could not be determined. With respects to the inappropriate alert display, the appropriate rate of physician's response was 42.1%. No incorrect/undetermined responses were found for the inappropriate alert display (Table 2). Data of Cohen's Kappa coefficients measuring the consistency between two clinical pharmacists show that the Kappa for alert display was 0.884 (95% CI: 0.86-0.91, $P < 0.0001$), and for physician's responses was 0.923 (95% CI: 0.89-0.94, $P < 0.0001$), indicating a satisfactory consistency between the evaluators (Table 3).

Main reasons for overriding alert might lie in evidence-based support, need for evaluation of the therapeutic benefits and potential risks, and need for ongoing monitoring of indicators. Figure 2 shows the counts of overridden alerts classified with the reasons. The top 3 overriding reasons were drug and disease contraindications (23.8%), drug and test value contraindications (23.3%) and compatibility issues (17.7%) (Figure 2). Table 4 shows some examples of

**Table 1. The demographic data in patients of the involved recorders**

| Items | Patients, *n* (%) |
|---|---|
| Gender | |
| Female | 727 (52.42%) |
| Male | 660 (47.58%) |
| Age (years) | |
| ≤ 49 | 195 (14.06%) |
| 50-59 | 190 (13.70%) |
| 60-69 | 383 (27.61%) |
| 70-79 | 350 (25.23%) |
| ≥80 | 269 (19.39%) |
| Severity (by Charlson comorbidity index) | |
| 0 | 66 (4.76%) |
| 1 | 51 (3.68%) |
| 2 | 142 (10.24%) |
| 3 | 152 (10.96%) |
| 4 | 201 (14.49%) |
| 5 | 208 (15.00%) |
| ≥ 6 | 567 (40.88%) |
| Number of alert overrides, *n* (%) | |
| 1 | 1282 (92.43%) |
| 2 | 97 (6.99%) |
| ≥ 3 | 8 (0.58%) |

**Table 2. Evaluations on appropriateness of alert generation and physicians' responses**

| Alert display | Physicians' responses, *n* (%) | | | |
|---|---|---|---|---|
| | Appropriate | Inappropriate | Non-decidable | Total |
| Appropriate | 572 (38.1%) | 267 (17.8%) | 30 (2.0%) | 869 (57.9%) |
| Inappropriate | 632 (42.1%) | 0 (0.0%) | 0 (0.0%) | 632 (42.1%) |
| Total | 1204 (80.2%) | 267 (17.8%) | 30 (2.0%) | 1501 (100%) |

**Table 3. Assessments of consistency of the two clinical pharmacists**

| | Alert display | | | Physicians' responses | | |
|---|---|---|---|---|---|---|
| | Value | S. E | Sig | Value | S. E | Sig |
| Kappa | 0.884 | 0.012 | < 0.0001** | 0.923 | 0.013 | < 0.0001** |

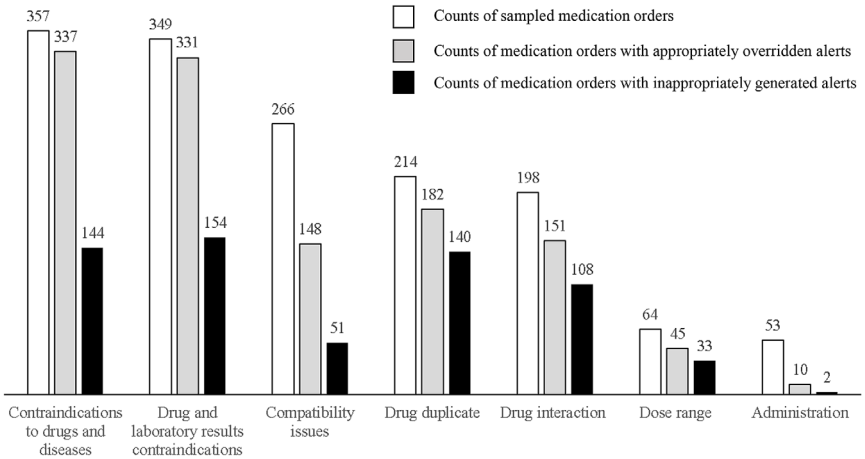


**Figure 2. Distribution map of overridden alert types**

**Table 4. Examples of generating alert and the responses of physicians**

| Type of alerts | Patients (Gender/Birth) | Diagnosis | Alerted order | Appropriateness of generation of alert | Appropriateness of physician's response | Comments |
|---|---|---|---|---|---|---|
| Drug and disease contraindications | M/2014 | Chronic kidney disease, hypokalemia | Potassium chloride injection: "Contraindicated for patients with renal insufficiency." Source: Drug package insert | Appropriate | Appropriate | Recommend by the Expert Consensus on the Management of Serum Potassium in Chronic Kidney Disease Patients in China (2020) |
| | F/1972 | Chronic kidney disease, hemodialysis | Ferrous succinate sustained-release tablets: "Contraindicated for patients with chronic kidney failure." Source: Drug package insert | Appropriate | Appropriate | Recommended by the Clinical Practice Guidelines for the Diagnosis and Treatment of Renal Anemia in China (2021) |
| | F/1939 | Anaphylactic shock, diabetes | Patients with diabetes are prohibited from using epinephrine injection solution. Source: Drug package insert | Appropriate | Appropriate | Currently under continuous monitoring |
| Drug and laboratory results contraindications | F/1931 | Community-acquired pneumonia | Torsemide injection: "Contraindicated for patients with hyponatremia. Attention required if the patient's serum sodium is below the normal value" Source: Drug Package Insert. | Appropriate | Appropriate | The patient had a blood sodium level of 135 mmol/L, indicating mild hyponatremia. Continuous monitoring of electrolytes was in progress |
| Compatibility issues | F/1955 | Breast cancer | Pirarubicin injection is incompatible with 0.9% Sodium chloride injection and should be avoided! Dissolve pirarubicin hydrochloride only with 5% dextrose injection or sterile water for injection to prevent the impact on potency or turbidity due to pH. Source: Drug package insert. | Appropriate | Not Appropriate | |
| Drug duplicate | M/1971 | Gastric cancer | Oxycodone hydrochloride prolonged-release tablets and morphine hydrochloride tablets both belong to the opioid alkaloid | Appropriate | Appropriate | Morphine used for the relief of breakthrough pain |
| Drug interactions | F/1970 | Thyroid nodule | Zhi Gancao (Processed Licorice) interacts with Seaweed, known as "Eighteen Incompatibilities"; Gancao antagonizes seaweed. Source: Chinese pharmacopoeia. | Appropriate | Appropriate | In the "Haizao Yuhu Decoction" (found in the "Orthodox Surgery" by Chen Shigong of the Ming Dynasty), the combination of Seaweed with Raw Licorice or Processed Licorice has the function of resolving phlegm, softening hardness, and dispersing goiter and tumors. |

**Table 4. Examples of generating alert and the responses of physicians (continued)**

| Type of alerts | Patients (Gender/Birth) | Diagnosis | Alerted order | Appropriateness of generation of alert | Appropriateness of physician's response | Comments |
|---|---|---|---|---|---|---|
| Dose range | F/1932 | Respiratory failure, somnolence | Nicardipine injection solution, when administered intravenously, has a maximum dose of 1.25 grams per administration (approximately 3.3 vials). The current dosage is 5 vials. Please be cautious! Source: Drug package insert. | Appropriate | Appropriate | For patients with somnolence, initially administer 0.375g intravenously at a slow rate. Subsequently, add 1.875-3.75g to 500 mL of fluid, and infuse intravenously at a rate of 25-30 drops per minute. Source: Internal medicine (5th edition) The family members refused to use the ventilator and signed the informed consent |
| Route of administration | F/1974 | Ovarian cancer | Carboplatin injection is prohibited for intraperitoneal infusion. Source: Drug package insert. | Appropriate | Appropriate | Recommended by the Expert Consensus on the Clinical Application of Intraperitoneal Hypothermic Perfusion Chemotherapy for Gynecological Malignant Tumors (2019) |

generation of alert and the responses of physicians. Total 417 drugs/administrations, including injection of potassium chloride, ferrous succinate sustained-release tablets, and injection of epinephrine, triggered alerts, which accounted for over 10% of alerts (Table 4). In light of the system-provided inputting alert override reasons, 88.3% of the alert overrides reasons were inputted as some meaningless characters or values, such as "123", "aaa", *etc*., which indicated that an obvious "alert fatigue" existed, which made the physicians become impatient to the alerts, as well as the following overrides.

## 4. Discussion

MRCDSS is useful to improve the safety of medication (*13*). However, if the system has flaws, such as oversensitivity, it might bring fatigue to the physicians and have a negative impact to the working efficiency (*1,4*), and potentially increase the adverse events of drugs (*5,6*). Constantly improvement of the algorithms is therefore indispensable, of those, understanding the *state quo* of the MRCDSS in practice is the first step. To the best of our knowledge, the present study is the first one to investigate the appropriateness of alerts generated by MRCDSS and the physician's responses in China. Our results show that 86.6% of alerts ≤ level 3 were overridden. Of the 1501sampled alerts, 80.2% were appropriately overridden. Of the sampled 1,501 alerts, 57.9% were appropriately generated and 42.1% were inappropriately generated. The top 3 alert types were drug and diagnosis contraindications (23.8%), drug and test value contraindications (23.3%), and compatibility issues (17.7%). In terms of the physicians' responses, 80.2% were appropriate, 17.8% were inappropriate, and 2.0% were undetermined. Most of the overrides reasons (88.3%) were inputted as meaningless characters or values, rather than cautiously inputted information. This phenomenon indicated that the physicians were quite impatient to the meaningless alerts, and had "alert fatigue". Our data suggested that the MRCDSS now using in China is far from satisfactory. More efforts on optimization of the system and algorithms are anticipant. We believe the findings of the present study are useful for better understanding the *state quo* of the MRCDSS in China.

### 4.1. Appropriateness of alert generation in MRCDSS in China

Our results show that 86.6% of alerts ≤ level 3 were overridden, and 57.9% of alerts were appropriately generated of the sampled 1501 alerts (Table 2), whereas 42.1% were inappropriate. The top 3 alert types were drug and diagnosis contraindications, drug and test value contraindications, and compatibility issues (Figure 2). In comparison with an analogous in Korea (*1*), our overridden rate was lower (86.6% *vs.* 92.9%). Because of

the difference of clinical guidance, pharmacopeia, habits of drug usage, and MRCDSS *per se* are quite different among different countries, these data might could not be compared directly. But the data in this study and in Korea were in a review paper (*3*), namely the ranges of overridden rate (46.2%-96.2%), and the appropriate rate (29.4%-100%). The potential reasons caused high overridden rate in our system might be: *i*) errors of trigger data, *ii*) lack of system functionality, *iii*) failure of unstructured text to be involved, *iv*) alerts content did not match drug instructions. Our data along with the previous studies (*1,3,4*) also supported that the MRCDSSs in practice by far are far from satisfactory. The most predominant problem is the oversensitivity of the system, that generates many useless and unnecessary alerts, which therefore must be overridden. However, the alerts can be reduced and become more specific by optimizing the knowledge base and updating the drug instruction. Some researchers developed a Disease-Drug Interaction Scoring Tool (DIST) based on available newest evidence, patient relevance and evidence credibility (*14*). Once this DIST were added and applied to the Kaiser Permanente's drug-disease knowledge base, the monthly alerts reduced from 32,045 to 1,168 (*15*). Since the drug instruction might be outdated in comparison with the newest evidence, constantly updating the knowledge base is indispensable. Another important issue is the severity of illness. An example of chronic kidney disease (CKD) can support this issue. Alerts of "use of contraindicated drugs" are more commonly triggered in patients with CKD stage 4 (*vs.* the other stages) due to the complicated pathophysiological states (*16*). To quantify the contraindications in drug and diagnosis, we suggest that disease, particularly the chronic diseases like hypertension, diabetes, and CKD, should be stratified by severity as mild, moderate and severe to improve the appropriateness of alerts. In addition, we found that several drugs, such as potassium chloride injection, ferrous succinate sustained-release tablets and epinephrine injection, accounted for more than 10% of overrides (Table 4). To reduce the alert override rate, selection of targeted interventions might be a solution. In a word, the MRCDSS requires timely maintenance, data updating, optimization of the algorithms, and fix of the bugs. Importantly, the sensitivity of triggering the alerts should be adjust to a proper level to avoid generation of inappropriate alerts.

### 4.2. Appropriateness of physicians' responses

Appropriate rate of the physicians' responses in this study were 80.2%, whereas 17.8% were inappropriate, and 2.0% were undetermined. The reliability of the physicians' responses was confirmed by assessments of the consistency of the two clinical pharmacists (Table 3). Our appropriate rate is lower than that of Korea study (92.4%) (*1*). The higher appropriate rate of physicians' response than alerts generation indicated that the MRCDSS so far is still in a developing state, which requires constantly optimization. The appropriateness of physicians' response is decided by two aspects. One aspect is the factor of physicians. Certainly, KAP of physicians can certainly affect the appropriateness, but both the present study and Korea study reported a relative higher rate of physicians' response, indicates that the professional ability of the physicians is reliable. Thus, the KAP factor might play a less role in the appropriateness of the physicians' responses. Another aspect is the factor of MRCDSS, we believe that the flaws of the MRCDSS may be a more remarkable factor to influence the appropriateness of physicians' responses. Based on the oversensitive nature of the MRCDSS so far, most of the alerts were meaningless and unnecessary. This will cause a "cry wolf" effect, and bring fatigue to the physicians, which make the physicians are prone to ignore the alerts, and impact the appropriateness of physicians' responses. The problem of "alert fatigue" has been a main limitation of MRCDSS which might lower the application effects of MRCDSS, and requires urgent resolution (*17*). Thus, appropriate sensitivity and accuracy of MRCDSS can reduce generation of useless alert and related alert fatigue, which is the improvement direction in developing and maintaining the MRCDSS. Additionally, Dekarske *et al.* found that physicians tend to choose predefined override reasons if the list of override reasons is available. Accordingly providing an appropriate list of override reasons might be helpful (*18*).

### 4.3. Recommendation and insights

On the basis of our results and our clinical experience, the following issues are recommended as for development and optimization of the MRCDSS system: *i*) High-level newest evidence, such as clinical guideline, must be imported to the system during each optimization, and be ensured if it can be implemented correctly. *ii*) Our data of appropriateness of both alert generation and physicians' responses supported that appropriate sensitivity and accuracy of generating alerts contribute to reduction of the useless alerts and avoiding the "alert fatigue" of physicians, which play a vital in the future development/ optimization of MRCDSS system. *iii*) Delays of data synchronization should be eliminated. Natural language processing is recommended being used to solve the problems such as lack of structured data partially. *iv*) The alert system should be periodically reviewed and maintained to reduce generation of useless alerts. *v*) Alert-review-rules should be constantly optimized. High-frequency alerts against certain drugs shall be noticed and evaluated. Accuracy of these alerts should be checked and useless alerts should be timely removed. All the flaws and bugs should be revised in time. *vi*) The human-computer interaction interface should be friendly. For example, the drop-down menu should be designed

to allow selection of the override reasons, which can be customized and revised as per the actual situation.

4.4. Strengths and limitations of the present study

This study has several strengths: *i*) This is the first study describing the *state quo* of MRCDSS actually in practice in China. *ii*) We sampled 1501 alert recorders from 12,659 overridden medical orders, which sample size is higher than the analogous published studies. *iii*) The consistency between two clinical pharmacists was evaluated using the Cohen's Kappa coefficients, the results indicate that the results of the appropriateness of the physicians' responses were reliable. *iv*) MRCDSS evaluated in this study is a commonly used system in hospitals in China (*19*). The results of the present study contribute to better understanding the *state quo* of MRCDSS in China and providing useful insights for developing and improving MRCDSS in the future.

Meanwhile, this study also has several limitations: *i*) This is a single-center study, the data of the present study were acquired in one hospital, which might have limited representativeness. *ii*) We conducted only an observational study, many important issues, like the relationship between adverse events and overridden alerts, illness severity and overridden alerts, were not investigated. All these limitations will be addressed in our following investigations.

## 5. Conclusion

This one-center, observational, one-center study evaluated the appropriateness of overridden alerts of MRCDSS in China. Our data indicated that the overridden rate was still high, and appropriateness of generation of alert was only 57.9%. These data indicated that the MRCDSS so far needs constantly optimization and timely maintenance. Of those, proper sensitivity to reduce triggering of useless alerts and generation of alert fatigue in physicians might play a vital role, which is highly anticipated.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
*Address correspondence to:*

Zhigao He, Department of Pharmacy, Longhua Hospital of Shanghai University of Traditional Chinese Medicine. 725, Wanping South Road, Xuhui District, Shanghai 200032, China.
E-mail: zhigaohe@hotmail.com

Li Yi, Department of Nephrology, Longhua Hospital of Shanghai University of Traditional Chinese Medicine. 725, Wanping South Road, Xuhui District, Shanghai 200032, China.
E-mail: liyi_1313@126.com

Tetsuya Asakawa, Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Bulan Road, Shenzhen, Guangdong 518112, China .
E-mail: asakawat1971@gmail.com

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# Beneficial impact of visual stimulation-based digital therapeutics on blood pressure control in non-hypertensive individuals

**Yiwen Jiang[1,§], Hong Liu[2,3,§], Lingrui Yang[4], Chen Wu[1], Feng Jiang[5], Yaosheng Wang[1,4,5,*]**

[1] Department of Cardiology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China;
[2] Business School, University of Shanghai for Science and Technology, Shanghai, China;
[3] Shanghai University of Medicine & Health Sciences, Shanghai, China;
[4] Clinical Research & Innovation Unit, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China;
[5] Chongming Hospital Affiliated to Shanghai University of Medicine and Health Sciences, Shanghai, China.

**SUMMARY** Hypertension-related diseases occur in both hypertensive and non-hypertensive individuals. However, few studies to date have explored blood pressure (BP) control in non-hypertensive individuals. This before-after study aimed to examine the impact of visual stimulation-based digital therapeutics (VS-DTx) on BP and heart rate (HR). Eighty-three eligible non-hypertensive participants were included in this study. The McNemar test and Paired Samples Wilcoxon Signed Rank Test were employed to assess decline rates and differences in BP and HR between the control phase and the intervention (using VS-DTx) phase. Pairwise correlation analysis was used to analyze the correlation between the two phases. This study found the systolic BP (SBP) and mean arterial pressure (MAP) in the VS-DTx phase showed a downward trend (66.2% *vs* 49.3%; 68.7% *vs* 55.4%). The mean SBP decreased from 114.73 mm Hg to 111.18 mm Hg, and the mean MAP decreased from 87.96 mm Hg to 84.88 mm Hg in the VS-DTx phase. Paired Samples Wilcoxon Test showed differences in both ΔSBP ($Z = -3.296$; $P < 0.01$) and ΔMAP ($Z = -2.386$; $P < 0.05$) (Δ is defined as the difference between baseline and post-stimulus). The pairwise correlations analysis revealed that VS-DTx affected the MAP reduction ($r = 0.33$; $P < 0.01$) between the browsing digital devices phase and the VS-DTx phase. The results indicated that VS-DTx may have a certain effect on BP, including SBP and MAP. This study preliminarily explored the possible effects of VS-DTx on BP, providing certain useful insights for future research in digital BP management.

***Keywords*** vagal nerve stimulation, visual stimulation, digital therapeutics, blood pressure, heart rate

## 1. Introduction

Hypertension, as a leading cause of mortality, poses a substantial public health challenge. According to the World Health Organization, over one billion individuals are affected by systemic hypertension, leading to 7.5 million deaths worldwide annually, representing 13% of total global mortality (*1,2*). Studies have demonstrated that hypertension, the most important and modifiable risk factor for ischemic heart disease, cerebrovascular disease, as well as chronic kidney disease in humans, has emerged as a global public health concern (*3,4*). It has been suggested that better blood pressure (BP) control leads to a reduced risk of future cardiovascular events. Systolic BP (SBP) of at least 110 mm Hg has been related to multiple cardiovascular and renal outcomes. It was associated with more than 10 million deaths and 212 million disability-adjusted life-years in 2015, a 1.4-fold increase since 1990 (*4,5*). This indicates that people suffer from hypertension-related diseases regardless of high or previously considered optimal (defined as SBP of less than 120 mm Hg and diastolic BP (DBP) of less than 80 mm Hg) BP. In this regard, maintaining a relatively low BP within the normal range may aid in the prevention of potential cardiovascular disease (CVD).

Current treatments for hypertension, encompassing pharmacological and non-pharmacological interventions, are not applicable to normotensive adults seeking to prevent hypertension. Guidelines at present do not advocate for medication usage to regulate blood pressure in individuals without hypertension (*6*). While certain non-pharmacological treatments like renal sympathectomy and invasive vagal nerve stimulation (VNS) have demonstrated efficacy, they are accompanied by notable side effects (*7*). The recent introduction of transcutaneous VNS (tVNS), a non-

invasive VNS therapy, poses challenges due to its intricate operation, substantial equipment prerequisites, and associated costs (*8*). Guidelines recommend certain preventive measures for hypertension, such as moderate exercise and a healthy diet. These measures have an effect on the regulation of BP levels but need to be long-lasting to realize their full potential. Consequently, an effective, non-pharmacological, and non-invasive daily method for blood pressure management is urgently required. A contemporary health management strategy labeled as "digital therapeutics (DTx)" has surfaced in recent years, leveraging digital platforms such as smartphone applications for the prevention and management of medical conditions (*9,10*). As a non-pharmacological and non-invasive BP management approach with great potential, DTx holds promise in opening a new horizon to help non-hypertensive individuals control their BP. There have been limited studies on this topic. Therefore, research is urgently needed to fill these gaps. This study presents a fresh outlook and preliminary exploration of the potential impact of DTx on BP levels.

The vagal nerve plays an essential role in maintaining physiological homeostasis. Given its innervation of the heart, VNS has been explored as a potential treatment for CVD (*11,12*). It has been confirmed to effectively improve left ventricular hemodynamics in heart failure patients, ameliorate post-myocardial infarction remodeling in the myocardium, and reduce the risk of atrial fibrillation, *etc*. (*12,13*). Studies have shown that a decrease in vagal cardiac tone is associated with and contributes to the development and maintenance of high BP (*14*). As a result, VNS has been investigated as an alternative treatment for hypertension. However, existing VNS treatment options are generally invasive, expensive, and associated with side effects (such as dysphonia, vocal hoarseness, dyspnea, paresthesia, and pain) (*8,15*). These operations are so complicated that they need medical support in the meantime–furthermore, the aforementioned treatments primarily target diagnosed hypertension and other CVDs (*16*). Indeed, there is a lack of a practical approach to assist non-hypertensive individuals in managing their BP.

Numerous studies have shown that both images and colors can influence human perception to varying extents (*17,18*). The optical images, with blurred patterns and moving plaids, are thought to induce VS and visual bistability (*19*). Visual bistability is a perceptual stimulus and is thought to potentially induce VNS (*19,20*). Building upon these theoretical foundations, we developed a smartphone application designed to stimulate the vagal nerve through the use of optical images. Through our study, we conducted a preliminary exploration of the effects of VS-DTx on BP and expect to provide a little useful guidance for the future development of non-invasive, non-pharmacological, cost-effective, and safe methods of BP control.

## 2. Methods

This before-after study involved participants from western and eastern China. It was conducted by the principles of the Declaration of Helsinki. All participants have read and signed the electronic form of informed consent before participating. The protocol was approved by the Ethics Committee of Chongming Hospital Affiliated to Shanghai University of Medicine and Health Sciences (CMEC-2022-KT-54).

### 2.1. Participant

Participants were recruited between January 2023 and February 2023. They were screened for eligibility after informed consent was obtained. A total of 95 individuals with a clinic-based SBP of 90-140 mm Hg and/or DBP of 60-90 mm Hg (from at least three separate visits) were enrolled. 83 participants, ranging in age from 18 to 65 years old (mean = 39.43; SD = 11.41), were assigned to the study, including 32 males and 51 females. The main reason for exclusion was not meeting the diagnostic criteria of normal BP. Before participating in the experiment, participants were informed about potential side effects, including possible dizziness and a decrease in BP due to VNS. Participants were required to abstain from taking antihypertensive medications or any other medications that could affect BP within the preceding two months. Individuals with any form of CVD, epilepsy, uncontrolled diabetes, severe mental illness, drug or alcohol abuse, pregnancy, and who have contraindications for the use of VNS were also excluded, as were those who had previously participated in another research trial. Additionally, Participants were instructed not to consume caffeine, smoke, or experience hyperkinesia on the day of the experiment.

### 2.2. Intervention

Prior to commencing the study, participants were required to rest in a quiet room for a minimum of 15 minutes. Following this, participants were asked to receive interventions, which included blank control and VS-DTx intervention. The intervention measure of the blank control was to have participants browse digital devices (view relaxing short video content) for five minutes as the browsing digital devices phase. The measure of the VS-DTx intervention was instructed to utilize the VS-DTx app for five minutes as the VS-DTx phase (see Figure 1A). Each participant underwent both blank control and VS-DTx intervention, separated by a 24-hour washout period. Due to the variations in BP at various times of the day, this would negate the impact of the control phase and ensure that both phases were carried out at the same time for two consecutive days (*21*). To achieve blinding, the order of the interventions received by participants was randomized. The study was

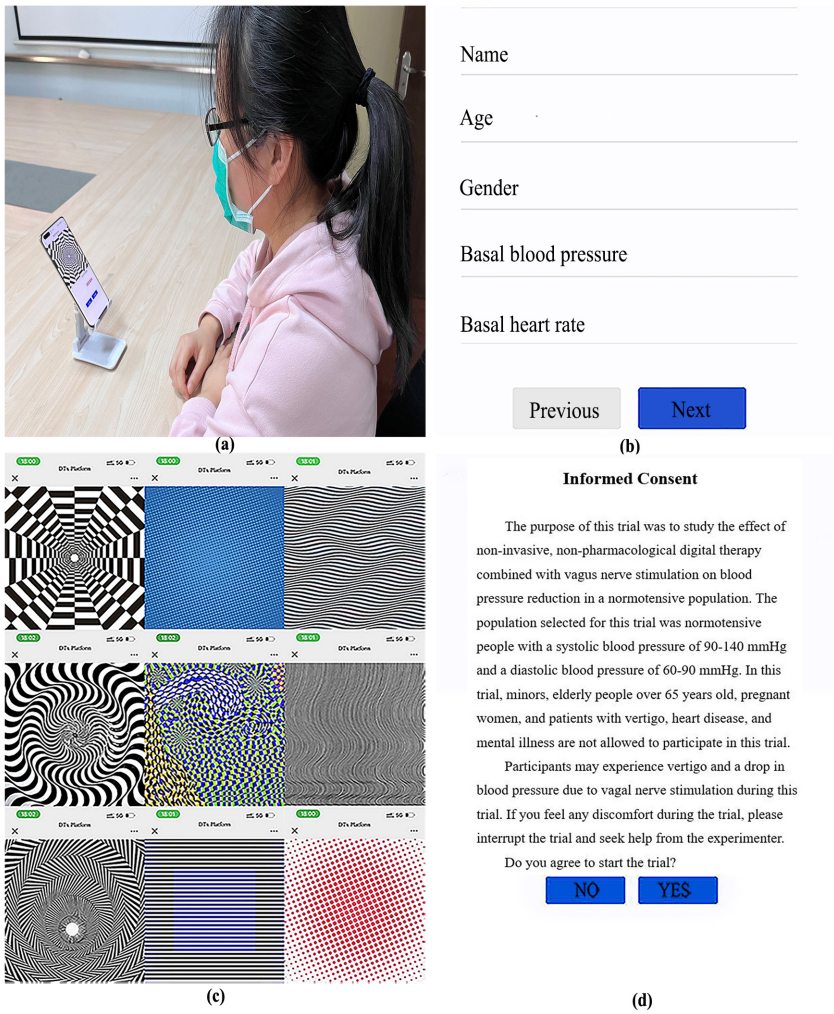


**Figure 1. The main interface of this app**. **(a)** Participant in VS-DTx phase. **(b)** The basic information. **(c)** The visual stimulus images. **(d)** Informed consent (English translation version).

conducted in a quiet room without any other distractions, and the digital devices running the app were positioned at a visual distance of 30 cm from each participant. This app (see Figures 1B-1D) contains basic information, informed consent, and VS images (which were selected from the optical art style).

## 2.3. Measures

The within-subjects approach used in this study has the advantage of effectively controlling the influence of subjects' variables on the results (*22*). The study included two phases: the browsing digital devices phase and the VS-DTx phase. The dependent variables are BPs, HR, ΔBPs, and ΔHR. BPs include SBP, DBP, and MAP. SBP and DBP are defined as the amplitude of the peak and the trough of the BP waveform, respectively. MAP represents the average BP within a single cardiac cycle (MAP = 1/3 SBP + 2/3 DBP), and HR is defined as the number of heartbeats in a minute (*23*). The process of measuring BP was performed according to a standard protocol recommended by the 2018 Chinese Guidelines for Prevention and Treatment of Hypertension (*24*). An assessor measured participants' BP and HR using a standard arm BP cuff and a sphygmomanometer in the brachial artery. The measurement was performed on each arm of the participants to obtain two sets of HR and BP values (two DBP values and two SBP values). The average of the two HR values, the two DBP values, and the two SBP values were calculated separately as the final HR, DBP, and SBP values, and the final MAP was calculated with the final SBP and final DBP. The automatic mode of the sphygmomanometer was used to measure BP without the need for intervention by the assessors other than to place the cuff and switch the device on.

## 2.4. Experimental procedure

According to previous studies, BP exhibits diurnal variations, with higher readings observed in the morning (*25*). To mitigate the potential impact of different times of day on the experimental outcomes, all subjects were measured within the predetermined period. The standard

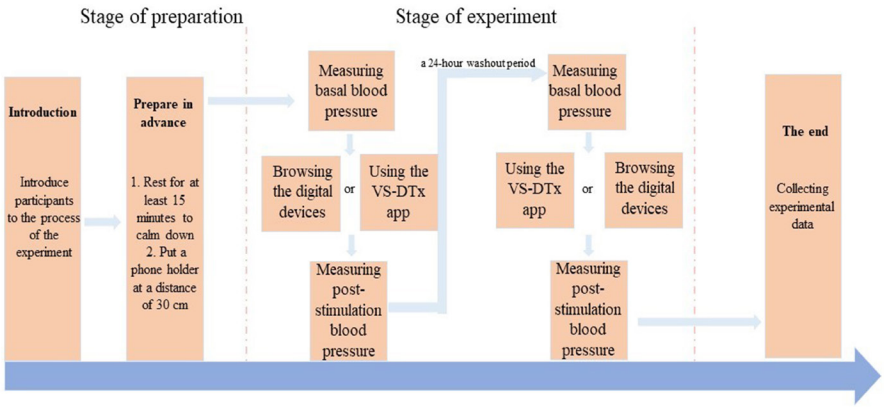


**Figure 2. Standard experimental procedure.**

**Table 2. Descriptive statistics physiological indexes for the different phases**

| Phase | Baseline browsing digital devices M (S.D) | post-stimulus browsing digital devices M (S.D) | Baseline VS-DTx M (S.D) | post-stimulus VS-DTx M (S.D) |
|---|---|---|---|---|
| SBP | 113.90 (11.45) | 113.77 (11.12) | 114.73 (11.15) | 111.18 (10.83) |
| DBP | 75.43 (7.32) | 73.89 (7.81) | 74.57 (7.88) | 71.73 (8.39) |
| MAP | 88.26 (8.19) | 87.18 (8.42) | 87.96 (8.42) | 84.88 (8.50) |
| HR | 75.84 (9.34) | 74.90 (9.07) | 75.34 (9.11) | 74.34 (9.75) |

**Table 1. Baseline characteristics of the participants**

| | |
|---|---|
| AGE (YEARS) | 39.45 ± 11.39 |
| MALE, N (%) | 32 (38.6) |
| BMI (KG/M$^2$) | 23.26 ± 3.65 |

experimental procedure is illustrated in Figure 2. On the first step of the experiment, participants received a comprehensive introduction to the experimental environment and procedure. Subsequently, they were instructed to sit quietly for a minimum of 15 minutes to allow any BP changes induced by tension to dissipate. BP and HR measurements were then taken to establish baseline values. After that, the participants sat in a chair, positioned at a visual distance of 30 cm in front of them. They were randomized to decide whether to receive the blank control or the VNS-DTx intervention first. After 5 minutes, post-stimulus BP and HR measurements were obtained. Following a 24-hour interval, a similar procedure was repeated. Participants were asked to perform another intervention that they had not previously received (using the VS-DTx app or browsing digital devices). Baseline and post-stimulation BP and HR measurements were recorded as well.

### 2.5. Statistical analysis

The analysis was performed using STATA statistical software (version 15.0, StataCorp, Texas, USA), with a *P*-value of < 0.05 indicating statistical significance. Data normality was assessed with the Shapiro-Wilk normality test. Continuous variables conforming to a normal distribution were compared using a Paired-Samples *T*-Test, whereas those that were not normally distributed were tested using the Paired Samples Wilcoxon Signed Rank Test. Differences in proportions were compared by using the McNemar test. Furthermore, pairwise correlations analysis was used to investigate the correlation between browsing digital devices and VS-DTx phases.

## 3. Results

The baseline characteristics are shown in Table 1 and the descriptive statistics for the physiological indexes for the different phases collected are shown in Table 2.

There were two phases in this study, including the browsing digital devices phase and the VS-DTx phase. McNemar test showed that the proportions of decrease in SBP, DBP, MAP, and HR in the VS-DTx phase were 66.2%, 61.4%, 68.7%, and 61.4%, respectively, whereas the proportions during the browsing digital devices phase were 49.3%, 55.4%, 55.4%, and 50.6%. And there were significant differences between the two phases for the ratio of SBP reduction ($\chi^2 = 4.45$; $P < 0.05$), and MAP reduction ($\chi^2 = 3.90$; $P < 0.05$), but it did not show any significant differences for DBP ($\chi^2 = 0.76$; $P > 0.05$) and HR ($\chi^2 = 1.88$; $P > 0.05$). Further paired comparisons were needed due to the finding of this study that there were overall significant differences in SBP and MAP, at least.

To investigate the differences in BP and HR reduction between the browsing digital devices phase and the VS-DTx phase, a set of Paired Samples Wilcoxon Test was performed. Significant differences were found

on both ΔSBP (Z = - 3.296; $P < 0.01$) and ΔMAP (Z = - 2.386; $P < 0.05$). The data further showed that the BP reductions after VS were greater than those from browsing digital devices. Moreover, a Paired Samples Wilcoxon test was performed to investigate ΔDBP and ΔHR. Results showed that there were no significant differences between the two phases on both ΔDBP ($P > 0.05$) and ΔHR ($P > 0.05$). Higher reductions of DBP and HR after VS were observed than those in the browsing digital devices phase. However, the differences were not significant. All these results are shown in Figure 3.

Furthermore, the scatter plot showed the linear correlation and the stability of the difference between the two phases (see Figure 4). According to the result of pairwise correlations, it is evident that the stability of the difference in MAP ($r = 0.33$; $P < 0.01$) is significant, indicating a high possibility for VS-DTx to have an impact on overall BP control.

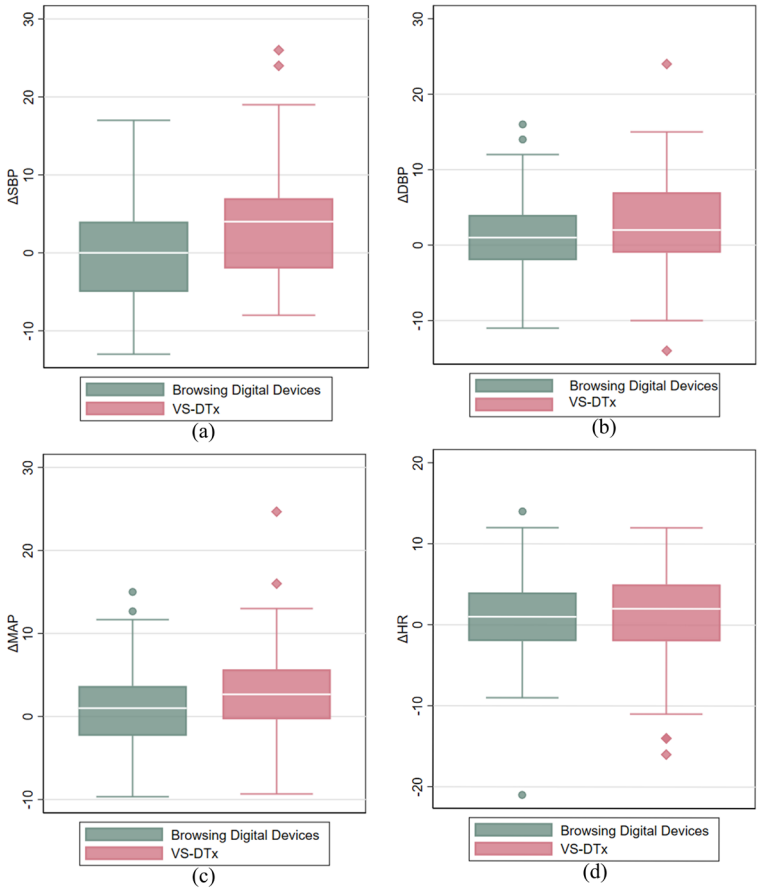


**Figure 3. The results of Paired Samples Wilcoxon Test on ΔSBP, ΔDBP, ΔMAP, ΔHR in different phases, and the results of the distribution of the median are shown in the figure. (a)** ΔSBP in browsing digital devices phase and VS-DTx phase (Z= -3.296; $^{**}P < 0.01$), very significant difference. **(b)** ΔDBP in browsing digital devices phase and VS-DTx phase ($P > 0.05$), no significant difference. **(c)** ΔMAP in browsing digital devices phase and VS-DTx phase (Z= -2.386; $^{*}P < 0.05$), significant difference. **(d)** ΔHR in browsing digital devices phase and VS-DTx phase ($P > 0.05$), no significant difference.

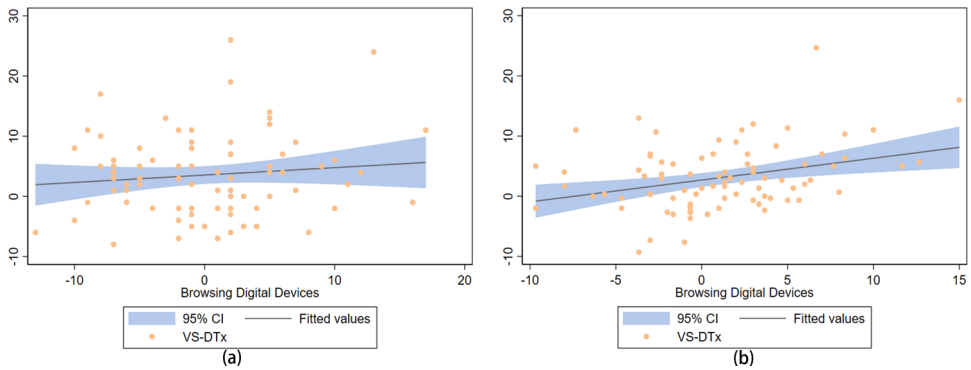


**Figure 4. Scatterplot depicting the correlation between browsing digital devices phase and VS-DTx phase. (a)** Scatterplot of ΔSBP. **(b)** Scatterplot of ΔMAP ($r = 0.33$; $^{**}P < 0.01$), very significant difference.

## 4. Discussion

VNS has exhibited remarkable efficacy in controlling BP in multiple studies. Early research in dogs in 1960 showed that VNS induced bradycardia and reduced atrial contractility, resulting in decreased ventricular filling and stroke volume, ultimately leading to BP reduction (*26*). Recent research has demonstrated that long-term activation of vagal parasympathetic pathways can restore autonomic balance and potentially serve, which is an effective treatment for hypertension in an animal model of neurogenic hypertension (*27*). In human subjects, VNS research has mainly focused on treating epilepsy since the late 1990s. As limited data from human trials is available, research on treating hypertension has only recently begun to grow (*28*). Early VNS devices necessitated the placement of bipolar electrodes encircling the cervical vagal nerve and the subcutaneous implantation of a generator in the chest wall. The surgical technique has been associated with a large number of surgical complications and side effects, including wound infection, cardiac arrhythmia under test stimulation, electrode malfunction, hoarseness, dysphagia, cough, and pain (*29*). Recently, tVNS has been used for BP control. It is a noninvasive device composed of electrodes placed on the skin of the external ear, and connected to a stimulating box (*30*). While tVNS effectively regulates BP, it does require complex parameter settings, specialized treatment equipment, and professional guidance (*8*). Neither the VNS techniques nor the pharmacological therapies detailed above are suitable for daily BP regulation in normotensive individuals. This study explored a novel non-invasive and non-pharmacological DTx approach, and we demonstrated that DTx had certain effects on BP. Its strength lies in its high acceptance, mild side effects, ease of use, and short treatment course. If it can be put into use in the future, users will be more inclined to adhere to it.

It is widely recognized that BP and HR are closely interrelated. As the heart pumps more blood per minute, it increases the lateral pressure on the vascular wall, leading to an elevation in BP. Conversely, when the HR slows down, the lateral pressure on the vascular wall from the blood flow decreases, resulting in a drop in BP. Simultaneously, changes in HR can be secondary to fluctuations in BP. A dip in BP triggers a response from the heart, causing it to be affected by negative feedback, leading to an increase in HR (*31,32*). In the present study, it was observed that HR did not exhibit a significant change in the VS-DTx phase compared to the browsing digital devices phase, while certain changes in BP value were noted. This finding suggested that the effect of VS-DTx on BP was independent of alterations in HR. Instead, it may directly impact SBP and MAP to affect BP.

In the present study, there was no significant change in DBP before and after the intervention. This could be attributed, in part, to the fact that DBP values are generally smaller compared to SBP (approximately 2/3 of SBP) and have a narrower range of variation after stimulation. Meanwhile, the intensity of VS on the vagal nerve is not as strong as direct stimulation (*e.g.*, invasive VNS and tVNS). Therefore, weak vagal nerve stimulation may not lead to a significant variation in DBP. Furthermore, the participants in this study used the app for only five minutes, which may also contribute to the lack of significant change. Chinese hypertension treatment guidelines place greater emphasis on SBP due to stronger evidence of its association with CVD endpoints (*33*). Also, MAP is an important indicator of the average BP level, as it is linearly correlated with all types of CVD endpoints (*34*). In the present study, VS-DTx primarily affected on SBP and MAP, indicating that the impact of VS-DTx on overall BP should not be disregarded, despite the insignificant change in DBP.

Numerous studies have consistently shown that maintaining BP at low levels (within normal ranges) is beneficial to health. One recent investigation found that each 5 mm Hg reduction in SBP was associated with a 10% relative risk reduction of major cardiovascular outcomes, including a 13% less risk for stroke, 7% for ischemic heart disease, 14% for heart failure, and 5% for death (*35*). A prospective study confirmed that people in the higher fitness category had lower BP than those in the lower fitness category (*36*). A recent study examined the effects of standard BP control and intensive BP control in the hypertensive population. Researchers found that lowering SBP to less than 120 mmHg (the intensive goal) resulted in a significant reduction in fatal and nonfatal cardiovascular events, as well as mortality from any cause, compared to the standard goal of less than 140 mm Hg (*37,38*). American College of Cardiology/American Heart Association (ACC/AHA) released guidelines for the management of hypertension in adults in 2017, defined hypertension as an SBP > 130 or a DBP > 80 mm Hg, and defined an SBP of 120-129 or a DBP of 70-79 mm Hg as elevated BP (*39*). The latest 2022 Chinese Clinical Practice Guidelines for Hypertension recommend lowering the diagnostic threshold for hypertension in Chinese adults from SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg to SBP ≥ 130 mm Hg and/or DBP ≥ 80 mm Hg (*6*). Although the benefits of intensive BP control are widely recognized, attention should also be given to BP control in non-hypertensive adults. In this present study, it was found that the mean SBP decreased from 113.77 mm Hg to 111.18 mm Hg, while the mean MAP decreased from 87.18 mm Hg to 84.88 mm Hg. The results also showed significant differences in both ΔSBP ($Z = -3.296$; $p < 0.01$) and ΔMAP ($Z = -2.386$; $p < 0.05$), indicating that VS-DTx had a certain impact on SBP and MAP. These findings suggest that non-hypertensive patients may benefit from VS-DTx.

With the advent of the digital age, there has been

an increasing addiction to mobile electronic devices. This shift highlights the need for a modern approach to health management. Clinical guidelines have provided recommendations to help non-hypertensive adults control their BP, such as maintaining a balanced diet, engaging in moderate exercise, controlling weight, and quitting smoking and alcohol consumption, as well as practicing meditation and yoga. While these methods are practical and effective, they can be slow to take effect, making adherence difficult. VS-DTx, a digital approach to health management, is ideally suited to be embedded in hand-held devices like cell phones and electronic watches. This advanced approach enables people to conveniently control their BP anytime and anywhere, especially younger populations who may have lower awareness and treatment rates for hypertension but are highly skilled in using electronic devices. The accessibility and convenience offered by this digital method empower individuals to take charge of their BP management in a more user-friendly manner. This study aimed to conduct a preliminary exploration investigation into the possibility of the impact of VS-DTx on BP, explore the potential of using VS-DTx to assist in controlling BP in non-hypertensive adults, and provide some new insights for future health management.

## 5. Limitations

Some limitations should be considered in the present study. First, the sample size was relatively small. Studies with larger sample sizes may be necessary to observe more striking differences. Second, the sampling was limited to two regions in China, which may restrict the generalizability of the results to the broader population. Moreover, the gender ratio in this study was not evenly balanced, with 32 males and 51 females, and this imbalance may potentially influence the findings. Furthermore, the study only focused on youth and middle-aged individuals. The sensitivity of BP and HR to VS may vary across different populations, and it is important to investigate the effects in diverse groups. Future trials should aim to include a wider range of individuals to enhance the generalizability and applicability of the findings. Additionally, this study was primarily intended to conduct a preliminary exploration of the effects of VS-DTx on BP. The study results can only indicate certain changes in some BP values and do not clarify the therapeutic effect of the method. In the future, building upon this study, more mature products can be developed to facilitate more comprehensive research for validation.

## 6. Conclusion

VS-DTx may have an impact on BP management, providing some useful insights for future research in digital BP management.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Yaosheng Wang, No.1665, Kongjiang Road, Yangpu District, Shanghai 200082, China.
E-mail: wangyaosheng@xinhuamed.com.cn

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# Bu-Shen-Ning-Xin decoction inhibits macrophage activation to ameliorate premature ovarian insufficiency-related osteoimmune disorder *via* FSH/FSHR pathway

**Hongmei Sun[1,2,3,4,§], Qing Qi[5,§], Xinyao Pan[1,2,3], Jing Zhou[1,2,3], Jing Wang[1,2,3], Lisha Li[1,2,3], Dajing Li[1,2,3], Ling Wang[1,2,3,*]**

[1] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;
[4] Hexi University, Zhangye, Gansu, China;
[5] Wuhan Business University, Wuhan, Hubei, China.

**SUMMARY** Limited studies are associated with premature ovarian insufficiency (POI)-related osteoimmune disorder currently. Bu-Shen-Ning-Xin decoction (BSNXD) displayed a favorable role in treating postmenopausal osteoporosis. However, its impact on the POI-related osteoimmune disorder remains unclear. The study primarily utilized animal experiments and network pharmacology to investigate the effects and underlying mechanisms of BSNXD on the POI-related osteoimmune disorder. First, a 4-vinylcyclohexene dioxide (VCD)-induced POI murine model was conducted to explore the therapeutical action of BSNXD. Second, we analyzed the active compounds of BSNXD and predicted their potential mechanisms for POI-related osteoimmune disorder *via* network pharmacology, further confirmed by molecular biology experiments. The results demonstrated that VCD exposure led to elevated follicle-stimulating hormone (FSH) levels, a 50% reduction in the primordial follicles, bone microstructure changes, and macrophage activation, indicating an osteoimmune disorder. BSNXD inhibited macrophage activation and osteoclast differentiation but did not affect serum FSH and estradiol levels in the VCD-induced POI model. Network pharmacology predicted the potential mechanisms of BSNXD against the POI-related osteoimmune disorder involving tumor necrosis factor α and MAPK signaling pathways, highlighting BSNXD regulated inflammation, hormone, and osteoclast differentiation. Further experiments identified BSNXD treatment suppressed macrophage activation *via* downregulating FSH receptor (FSHR) expression and inhibiting the phosphorylation of ERK and CCAAT enhancer binding proteins β. In conclusion, BSNXD regulated POI-related osteoimmune disorder by suppressing the FSH/FSHR pathway to reduce macrophage activation and further inhibiting osteoclastogenesis.

***Keywords*** premature ovarian insufficiency, Bu-Shen-Ning-Xin decoction, osteoimmune disorder; network pharmacology, macrophages, osteoclastogenesis

## 1. Introduction

Premature ovarian insufficiency (POI), commonly called premature ovarian failure before, is a disease characterized by menopause or sparse menstruation, elevated follicle-stimulating hormone (FSH), and loss of oocytes and folliculogenesis, affecting 3.7% or more of women under age 40 (*1,2*). POI involves a higher risk of infertility, osteoporosis, cardiovascular disease, and a decline in cognitive function (*3-5*); however, studies usually focus on the poor reproductive potential in POI (*3,4*), and little is associated with POI-related osteoporosis currently. Therefore, studies are needed to explore the pathogenesis of POI-related osteoporosis, and strategies are imperative for the disease.

Elevated FSH levels, pointing to the decline of ovarian function, affected the bone immune microenvironment. FSH and bone mineral density (BMD) have a negative correlation (*6*). Likewise, the biochemical markers of bone turnover, including osteocalcin and serum bone-specific alkaline phosphatase, were positively correlated with sharply elevated FSH (*7*), which can enhance bone

resorption independent of estrogen (*8,9*). Mechanically, FSH stimulates tumor necrosis factor (TNF) production from the monocytes to enhance osteoclast formation, involving the MEK/ERK and CCAAT enhancer binding proteins (C/EBP) β signaling pathways (*10,11*).

Traditional Chinese medicine (TCM) has rich experience and unique advantages in treating POI due to its multi-component and multi-target action. Several studies highlighted the clinical efficacy of TCM compounds on POI or osteoporosis (*12,13*). Bu-Shen-Ning-Xin decoction (BSNXD), formulated on clinical experience over the decades and comprising eight herbal medicines, has been used for treating postmenopausal osteoporosis (*14*). In our previous works, BSNXD ameliorated postmenopausal osteoporosis by blocking the nuclear factor of activated T-cells 1 (NFATc1) pathway to inhibit osteoclast differentiation (*15,16*). Moreover, BSNXD could modulate receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin imbalance by regulating the immunocyte function (*17*). Nonetheless, the effects and mechanisms of BSNXD in regulating POI-related osteoimmune disorder are unclear.

This work explored the effects of BSNXD in a 4-vinylcyclohexene dioxide (VCD)-induced murine model, which successfully simulates a POI-related osteoimmune disorder (*18,19*). Then, we analyzed the active compounds of BSNXD and predicted its potential mechanisms on the POI-related osteoimmune disorder *via* network pharmacology, which explores the herbs' pharmacological mechanisms based on system biology and bioinformatics (*20*). Finally, we utilized molecular biology experiments to validate the molecular mechanism.

## 2. Materials and Methods

### 2.1. Animal experiments

#### 2.1.1. Reagents

4% paraformaldehyde was purchased from Shanghai USEN Biological Technology Co., Ltd. (Shanghai, China). Estradiol (E2) parameter assay kit was obtained from R&D System, Inc. (Minneapolis, MN, USA). An enzyme-linked immunosorbent assay kit for FSH was obtained from Cloud-Clone Corp. (Wuhan, China). Tissue RNA extraction kits, reverse transcription-PCR kit, and SYBR Green quantitative PCR assay mix were purchased from HiFun Biotechnology Co., Ltd. (Shanghai, China). Anti-CD16/CD32, PE anti-mouse MHCII, PE anti-mouse CD86, FITC anti-mouse/human CD11b, and APC anti-mouse F4/80 antibodies were provided by Biolegend, Inc. (CA, USA). APC-Cy7 anti-mouse CD45 and BV605 anti-mouse CD3e antibodies were obtained from BD Biosciences (NJ, USA). LIVE/DEAD™ fixable dead cell stain kit and FSH receptor (FSHR) polyclonal antibody were obtained from Thermo Fisher Scientific, Inc. (MA, USA). RIPA and protease inhibitors were obtained from New Cell & Molecular Biotech Co., Ltd. (Suzhou, China). pC/EBPβ and pERK1-T202/Y204 rabbit antibodies were purchased from ABclonal Technology Co., Ltd. (Wuhan, China). Toluidine blue staining solution and tartrate-resistant acid phosphatase (TRAP) dye solution kit were acquired from Servicebio Technology Co., Ltd. (Wuhan, China). Rabbit antibody against tubulin and goat anti-rabbit secondary antibodies were purchased from Proteintech Group, Inc. (Rosemount, IL, USA).

#### 2.1.2. Preparation of BSNXD extracts

Chinese medicine formula granules in BSNXD were obtained from Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). The composition and dosage of BSNXD are listed in Table 1. All voucher specimens were deposited and quantified for quality control at the Obstetrics and Gynecology Hospital of Fudan University. Our previous study analyzed the UPLC fingerprint and the multi-component content of BSNXD (*14*).

#### 2.1.3. Animals and treatment

Thirty female C57BL/6 mice aged 6-8 weeks (Vital River Laboratory Animal Technology Co., Ltd., Zhejiang, China) were adaptively fed in carbonate plastic cages in the Fudan University laboratory (SPF grade) animal room for one week. The environment was kept temperature- and humidity-controlled. The mice were fed a commercial product (Jiangsu Synergetic Pharmaceutical Bioengineering Co., Ltd.). All procedures are carried out in accordance with the requirements of the Laboratory Animal Care Principles (National Institutes of Health Publication No. 85-23, revised 1985) and guidelines for care and use of Fudan University Laboratory Animal (approval number: 2022120016S).

The mice were randomly divided into the control, model, and BSNXD groups ($n$ = 10 in each group). VCD (160 mg/kg/day) was injected intraperitoneally for 15 consecutive days to establish the POI model (*18*). The model and BSNXD groups were given VCD while the control group was given saline for 15 days. At the same

**Table 1. Composition and dosage of BSNXD**

| Chinese name | Latin name | Content (g) |
|---|---|---|
| Di-Huang | *Rehmannia glutinosa* (Gaertn.) DC. | 15 |
| Zhi-Mu | *Anemarrhena asphodeloides* Bunge | 15 |
| Huang-Bai | *Phellodendron chinense* C.K.Schneid. | 9 |
| Gou-Qi | *Lycium chinense* Mill. | 15 |
| Tu-Si-Zi | *Cuscuta chinensis* Lam. | 12 |
| Yin-Yang-Huo | *Epimedium brevicornu* Maxim. | 12 |
| Suan-Zao | *Ziziphus jujuba var. spinosa* (Bunge) Hu ex H.F.Chow. | 9 |
| Ze-Xie | *Alisma plantago-aquatica* L. | 12 |

time, the mice in the BSNXD group were intragastrically administered with BSNXD (1.287 g/kg) daily, and the mice in the control and model groups were given sterile water. Animals were sacrificed under fluoroethane anesthesia on the 30th after the last injection of VCD. Then, ovaries, blood, femurs, tibia, vertebrae, spleen, and mesenteric lymph nodes were collected for further study. The murine body weights were recorded weekly.

2.1.4. Serum FSH and E2 levels measurement

Blood was collected through cardiac puncture, solidified in 2 hours at room temperature, and then centrifuged to obtain serum. In accordance with protocols from the manufacturers, serum E2 and FSH levels were detected with an E2 parameter assay kit and an enzyme-linked immunosorbent assay kit for FSH, respectively.

2.1.5. Hematoxylin and eosin (H&E) staining

The murine ovarian was exfoliated, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five μm paraffin-embedded samples were then sectioned for further H&E staining. H&E-stained segments were observed under an optical microscope (Nikon, Japan) to analyze the morphological changes and follicle count according to the classification standard (*21*).

2.1.6. Micro-computed tomography (micro-CT) scanning and analysis

Murine femurs and vertebrae were fixed with 4% paraformaldehyde. Then, the tissues were scanned and analyzed by micro-CT (SKYSCAN 1176 scanner, Bruker Corporation) at 50 kV voltage and 500 μA current with a scanning parameter of 9 μm per layer. The mouse femur and fourth lumbar vertebra were used for analysis. The bone microstructure parameters were obtained according to the relevant three-dimensional (3D) images in the CTAn software (Bruker micro-CT, Kontich, Belgium) (*22*).

2.1.7. Immunohistochemistry

The fixed murine tibias were proceeded for decalcification with ethylenediaminetetraacetic acid for the TRAP staining, following the steps required by the manufacturer. The undecalcified murine tibias were used for the toluidine blue staining following the instructions. Two pathologists independently and double-blindly observed histological changes using a microscope (OLYMPUS, Tokyo, Japan).

2.1.8. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the homogenized bone tissue using a tissue RNA purification kit. The cDNA was synthesized using a reverse transcription kit. We performed quantitative real-time PCR in triplicate with an SYBR premix ex taq kit on an Applied Biosystems 7900 HT system (Foster City, CA, USA). The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Mouse primers listed in Table 2 were synthesized by Huagene Biotech (Shanghai, China).

2.1.9. Flow cytometry

Single-cell suspensions from spleen, mesenteric lymph nodes, or bone marrow were stained with a LIVE/DEAD™ fixable yellow dead cell stain kit and blocked with anti-CD16/CD32 antibody. Then, the cells were washed in phosphate-buffered saline solution and incubated with antibody cocktails. The antibody cocktails included PE anti-mouse MHCII, FITC anti-mouse/human CD11b, PE anti-mouse CD86, APC anti-mouse F4/80, APC-Cy7 anti-mouse CD45, or BV605 anti-mouse CD3e. The stained cells were analyzed on a

**Table 2. Primers used in this study**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *Fshr* | CCTTGCTCCTGGTCTCCTTG | CTCGGTCACCTTGCTATCTTG |
| *Trap* | CAAGAACTTGCGACCATTGTTA | ATCCATAGTGAAACCGCAAGTA |
| *Ctsk* | CTGAGAATGTGGCTGTGGAG | TACCCTCTGCATTTAGCTGCCT |
| *Mmp9* | GCAGAGGCATACTTGTACCG | TGATGTTATGATGGTCCCACTTG |
| *c-fos* | TCTCTAGTGCCAACTTTATCCC | GAGATAGCTGCTCTACTTTGCC |
| *Nfatc1* | GAGAATCGAGATCACCTCCTAC | TTGCAGCTAGGAAGTACGTCTT |
| *C/ebpβ* | CCAAGCCGAGCAAGAAGC | AGGGCGAACGGGAAACCG |
| *Rankl* | CAAGATGGCTTCTATTACCTGT | TTGATGCTGGTTTTAACGAC |
| *Tnfα* | TATGGCCCAGACCCTCACA | GGAGTAGACAAGGTACAACCCATC |
| *Il6* | CTTCTTGGGACTGATGCTGGTGAC | TCTGTTGGGAGTGGTATCCTCTGTG |
| *Il1β* | AAAAAGCCTCGTGCTGTCGG | GTGGGTGTGCCGTCTTTCAT |
| *Ccl2* | TTAAAAACCTGGATCGGAACCAA | GCATTAGCTTCAGATTTACGGGT |
| *β-actin* | GGCTGTATTCCCCTCCATCG | CCAGTTGGTAACAATGCCATGT |

Ccl: chemokine (CC-motif) ligand; Cebp: CCAAT enhancer binding protein; Ctsk: cathepsin K; Fshr: follicle-stimulating hormone receptor; IL: interleukin; Mmp9: matrix metalloprotein 9; Nfatc1: nuclear factor of activated T-cells 1; Rankl: receptor activator of nuclear factor-κB ligand; Tnf: tumor necrosis factor; Trap: tartrate-resistant acid phosphatase.

CytoFLEX (Beckman Coulter, Inc., CA, USA). FlowJo software (Tree Star, Ashland, OR, USA) was utilized to analyze data.

2.1.10. Western blotting

The supernatant was collected from the lysed bone tissue and quantified using a BCA protein detection kit. The protein samples were separated by electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-FSHR, anti-pC/EBPβ, anti-pERK, or anti-tubulin primary antibodies at 4℃ overnight after being blocked by 5% skim milk powder solution. The next day, the membrane was washed with TBST solution and incubated with a secondary antibody at room temperature. Protein-binding bands are displayed using an ECL reagent on an Amersham Imager 600 (GE Healthcare Life Sciences, MA, USA). Image J (NIH, USA) was performed for quantitative analysis.

2.2. Pharmacological network analysis

2.2.1. Screening the active chemical compound and potential targets of BSNXD

We screened the potential active ingredients of BSNXD under the following filter criteria: Oral bioavailability ≥ 30% and Drug likeness ≥ 0.18 in the HERB (*http://herb.ac.cn*) and TCM systematic pharmacology database and analysis platform (TCMSP, *https://www.tcmsp-e.com/tcmsp.php*). The bioactive compound-related targets were collected from TCMSP and standardized through the UniProt database (*https://www.uniprot.org*). Then, we imported active components and associated gene data into Cytoscape 3.7.2 to build a component-target gene network.

2.2.2. Collection of potential targets of POI-related osteoimmune disorder

The POI and osteoimmune disorder-related targets were obtained from the Disgenet (*https://www.disgenet.org/home/*), OMIM (*https://www.omim.org/*), and GeneCards (*https://www.genecards.org*) databases with the keyword "premature ovarian insufficiency" and "osteoimmune disorder." The overlapped genes were chosen as the targets of BSNXD against POI-related osteoimmune disorder using Venny 2.1.0 (*http://www.liuxiaoyuyuan.cn/*).

2.2.3. Network construction

The overlapped targets were inputted into the STRING database (*https://cn.string-db.org/*). The species was set as "homo sapiens," and the confidence score was set to > 0.4. A protein-protein interaction (PPI) network of the overlapped targets was re-edited and visualized in the Cytoscape 3.7.2 software. Topology attribute parameters are calculated using Cytohubba and cytoNCA plugins, which were used to obtain the top 20 targets and rebuild the critical network.

2.2.4. Enrichment analysis

The overlapped targets were fed into the Metascape database (*https://metascape.org/*) for the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. The involved biological processes and signaling pathways were visualized by bubble charts and bar charts with $P$-value < 0.05, enrichment factor > 1.5, and minimum count of 3.

2.3. Statistical analysis

Date are displayed as mean ± SD. Comparisons were evaluated by $t$-test or one-way ANOVA followed by Dunnett's multiple comparisons tests using GraphPad Prism version 8.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). A $P$-value less than 0.05 was statistically significant.

**3. Results**

3.1. BSNXD slightly ameliorated the ovarian dysfunction in the VCD-induced POI model

To explore the effects of BSNXD, we established a VCD-induced POI murine model. VCD and BSNXD had little impact on body weight (Figure 1A). Since the disorder of sex hormones plays a crucial role in POI progression, the levels of serum FSH and E2 were measured to investigate the murine ovarian function. As shown in Figures 1B-1C, serum FSH and E2 levels increased sharply when mice were exposed to VCD. BSNXD slightly downregulated the serum level of FSH; however, the levels of serum FSH and E2 remained indistinguishable between the model and BSNXD groups (Figures 1B-1C). VCD selectively destroys the primordial and primary follicles, gradually depleting the murine follicle pool (*18*). At 30 days after the last VCD injection, we measured the wet weight of the ovaries and calculated the ovarian index. VCD exposure decreased the murine ovarian index (Figure 1D) and led to a 50% reduction in the primordial follicles and a decrease in other types of follicles (Figure 1E), indicating VCD successfully induced ovarian failure. BSNXD treatment markedly improved the ovarian index (Figure 1D) and saved part of the primordial and primary follicles (Figures 1E-1F).

3.2. BSNXD improved the bone microstructure parameters in the murine vertebrae

Long-term elevated FSH levels contribute to

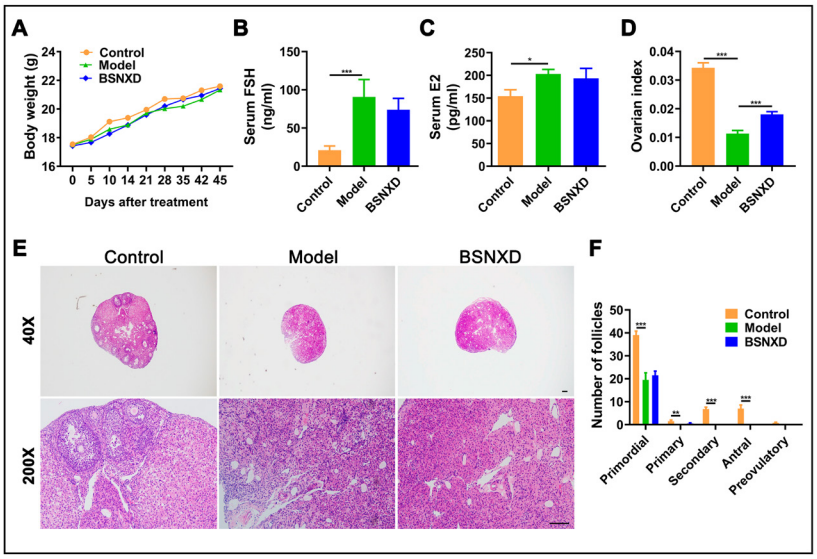


**Figure 1. The effects of BSNXD on body weight, serum hormones, and ovaries. (A)** Body weight. **(B-C)** The serum levels of FSH **(B)** and E2 **(C). (D)** Ovarian index. **(E)** Representative images of H&E-stained ovarian sections at different magnifications. Scale bars are 100 μm. **(F)** The follicle numbers were quantified in ovaries. $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

osteoporosis risk in women with menopause transition (*9*). To investigate whether VCD exposure induced bone morphological changes and reduced bone density, one of the common POI complications, we utilized micro-CT scanning to reconstruct clear 3D images of the murine vertebrae and femur. As shown in Figure 2A, the trabecular bone structure of the control mice was clear, rich, and continuous, connecting into a network; however, an enlarged trabecular separation (Tb.Sp) and trabecular bone volume fraction were observed in the model mice. Notably, BSNXD treatment decreased the bone parameters, including bone surface/bone volume (BS/BV, Figure 2B), Tb.Sp (Figure 2F), structure model index (SMI, Figure 2G), and trabecular bone pattern factor (Tb.Pf, Figure 2H). Meanwhile, BSNXD treatment markedly elevated trabecular thickness (Tb.Th, Figure 2E) and mildly raised the values of bone volume/tissue volume (BV/TV, Figure 2C) and trabecular number (Tb. N, Figure 2D). Next, we measured the murine spine BMD to evaluate bone loss. There was a mild reduction of spine BMD in the VCD-induced mice, but BSNXD treatment slightly improved the spine BMD (Figure 2I).

### 3.3. BSNXD improved the bone microstructure parameters in the murine femur

We also further analyzed the bone microstructure of the murine femur (Figures 3A-3B). Apparent voids among bone trabeculae in the cancellous bone were observed in the murine femur in the model group. The values of BS/BV (Figure 3C), Tb.Sp (Figure 3G), SMI (Figure 3H), and Tb.Pf (Figure 3I) increased while the values of BV/TV (Figure 3D) and Tb.Th (Figure 3F) decreased in the model group, showing structural changes in the trabecular bone after VCD exposure. BSNXD treatment significantly reverses these parameters' change, improving the bone microstructure of the murine femur. There were no indistinguishable changes in the cancellous BMD (Tb.BMD, Figure 3J) and cortical BMD (Ct.BMD, Figure 3K) among the three groups, probably due to the short period of the experiment.

### 3.4. BSNXD inhibited the differentiation and maturity of the osteoclast

Bone remodeling depends on the balance of the number and function of osteoblasts and osteoclasts (*23*). To determine the impact of BSNXD on osteoblasts or osteoclasts, we quantitatively analyzed bone turnover parameters and histological morphology *via* toluidine blue and TRAP staining. The bone histomorphometric parameters indicated an increased number of osteoblasts and osteoclasts after VCD treatment (Figures 4A-4C). BSNXD could reduce the osteoclast number (Figures 4A, 4C) but have no obvious inhibitory effect on the osteoblast number (Figures 4A-4B). Next, to investigate whether BSNXD inhibits osteoclast differentiation, we induced each group's murine bone marrow-derived macrophages to differentiate *in vitro*. The number of $TRAP^{+}$ cells increased in the model group on day 7 according to the number of nuclei (Figure 4D). Meanwhile, BSNXD treatment decreased the number of $TRAP^{+}$ multinucleated cells (Figure 4D). We also detected the gene expression related to osteoclastogenesis and osteoclasts' function. BSNXD treatment led to significantly reduced gene expression of *Trap*, matrix metalloprotein 9 (*Mmp9*), cathepsin K (*Ctsk*), *c-fos*, *Nfatc1*, and *Rankl* compared with the model group (Figure 4E), presenting a significant impact of BSNXD on osteoclastogenesis and osteoclast maturity.

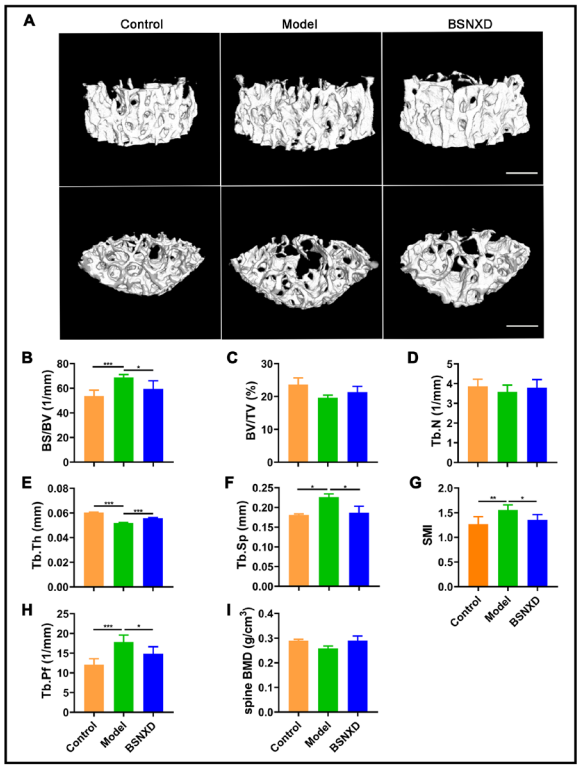


**Figure 2. BSNXD treatment protected against bone loss in the lumbar vertebra in the VCD-induced POI model.** **(A)** Representative 3D images of the 4th lumbar vertebra. Scale bars are 0.1 mm. (B-I) Comparative analysis of the bone structural parameters: **(B)** BS/BV, **(C)** BV/TV, **(D)** Tb.N, **(E)** Tb.Th, **(F)** Tb.Sp, **(G)** SMI, **(H)** Tb.Pf, and **(I)** spine BMD. $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

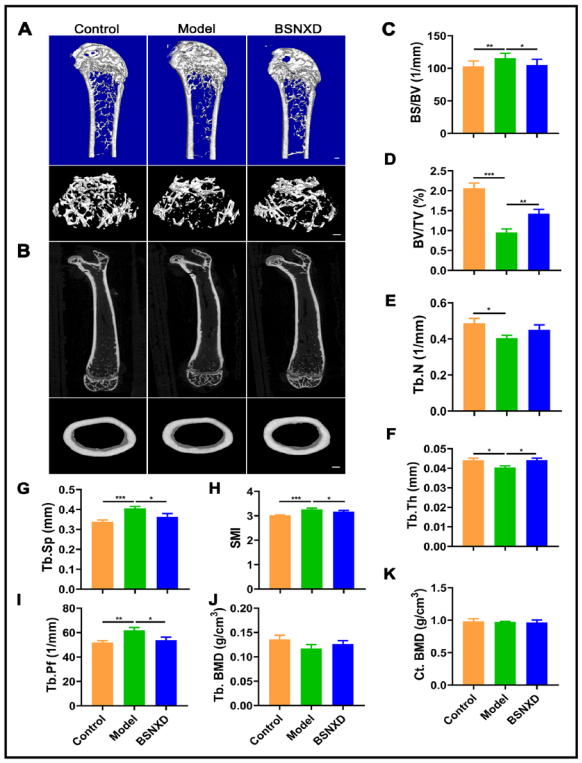


**Figure 3. BSNXD treatment ameliorated the bone microstructure parameters in the femoral bone in the VCD-induced POI model.** **(A-B)** Representative 3D reconstruction images of **(A)** cancellous and (B) cortical bone in the femur. Scale bars are 0.25 mm. **(C-K)** Comparative analysis of the trabecular parameters of the femur: **(C)** BS/BV, (D) BV/TV, **(E)** Tb.N, **(F)** Tb.Th, **(G)** Tb.Sp, **(H)** SMI, **(I)** Tb.Pf, **(J)** Tb.BMD, and **(K)** Ct.BMD. $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

### 3.5. Network pharmacology predicted potential mechanisms of BSNXD regulating the POI-related osteoimmune disorder

#### 3.5.1. The intersection targets between BSNXD and POI-related osteoimmune disorder

To elaborate on the underlying therapeutic mechanism, we applied network pharmacology to determine the potential candidate compounds of BSNXD, which consists of the dried root of *Rehmannia glutinosa* (Gaertn.) DC., rhizomes of *Anemarrhena asphodeloides* Bunge, *Phellodendron chinense* C.K.Schneid., seed of *Lycium chinense* Mill., *Cuscuta chinensis* Lam., *Epimedium brevicornu* Maxim., seed of *Ziziphus jujuba var. spinosa* (Bunge) Hu ex H.F.Chow., and *Alisma plantago-aquatica* L. (Table 1). One hundred and fifty-three candidate compounds of BSNXD were obtained based on the oral bioavailability and drug likeness (Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=197*), some of which, including quercetin, kaempferol, beta-sitosterol, and anhydroicaritin, have been identified in the BSNXD fingerprint analysis (*14*). Figure 5A shows the components-targets network of BSNXD. Two hundred forty-three compound targets were sorted out using the TCMSP and HERB databases (Figure 5B). A total of 1264 POI and osteoimmune disorder-related targets were obtained using the OMIM, GeneCards, and Digenst databases. The Venn analysis diagrams shows the intersection targets of BSNXD against POI-related osteoimmune disorder, illustrating 127 overlapping targets (Figure 5B).

#### 3.5.2. PPI network construct

Next, we utilized the 127 proteins to construct a preliminary network in the STRING database and obtained the PPI network, which was re-edited by Cytoscape software (Figure 5C). The node degree average is 45. The top 20 hub targets were screened out (Figure 5D), representing a greater probability that the active ingredients will act on these targets.

#### 3.5.3. GO and KEGG analysis

To assess the involved signaling pathway regulated by BSNXD, we performed GO and KEGG enrichment analysis for the top 20 hub targets using the Metascape database. As shown in Figure 5E, the core genes were enriched to 687 biological process entries, 16 cellular

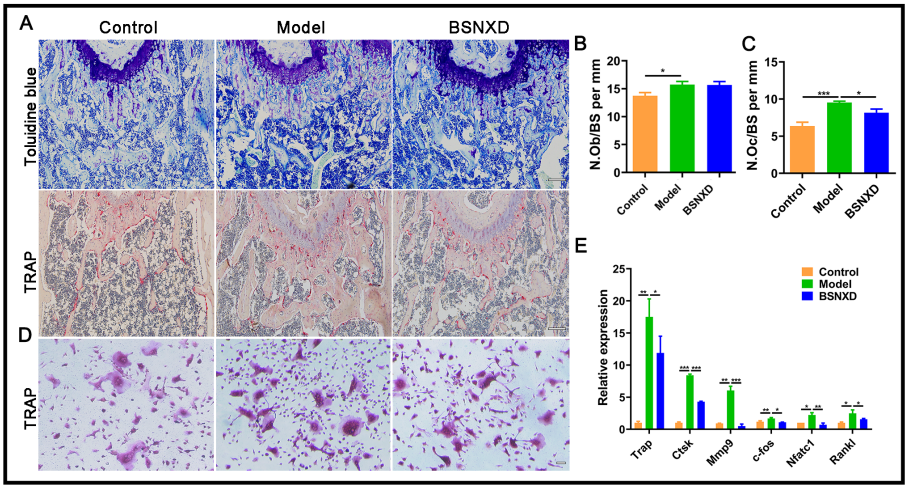


**Figure 4. BSNXD treatment inhibited osteoclastogenesis in the VCD-induced POI model**. **(A)** Histological analysis of the tibia bones, including toluidine blue and TRAP staining. **(B-C)** Quantitative analysis of **(A)**: **(B)** N.Ob/BS and **(C)** N.Oc/BS. Scale bar represents 100 μm (toluidine blue and TRAP staining). Murine bone marrow-derived macrophages from each group were cultured for RNAKL-induced osteoclastogenesis. **(D)** Representative images of TRAP staining after seven days of osteoclast differentiation. Scale bars present 50 μm. **(E)** The gene expression of *Trap, Ctsk, Mmp9, c-fos, Nfatc1*, and *Rankl* in murine tibial bone. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

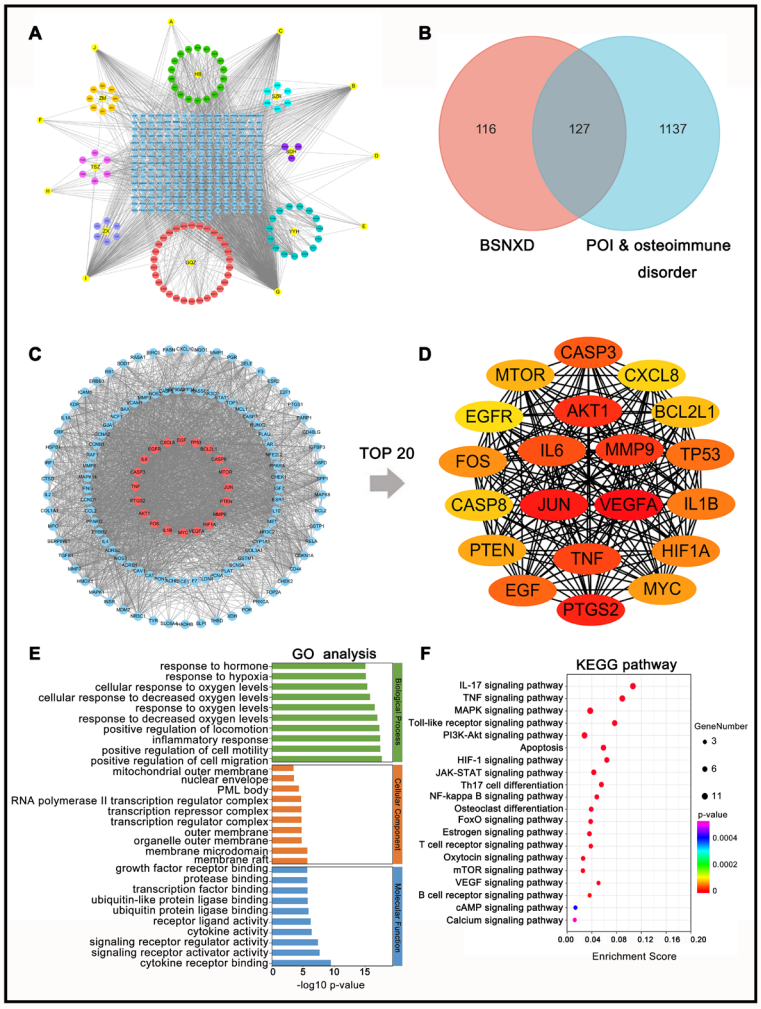


**Figure 5. Network pharmacology predicted the action mechanisms of BSNXD on POI-related osteoimmune disorder**. **(A)** Components-targets network. The blue diamond in the middle represents the compound target; the eight circles in different colors are different kinds of herbs and their active compounds in BSNXD; the outermost yellow circles are the common targets of the eight herbs. **(B)** The regulatory genes of BSNXD against POI-related osteoimmune disorder. **(C)** PPI network of the core regulatory targets. Red circles represent the top 20 targets, and blue circles represent other targets after the re-edition. **(D)** PPI network of the top 20 targets. The oval defines targets, and the one with the darker color defines a greater degree of centrality. **(E)** The GO pathway enrichment analysis: Green columns represent biological process; orange columns represent cellular composition; blue columns represent molecular function. **(F)** The KEGG pathway enrichment analysis.

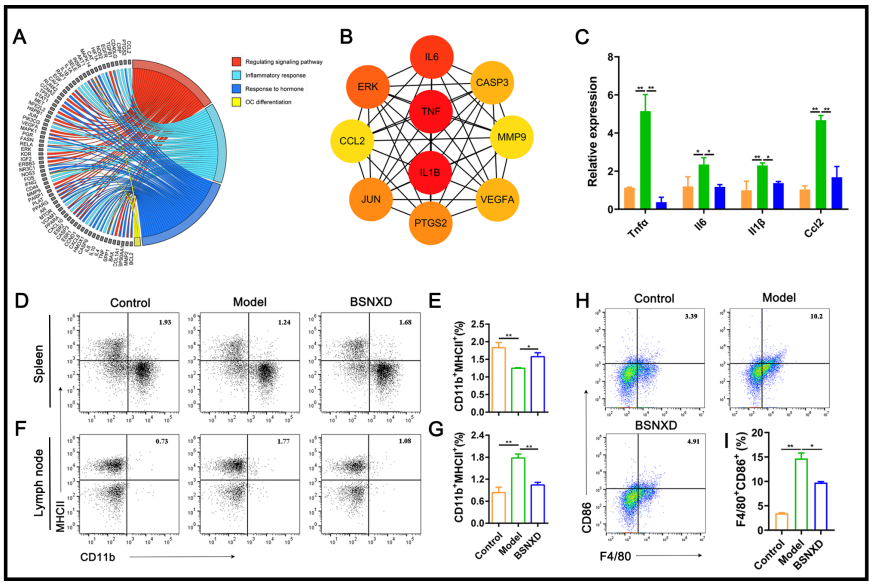


**Figure 6. BSNXD treatment regulated osteoimmune disorder via inhibiting macrophage activation. (A)** GO chord analysis obtained the intersection targets among response to inflammation, response to hormone, and osteoclast differentiation. **(B)** Hub targets were identified from **(A)**. **(C)** The gene expression of *Tnfa, Il6, Il1β*, and *Ccl2* in murine tibial bone. **(D-G)** Flow cytometric analysis: $CD11b^{+}MHCII^{+}$ cell percentage in **(D-E)** spleen and **(F-G)** mesenteric lymph nodes; **(H-I)** $CD3^{-}CD11b^{+}F4/80^{+}CD86^{+}$ cell percentage in bone marrow. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

composition entries, and 36 molecular function entries. The top 20 biological processes involve signaling receptor regulator activity, signaling receptor activator activity, receptor ligand activity, cytokine activity, and positive regulation of cell migration. One hundred sixteen signaling pathways related to BSNXD against POI-related osteoimmune disorder were enriched. The top 20 pathways include the TNF signaling pathway, MAPK signaling pathway, osteoclast differentiation, and other signaling pathways (Figure 5F), suggesting the underlying mechanisms of BSNXD were closely associated with inflammation response and osteoclast differentiation.

### 3.6. BSNXD regulated macrophage activation in the VCD-induced POI mice

VCD exposure leads to increased FSH levels, playing a vital role in bone loss during menopause (*9,24*). Considering FSH evokes an inflammation response (*10,11*), we hypothesized that BSNXD may lead to inflammation regression to balance osteoclast differentiation and function. Thus, we performed bioinformatics analysis to assess the intersection targets among response to inflammation, response to hormone, and osteoclast differentiation (Figure 6A). Ten hub genes were screened using the Cytoscape software (Figure 6B); in particular, BSNXD treatment downregulated inflammatory factors, including *Tnfα*, interleukin (*Il*)6, *Il1β*, and chemokine (CC-motif) ligand (*Ccl*) 2 (Figure 6C). CCL2, known as monocyte chemotactic protein 1, leads to the migration and infiltration of inflammatory cells like monocytes/macrophages to the site of inflammation (*25*). Thus, we explored

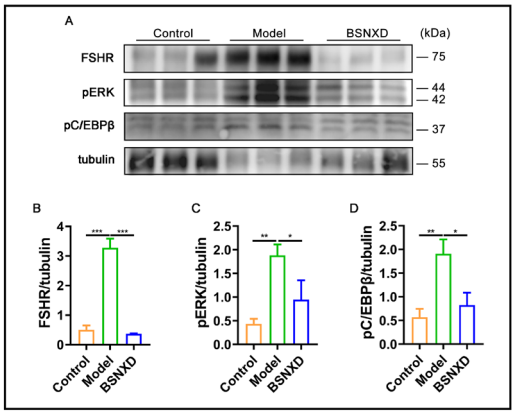


**Figure 7. BSNXD treatment inhibited the FSH/FSHR pathway**. **(A)** Western blotting of proteins from the murine bone tissue. **(B-D)** Quantifications of the protein expressions: **(B)** FSHR, **(C)** pC/EBPβ, and **(D)** pERK. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the proportions of macrophages and monocytes, a primary source of *Tnfα*, *Il6*, and *Il1β*. Interestingly, we found a significant expansion of activated monocytes ($CD11b^{+}MHCII^{+}$) in the mesenteric lymph nodes and macrophages ($CD3^{-}CD11b^{+}F4/80^{+}CD86^{+}$) in the bone marrow but a reduction of activated monocytes in the spleen in the model mice. BSNXD reversed the changes, demonstrating its role in cell migration. These findings indicated that inflammation regulation of BSNXD was responsible for the POI-related osteoimmune disorder.

### 3.7. BSNXD inhibited macrophage activation by antagonizing the FSH/FSHR pathway

FSH binds to FSHR to activate macrophages by regulating downstream pathways and transcription

factor C/EBPβ (*11*). Western blotting indicates elevated FSHR, pERK, and pC/EBPβ levels after VCD exposure (Figure 7), mainly due to the increased FSH levels. Notably, BSNXD decreased the FSHR expression and inhibited the phosphorylated level of ERK and C/EBPβ (Figures 7A, 7C-7D), indicating BSNXD suppressed the macrophage activation *via* regulating the FSH/FSHR pathway.

## 4. Discussion

Premenopause before the age of 40 years is linked to premature osteoporosis and fracture risk (*26,27*), attracting more attention to women with POI-related osteoporosis. In this work, BSNXD restrained osteoclast differentiation to ameliorate osteoimmune disorder in the VCD-induced murine POI model. Then, network pharmacology predicted the potential mechanisms of BSNXD against POI-related osteoimmune disorder involving TNFα and MAPK signaling pathways. The potential targets involving response to inflammation, response to hormone, and osteoclast differentiation were further validated by molecular biology experiments. Our results demonstrated that BSNXD inhibited the macrophage activation to reduce osteoclastogenesis by inhibiting the FSH/FSHR pathway.

VCD can selectively destroy the primordial and primary follicles to deplete the follicle reserve pool, leading to ovarian dysfunction (*28,29*). We successfully replicated a POI-related osteoimmune disorder murine model, distinguished by elevated serum FSH level, increased follicle destruction, macrophage activation, and impaired bone microstructure in the vertebrae and femur after VCD exposure. Despite follicle depletion in the POI mice, the ovaries appear to produce estradiol, leading to obviously increased E2 levels. It is consistent with the previous study that serum E2 levels were raised or remained unchanged in the early stage in the VCD-induced mice (*30,31*), mainly owing to remaining functional granulosa cells secreting E2 (*32*). Compared to ovariectomy, VCD modeling preserves part of the ovarian tissue and indicates a slow change of hormone levels, visually imitating human POI. Although we did not observe remarked changes in the trabecular and cortical BMD 30 days after the final VCD injection, there were impaired bone microstructures, including increased Tb.Sp and decreased BV/TV, Tb.Th, and Tb.N in the murine vertebrae or femur, inconsistent with the literature reports (*18*). It may be because a 45-day period in the VCD-induced POI mice is still in its early stages, only showing an osteoimmune disorder accompanied by a high bone turnover. Accelerated bone remodeling is usually characterized by an augmented number of osteoblasts and osteoclasts (*23*). Inspiringly, BSNXD treatment ameliorated the impaired bone microstructures, mainly due to the reduced number of osteoclasts but not osteoblasts. Considering c-fos/NFATc1 signaling is a confirmed target to inhibit osteoclast differentiation (*33,34*), we identified that BSNXD treatment inhibited osteoclastogenesis *via* suppressing the osteoclastic mRNA expression (*Nfatc1*, *Ctsk*, *Trap*, *Mmp9*), consistent with our previous work (*15*).

Network pharmacology is an effective tool to reveal the pharmacology mechanism of the TCM formula (*35*). Based on this, we utilized network pharmacology to evaluate the potential mechanisms of BSNXD against the POI-related osteoimmune disorder. We filtered 153 bioactive compounds and predicted the top 20 core targets, including TNF, IL6, and IL1β, which may play central roles in BSNXD treating the POI-related osteoimmune disorder. The GO and KEGG analysis indicated that the mechanisms of BSNXD in relieving the POI-related osteoimmune disorder were possibly associated with the TNF and MAPK signaling pathways. Previous studies have confirmed that some BSNXD-related bioactive components inhibited osteoclastogenesis. For example, alisol C 23-acetate treatment lowered serum levels of TNFα, IL6, and IL1β and inhibited RANKL-induced osteoclast differentiation and function against osteoporosis (*36*). The top two active ingredients derived from BSNXD are quercetin and kaempferol, which are efficacious in suppressing osteoclastogenesis and bone resorption (*37*). Further studies confirmed these anti-inflammatory properties could inhibit osteoclastogenesis (*38,39*). These studies suggest that BSNXD might inhibit osteoclast differentiation by regulating inflammation.

Emerging evidence shows that menopausal transition prompts chronic low-grade inflammation due to FSH or estrogen changes (*9,40,41*). VCD can increase serum FSH levels, which causes hypogonadal bone loss independent of estrogen (*10*). FSH stimulates macrophages to produce TNF, which enhances osteoclast over-formation and causes bone remodeling disorder (*9*). Our results showed an increased ratio of activated monocytes from the mesenteric lymph nodes and bone marrow but a reduced ratio of activated monocytes from the spleen, accompanied by a high expression of CCL2, probably resulting in activated monocyte migration. Next, we utilized bioinformatics analysis to minify the potential targets of BSNXD ameliorating POI-related osteoimmune disorder. Of note, ten molecules, including TNF, IL1β, IL6, MMP9, CCL2, ERK, JUN, CASP3, PTGS2, and VEGFA, were identified, among which TNFα and IL1β are bone-reabsorbing cytokines (*42*). BSNXD treatment suppressed the bone gene expression of TNF, IL1β, IL6, CCL2, and MMP9 and regulated the ratio of activated macrophages in the different tissues, showing vital impacts of BSNXD on the activation and migration of monocytes/macrophages. FSH binds to FSHR on the surface of monocytes/macrophages to induce MEK/Erk and C/EBPβ phosphorylation, stimulating TNF production to enhance osteoclast differentiation (*10,11*). As we speculated, BSNXD

downregulated the FSHR expression and inhibited the ERK and C/EBPβ phosphorylation in the POI-relative osteoimmune disorder, indicating BSNXD suppressed the macrophage activation *via* regulating the FSH/FSHR pathway.

In summary, BSNXD suppressed osteoclast differentiation by regulating the inflammatory response. More specifically, BSNXD inhibited the macrophage activation to reduce osteoclastogenesis by downregulating the FSHR expression and inhibiting the ERK and C/EBPβ phosphorylation. However, considering that VCD can induce osteoporosis after 75 days or more treatment, the effects of BSNXD on the disease's different stages need to be studied. Moreover, the direct or indirect action of how BSNXD regulates macrophage activation to inhibit osteoclast differentiation needs further elaboration.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

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Original Article

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# Yishen Huatan Huoxue decoction and quercetin ameliorate decidualization dysfunction in polycystic ovary syndrome: A comprehensive investigation combining clinical trial and experimental studies

**Jing Wang[1,2,3,§], Lisha Li[1,2,3,§], Jing Zhou[1,2,3], Xinyao Pan[1,2,3], Qing Qi[1,2,3], Hongmei Sun[1,2,3], Ling Wang[1,2,3,*]**

[1] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China.

**SUMMARY** Polycystic ovary syndrome (PCOS) is a common gynecological endocrine disorder characterized by a complex pathogenesis and limited treatment options. Yishen Huatan and Huoxue decoction (YHHD), as a traditional Chinese Medicine formula, has shown effectiveness in treating PCOS. However, the specific mechanisms by which YHHD exerts its therapeutic effects remain unclear. In this study, we performed to investigate the therapeutic effects of YHHD and quercetin on dehydroepiandrosterone-induced PCOS mice, and examine the effect of quercetin on the decidualization of T-HESCs under hyperinsulinemic conditions. The results showed that YHHD could reduce early miscarriage rates in PCOS patients and significantly improved glucose metabolism disorders, sex hormone levels, and the estrous cycles in PCOS mice. Quercetin could alleviate effect of high insulin levels and restore the low expression of insulin receptor substrate1/2 (IRS1/2) and glucose transporte 4 (GLUT4) in T-HESCs, demonstrating its potential to mitigate hyperinsulin-induced decidualization dysfunction *via* the GLUT4 signaling pathway mediated by IRS1/2. This study provides valuable molecular insights of YHHD and highlight the therapeutic potential of quercetin in treating decidualization dysfunction in PCOS.

***Keywords*** Quercetin, polycystic ovary syndrome, decidualization, traditional Chinese medicine

## 1. Introduction

Polycystic ovary syndrome (PCOS) is a multifaceted clinical syndrome that mainly impacts women of reproductive age, resulting in endocrine abnormalities and infertility (*1-3*). This disorder is characterized by hyperandrogenism, ovulation dysfunction, polycystic ovarian changes, and insulin resistance (*4,5*). While ovulation dysfunction is the leading cause of infertility among PCOS patients, poor pregnancy success rates suggest that other pathologies may be involved, particularly in the endometrial microenvironment (*6,7*). Successful pregnancies heavily rely on decidual tissue, which facilitates blastocyst implantation, invasion, and communication between the mother and fetus (*8*). Despite the extensive studies on decidualization (DE) regulation mechanisms, especially those associated with the pathogenesis of PCOS, further research is necessary to identify additional factors.

Traditional Chinese Medicine (TCM) literature has described conditions similar to PCOS in chapters related to metrorrhagia, amenorrhea, abdominal pain, and infertility. The treatment regimen centers around rectifying the balance among kidney essence, Tiangui, Chongren, and uterus (*9*). Although various medications have been proposed for treating PCOS, they have limitations such as adverse effects, low compliance, low efficacy, and contraindications in certain instances (*10*). Given its effectiveness and safety, TCM could be an alternative or complementary treatment option for PCOS management (*11*). Yishen Huatan and Huoxue decoction (YHHD) is a TCM formulation that reportedly improves insulin resistance, regulates metabolism, increases blood flow, and eliminates blood stasis. This study has shown that YHHD has the potential to regulate ovarian haemodynamics, serum hormone levels, and

menstruation in PCOS patients, thereby normalizing endocrinology and improving menstrual irregularities, ovulation, and pregnancy rates. However, the biological mechanisms underlying the effectiveness of treatment options for PCOS are not fully understood. Quercetin (Que), a common flavonoid present in plant-based foods, contributes to glucose homeostasis regulation. However, the precise role of quercetin in the regulation of insulin action is not yet fully understood (*12*). This study aims to investigate how quercetin affects insulin-mediated glucose transporter translocation under hyperinsulinemic conditions and the specific molecular mechanisms underlying YHHD's promotive effect on decidualization. The study aims to evaluate the efficacy of YHHD in treating PCOS, shedding light on its underlying endocytic mechanisms and providing potential avenues for future research and treatment of the condition.

## 2. Materials and Methods

### 2.1. The clinical trial validates YHHD for PCOS

This retrospective trial was conducted at the Obstetrics and Gynaecology Hospital of Fudan University between 1 September 2018 and 30 September 2022. The study received approval from the hospital ethics committee (No. 2024-47) and followed the guidelines established in the Declaration of Helsinki for research involving human subjects (*13*). The diagnostic criteria of PCOS followed the Rotterdam criteria (*14*): *i* Occasional ovulation or anovulation; *ii* Clinical and/or biochemical indicators suggest hyperandrogenism; *iii* Ultrasonography of ovarian polycystic changes. The exclusion criteria included: *i* Uterine amenorrhea, primary amenorrhea, adrenal tumor, Cushing's syndrome, hyperprolactinemia, hyperthyroidism or hypothyroidism, Sheehan's syndrome, mental factors, emaciation, anorexia, and other hypothalamic amenorrhea, and sex chromosome abnormalities; *ii* Complications with genital malformation, congenital gonadal hypoplasia, tubal obstruction, and other organic diseases; *iii* Cardiovascular, pulmonary, and other serious primary diseases; *iv* Mental and neurological diseases such as hemorrhage, epilepsy, carbuncle, psychosis, and neurosis; *v* Those who are allergic to the test drug or have a severe allergic constitution; *vi* Those who have taken hormonal drugs such as contraceptives, ovulation promoting drugs, glucocorticoids, or related therapeutic drugs in recent months; *vii* Breastfeeding in the past 6 months.

According to the results of the two prior studies (*15,16*), the combined miscarriage rate was found to be 14.8% in the progestogen group, whereas the rate was 27.1% in the control group. Based on an 80% statistical power and a type I error rate of 0.05, a sample size of 200 women in each group was determined to be necessary. To account for potential drop-outs, our target was to recruit a total of 660 women, with 220 women in each group. The YHHD group received YHHD treatment obtained from the Fudan University Obstetrics and Gynaecology Hospital for a treatment duration of 14-28 days. The primary outcome of this study was the incidence of early miscarriage, which was defined as occurring before the 20th week of gestation.

### 2.2. Animal experimental protocols and testing

The study used female C57BL/6 mice of three weeks old, obtained from Zhejiang Viton Lihua Laboratory Animal Technology Co., with Specific Pathogen-Free (SPF) grade. The experimental procedures utilized in this study were approved by the Ethics Committee of the Obstetrics and Gynaecology Hospital of Fudan University. All the animals were acclimated under standard laboratory conditions (ventilated room, 25 ± 1°C, 60 ± 5% humidity, 12 h light/dark cycle) and had free access to standard water and food. After a one-week acclimation period, the mice, with an average body weight of 13.72 ± 2.42 g. The mice were randomly assigned to one of the four groups: Control, dehydroepiandrosterone (DHEA) (Aladdin Biochemical Technology Co., Ltd, Shanghai, China), DHEA + YHHD, and DHEA + quercetin (50 mg/kg) (Aladdin Biochemical Technology Co., Ltd, Shanghai, China). The DHEA-treatment was administered for 20 consecutive days. For the control group ($n = 6$), mice were administered a daily subcutaneous injection of 0.1 ml of sesame oil (Sigma-Aldrich Inc., UNITED STATES). The models of PCOS assigned to the DHEA group ($n = 20$) were given a daily subcutaneous injection of DHEA (6 mg/100 g) administered once daily. On day 21 of DHEA modelling, the mice in the DHEA group were screened for estrous cycle, hormone level, and body weight and were thereafter subjected to treatment with YHHD and quercetin, respectively. Their reproductive and metabolic functions were assessed 22 days after commencing treatment, and evaluations were continuous until the animals were euthanized. Additionally, their weight was monitored every other day during the treatment period. To ensure precise and consistent results, all samples were analyzed in triplicate.

### 2.3. Estrous cycle

To determine the stages of the estrous cycle, vaginal smears were obtained every day at 9 AM beginning from the 13th day following the initial DHEA injection and collected for seven successive days.

### 2.4. Glucose and insulin tolerance test

The mice fasted for 12 hours and had unrestricted access to water. The subsequent morning, tail vein

blood samples were obtained to determine fasting blood glucose (FBG). They were then administered 20% glucose solution until their stomachs were filled and blood glucose levels were measured at intervals of 15, 30, 60 and 90 minutes post-administration (Sinocare, China). The Oral Glucose Tolerance Test (OGTT) curve was generated, and the overall area under the curve (AUC) was calculated. Blood glucose and serum insulin levels were assessed, and the homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the following formula: fasting blood glucose [mmol/L] × fasting serum insulin [μU/mL]/22.5.

2.5. Hormonal analysis of serum

On day 21 of DHEA intervention and day 41 of treatment, mice were given anesthesia, and blood samples were collected from the retro-orbital plexus following an overnight fast. The serum concentrations of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone (T), and insulin were measured using a mouse ELISA Kit (R&D Systems, USA).

2.6. Artificial decidualization

To induce artificial decidualization on the 4th day of pseudopregnancy, 20 μL of sesame oil was injected into one uterine horn, while the non-injected contralateral horn served as a control. On the 8th day of pseudopregnancy, the deciduoma was evaluated.

2.7. Molecular structure docking analysis

The two-dimensional structure of the active ingredient was obtained from the PubChem (*https://pubchem.ncbi.nlm.nih.gov/*) website and converted to a minimum free energy three-dimensional structure using the ChemBio3D software. The three-dimensional structures of the core targets were obtained from the PDB database, and proteins as well as drug components were converted to PDBQT format files using "Auto Dock Tools" software.

2.8. Treatment of T-HESCs

T-HESCs were obtained at the Institution of Obstetrics and Gynecology Hospital of Fudan University, and quercetin was dissolved in concentrations of 5, 10, and 20 μM for cell treatment. To simulate hyperinsulinemia seen in PCOS patients, T-HESCs were treated with insulin (Sigma-Aldrich Inc., UNITED STATES) at concentrations of 10, 50, 100, 200, and 500 nM for three days. To determine if the Insulin Receptor Substrate 1/2 (IRS1/2) inhibitor NT157 (TargetMol Chemicals Inc., Boston) could block the effects of quercetin, T-HESCs were pre-decidualized for three days and treated with 6 μM NT157 for one hour before being exposed to 50 nM insulin and quercetin treatment for three days. T-HESCs were then induced for decidualization *in vitro* by treating them with 8-Br-cAMP (50 mM, Sigma, MO, USA), progesterone (100 ng/ml, Sigma, MO, USA), and estradiol (10 ng/ml, Abcam, CA, USA) for a further three days.

2.9. Cells viability assays

The cells were incubated with a medium containing quercetin at concentrations of 0, 5, 10, 20, 50, 100, and 200 μM for 24, 48, and 72 hours. After the incubation period, the cells were subjected to a CCK-8 assay (Yeasen Biotechnology Co., Ltd.) and viable cells were identified and quantified by measuring absorbance at 450 nm.

2.10. Cell apoptosis assays

To analyze apoptosis, $3 \times 10^3$ cells were stained with Annexin V and propidium iodide (Biolegend Inc.), and the percentage of apoptotic cells was determined with flow cytometry (CytoFLEX; Beckman Coulter, Inc.).

2.11. Wound healing assays

To examine the impact of quercetin on cell migration, T-HESCs were exposed to serum-free Dulbecco's Modified Eagle Medium (DMEM) (GENOM BIO, Zhejiang, China) supplemented with 0, 5, 10, or 20 μM quercetin in 1.25% v/v Dimethyl sulfoxide (DMSO) (Sango Biotech Co.,Ltd.) (500 μL/well). A scratch was then made in the cell monolayer at 0 h, and fresh medium was added. The T-HESCs were then cultured for an additional 3 days while images were captured at consistent locations every 24 h over the 72-h period. The images were analyzed using Image J software to assess cell migration.

2.12. RT-qPCR

RNA extraction was carried out from the treated cells using the RNAiso Plus kit (Takara Biomedical Technology (Beijing) Co. Ltd.), and the cDNA was synthesized utilizing the PrimeScript™ RT kit (Perfect Real Time (Takara)). The list of target genes and primers used can be found in Table 1.

2.13. Statistical data analysis

The data that exhibited a normal distribution were presented as mean ± standard deviation (mean ± SD), and their statistical significance was evaluated using a one-way analysis of variance (ANOVA). The Student's *t*-test and ANOVA were employed to determine the significance of the outcomes, and these analyses were conducted utilizing GraphPad Prism v.8.

## 3. Results

### 3.1. YHHD reduces early miscarriage rates in PCOS patients

Between September 1, 2018, and December 30, 2022, a total of 660 patients diagnosed with PCOS were enrolled in the study after meeting the requisite inclusion and exclusion criteria. Baseline clinical and hormonal characteristics were similar across all groups (Table 2). The rate of early miscarriage rate was 12.19%, 24.44% and 16.62% in the groups receiving dydrogesterone (10 mg, Abbott Biologicals B.V) and YHHD, YHHD, and dydrogesterone alone, respectively. Notably, the combination of dydrogesterone and YHHD was found to be significantly associated with a lower rate of early miscarriage than YHHD alone ($P < 0.05$).

### 3.2. YHHD ameliorated the symptoms in DHEA-induced PCOS mice

To investigate the potential therapeutic efficacy of YHHD in treating PCOS, we conducted an experiment to assess its impact on body weight, estrous cycles, histological changes, and serum hormone levels in three groups. According the protocol (Figure 1A), our findings demonstrated a significant increase in weight gain in PCOS mice when compared to the normal group, which was effectively reduced by YHHD treatment (Figure 1B). The estrous cycle of PCOS mice was closely monitored to confirm the efficacy of YHHD treatment, and our findings demonstrated that PCOS mice had disrupted estrous cycles, this disruption was characterized by prolonged metaestrous and diestrous (M/D) phases, as well as a reduction in proestrus and estrous phases (Figure 1C). Following administration of YHHD for two weeks, there was a gradual normalization of the estrous cycle. Compared with the normal control group, HE staining of ovarian tissue showed numerous vesicular follicles in PCOS group, with a significant reduction of granulosa cell layer. After YHHD treatment, a large number of luteal structures and all levels of growing follicles were seen in the ovarian tissue, and the normal ovarian structure was restored (Figure 1D). Moreover, YHHD treatment effectively reversed the abnormal hormone levels (Figure 1E). Compared to the control group (0.97 ± 0.73), the results showed a significant increase in HOMA-IR score in PCOS group (5.41 ± 0.62) (Figure 1F). Intriguingly, the HOMA-IR score decreased significantly (1.31 ± 0.68) ($P < 0.05$) in PCOS mice treated orally with YHHD. This indicates that YHHD has the potential to treat insulin resistance in PCOS mice. The control and YHHD group mice exhibited a peak increase in FBG levels prior to the 30-minute point of glucose loading, which then dropped to levels near-normal at 120 minutes. The oral administration of YHHD significantly reduced blood glucose levels in PCOS mice from 60 minutes onwards ($P < 0.05$) compared to PCOS mice (Figure 1F). This finding suggests that YHHD has the potential to improve glucose tolerance in PCOS mice by reducing insulin resistance. Overall, the results suggest that YHHD has a

**Table 1. Primers and probes for real-time RT-qPCR**

| Gene | Primer sequence (5′–3′) | Product size (bp) |
|---|---|---|
| *IGFBP-1* | AGCACGGAGATAACTGAGGAGGAG<br>GTTGGTGACATGGAGAGCCTTCG | 129 |
| *PRL* | GCAGATGGCTGATGAAGAGTCTCG<br>GCAGTTGTTGTTGTGGATGATTCGG | 130 |
| *FoxO1* | TGTCCTACGCCGACCTCATCAC<br>GCACGCTCTTGACCATCCACTC | 96 |
| *IRS-1* | AGTGGAGAGCAGCGGTGGTAAG<br>AGTAGTAGGAGAGGACTGGCTTGTG | 146 |
| *IRS-2* | CTCTGCCTCGCTGGATGAATACAC<br>GATGTCTCCGTAGTCCTCTGGGTAG | 133 |
| *GLUT4* | CCTTGGTCTCGGTGTTGTTGGTG<br>AGGAGCAGAGCCACAGTCATCAG | 109 |
| *GLUT12* | GGTTGGAGTCGTCAAGGTCATTAGC<br>GCCATCACAGAGGAGCCAATGC | 99 |

**Table 2. The comparison of the clinical data and pregnancy result of the patients with PCOS after treatment**

| Parameters | YHHD $N = 220$ | Dydrogesterone $N = 220$ | Dydrogesterone + YHHD $N = 220$ | *P* values |
|---|---|---|---|---|
| Age median (years) | 27.2 (5.2) | 30.4 (2.1) | 28.5 (5.3) | 0.842 |
| BMI (Kg/m$^2$) | 23.8 (5.1) | 25.9 (4.1) | 22.9 (3.8) | 0.604 |
| FSH (mIU/mL) | 6.56 (2.3) | 5.86 (3.2) | 6.32 (4.7) | 0.674 |
| LH (mIU/mL) | 7.43 (4.6) | 10.85 (5.3) | 6.74 (3.2) | 0.053 |
| E2 (mIU/mL) | 48.8 (22.3) | 55.83 (33.4) | 36.32 (42.6) | 0.056 |
| Testosterone (ng/mL) | 0.56 (0.33) | 0.86 (0.32) | 0.32 (0.27) | 0.042 |
| SHBG (nmol/mL) | 56.54 (15.3) | 45.49 (23.2) | 58.56 (24.5) | 0.065 |
| Glucose 0 minute (mmol/L) | 5.1 (2.3) | 6.1 (1.4) | 4.4 (2.1) | 0.063 |
| Glucose 120 minute (mmol/L) | 7.8 (2.1) | 8.4 (3.2) | 6.5 (4.3) | 0.058 |
| Insulin 0 minute (μU/mL) | 10.53 (6.15) | 12.11 (5.43) | 9.56 (2.13) | 0.051 |
| Insulin 120 minute (μU/mL) | 39.13 (14.15) | 65.32 (24.34) | 24.15 (23.13) | 0.032 |
| Early abortion rate (%) | 24.44 (15.27) | 16.62 (14.05) | 12.19 (14.56) | 0.042 |

All the data as expressed the mean.

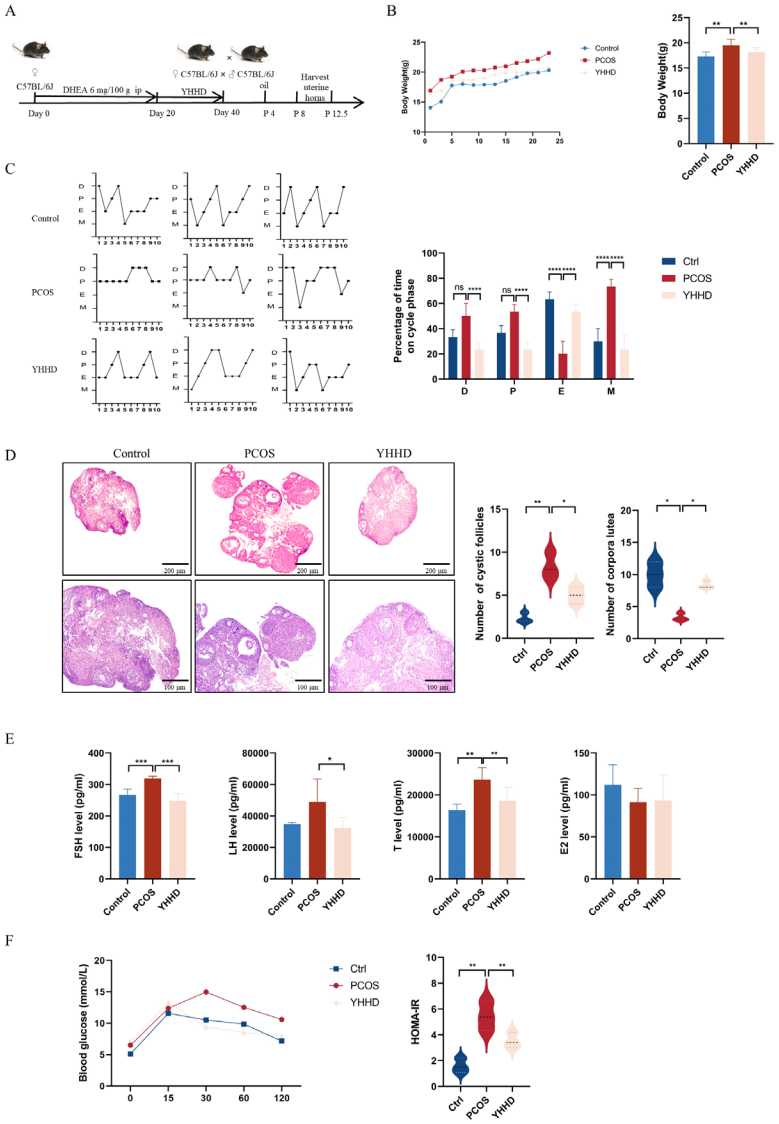



**Figure 1. To depicts the effect of YHHD on DHEA-induced PCOS in mice. (A)** The results showed the control groups and treatment schedule. **(B)** The effect of YHHD on body weight in PCOS mice ($n = 6$). **(C)** The representative estrous cycles and quantifies the changes in estrous cycle ($n = 6$). **(D)** The representative ovary tissue samples stained with H&E, and the quantification of cystic follicles and corpus luteum ($n = 6$). The scale bar represents 1mm. **(E)** The serum levels of sex hormone indicators including T, E2, LH, and FSH, which were determined using ELISA kits ($n = 6$). **(F)** Effect of YHHD on HOMA-IR and OGTT. The data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* the PCOS group.

significant therapeutic effect in relieving the endocrine abnormalities associated with PCOS.

### 3.3. YHHD improves decidualization dysfunction in PCOS mice

To investigate the impact of YHHD on decidualization in PCOS mice, we evaluated the artificially induced decidual response of different groups of female C57BL/6J mice. The control group showed a decidual response within 8 days of artificial stimulation, as demonstrated by the increase in the size of the decidual angle (Figure 2A, left). The YHHD group exhibited a decidual reaction compared to the PCOS group (Figure 2A, right). The outcomes demonstrated that the weight of stimulated uterine horns in the PCOS group was significantly lower than that in the control and YHHD groups, indicating that the decidual response was lower in PCOS mice. These findings suggest that YHHD treatment ameliorates the decidual response in PCOS mice, resulting in improved uterine wet weight gain (Figure 2A). Moreover, compared with the normal control group and YHHD group, the uterine thickness of PCOS group became thin, the glands was not tight and the number was significantly reduced, and the gland cavity was smaller (Figure 2B). The expression of decidual-related genes, bone morphogenetic protein-2 (*BMP-2*), *Prl8a2*, Heart And Neural Crest Derivatives Expressed 2 (*HAND2*), and Wnt Family Member 4 (*Wnt4)* was significantly reduced in the stimulated uterus of PCOS mice compared to that in control mice (Figure 2C). However, the expression levels of these decidual-

related genes were notably elevated in the YHHD-treated group compared to the PCOS group, suggesting amelioration of decidualization dysfunction in PCOS mice subsequent to YHHD intervention. These results suggest that YHHD has the potential to enhance the process of decidualization in PCOS mice by modulating the expression of decidual-related genes.

### 3.4. The therapeutic effect of quercetin on PCOS mice

The Table 3 displays the top three pharmacological components based on degree values, the top three compounds in the network, quercetin, daidzein, and kaempferol had 128, 62, and 53 targets, respectively, which are crucial for the treatment of PCOS. Therefore, we chose quercetin for the subsequent experiments. The mice were treated with quercetin for 2 weeks, and the effects on reproductive and metabolic parameters were evaluated (Figure 3A). Relative to the PCOS group, quercetin significantly reduced body weight in the PCOS mice ($P < 0.001$) (Figure 3B). The results showed that quercetin treatment substantially improved reproductive parameters, such as restoring estrous cyclicity (Figure 3C), promoting follicle maturation, and increasing the number of corpus luteum (Figure 3D). The results have found that quercetin can modulate DHEA-induced

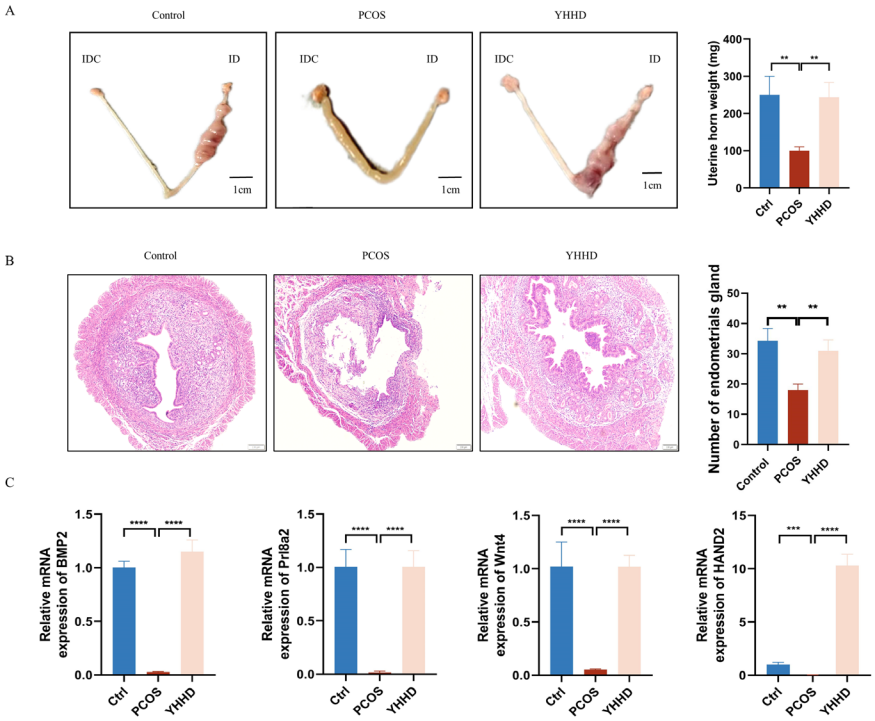
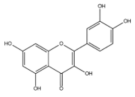
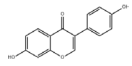
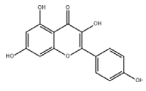



**Figure 2. YHHD improves impaired decidualization in PCOS mice. (A)** The gross morphology of the uterus was evaluated in all three groups of mice on day 5 after induction of artificial decidualization. The ratio of the mean weight of the stimulated uterine horns was measured in the three groups of mice ($n = 6$) and presented as a histogram. **(B)** Histological analysis of uterine sections from the three groups of stimulated mouse were performed using hematoxylin and eosin staining on day 5 after the induction of artificial decidualization. **(C)** The mRNA expression levels of the decidualization-related genes *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4* were measured by real-time PCR in the uterus of the three groups of mice 5 days after oil infusion. The data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, $^{****}P < 0.0001$ *vs.* the PCOS group.

**Table 3. Networks analysis of major compound targets in YHHD and their topological properties**

| MOLID | Chemical composition | Degree | Molecular mass | Compound source | Structure |
|---|---|---|---|---|---|
| MOL000098 | Quercetin | 128 | 302.24 | Huangbai Danpi Gouqizi Tusizi Xianlingpi Huangqi Chuanxiong Changpu Gegen |  |
| MOL000390 | Daidzein | 62 | 254.24 | Huangqi |  |
| MOL000422 | Kaempferol | 53 | 286.24 | Baishao Zhimu Danpi Tusizi Xianlingpi Huangqi Changpu |  |

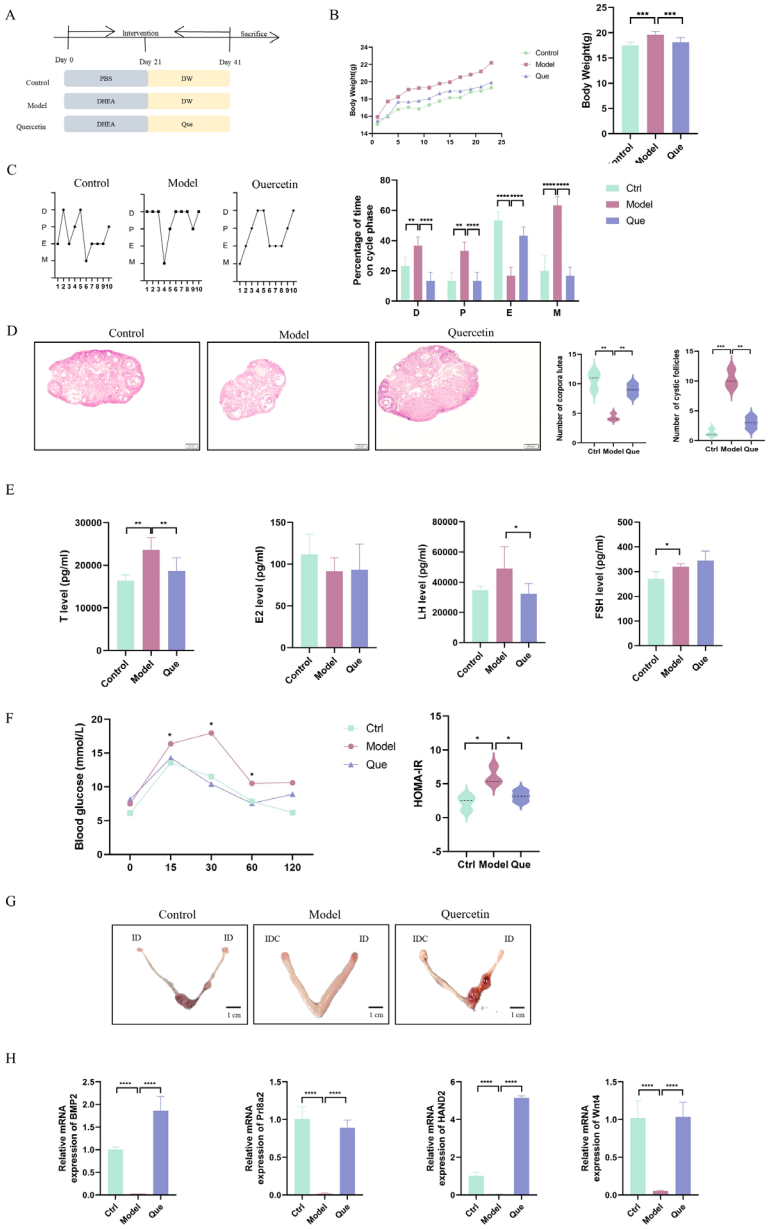



**Figure 3. Effect of quercetin on DHEA-induced PCOS in mice. (A)** The experimental plan was presented. **(B)** Quercetin was found to have an effect on body weight in PCOS mice ($n = 6$). **(C)** Representative estrous cycles were shown, and changes in estrous cycles were quantified ($n = 6$). **(D)** Representative ovarian tissue samples were stained with H&E, and the number of cystic follicles and corpus luteum was quantified ($n = 6$). **(E)** Serum levels of sex hormone markers, including T, E2, LH, and FSH, were measured using ELISA kits ($n = 6$). **(F)** Quercetin was found to have an effect on HOMA-IR and OGTT. **(G)** The gross morphology of the uterus was evaluated in all three groups of mice on day 5 after induction of artificial decidualization. **(H)** The mRNA expression levels of the decidualization-related genes *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4* were measured by real-time PCR in the uterus of the three groups of mice 5 days after artificial decidualization. The data are shown as mean ± SEM, and statistical significance was observed when compared to the PCOS group as indicated by the '*' (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

changes in hormones, such as LH and T (Figure 3E). In addition, quercetin treatment also improved metabolic parameters, such as reducing the levels of insulin resistance (Figure 3F). The outcomes indicated that the mice in the quercetin group displayed a significantly more robust decidual reaction compared to the PCOS group (Figure 3G). The expression levels of decidual-related genes, including *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4*, were assessed as indicators of decidualization within the stimulated uterus of PCOS mice. The expression of these genes was significantly reduced in the PCOS group when compared to the control group (Figure 3H). In contrast, the expression of these decidual-related genes in the quercetin group was significantly greater than that in the PCOS group, implying a reversal of the decidualization dysfunction observed in PCOS mice following quercetin treatment. Overall, the ability of quercetin to improve various hormonal parameters and metabolic markers suggests that it may have potential benefits in promoting successful pregnancy outcomes in

women with PCOS.

### 3.5. Quercetin improves cell proliferation, migration and inhibits cell apoptosis

Within our study, we conducted a series of *in vitro* assays to explore the potential impact of quercetin on T-HESCs. We utilized flow cytometry to evaluate T-HESC apoptosis and identified no significant increase in apoptosis induction within the quercetin (5 μM, 10 μM, 20 μM) group when compared to the control group ($P > 0.05$) (Figure 4B). However, treatment with 50 μM and 100 μM quercetin resulted in an increase in the percentage of T-HESCs with positive Annexin V staining. Furthermore, we utilized CCK-8 proliferation assays to demonstrate that T-HESC cellular activity was inhibited in a dose-dependent manner following treatment with various concentrations of quercetin for 24, 48, and 72 hours (Figure 4C). The quercetin (100 μM, 200 μM) group showed a significant decrease in cell survival rate (CSR) at 24, 48, and 72 h. Quercetin (50 μM, 100 μM, 200 μM) showed significant growth inhibition after 72 h of intervention while the quercetin (10 μM, 20 μM) group did not show any effect on cell viability at the three time periods. Additionally, wound healing assays revealed that quercetin improved the cells' ability to migrate (Figure 4D). The percentage of wound closure values for cells treated with serum-free DMEM control, decidualization group, quercetin at 5 μM, 10 μM, 20 μM were 50.00%, 58.06%, 45.00%, 46.15%, and 28.57%, respectively. Compared to 8-Br-cAMP (0.5 mM), progesterone (100 ng/mL) and estrogen (10 ng/mL) (DE) for 72 h, treatment of 5, 10 and 20 μM quercetin increased the expression

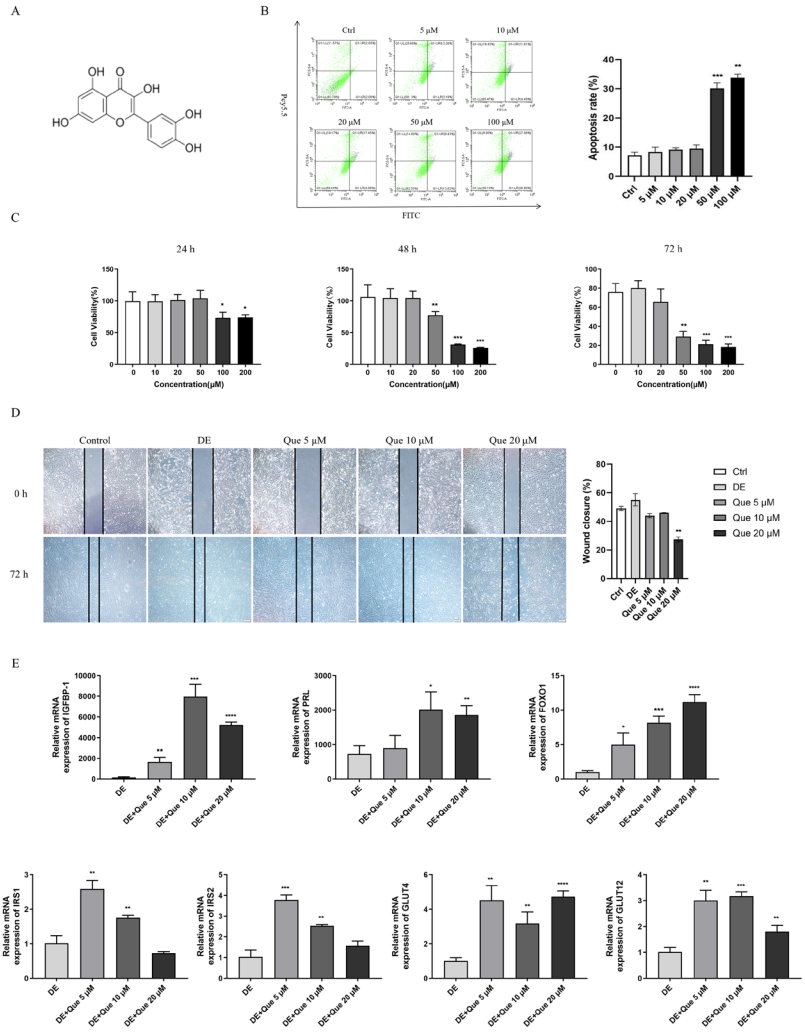



**Figure 4. Effect of quercetin on the viability of T-HESCs. (A)** Chemical structure of quercetin. **(B)** Apoptotic effects of quercetin, dot plots and percentage cell distribution of T-HESCs cells. Data are expressed as mean ± SD. **(C)** Cytotoxic effects of quercetin in T-HESCs were determined by CCK-8 assay at different concentrations for 24, 48, 72 h. **(D)** Quantitative analysis of the migration area reported as % wound closure. Representative images of T-HESC cells in a wound scratch assay. The images were taken immediately after the scratches had been made and then after 24, 48, 72 h in the presence and absence of quercetin. **(E)** Expression of decidualization markers IGFBP1, PRL, FOXO1 and IRS-associated and GLUT mRNA were determined using RT-qPCR analysis. Scale bars represent 200 μm. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Three independent experiments were performed in at least triplicate and data are expressed as mean ± SD. Que, quercetin.

of decidualization markers (IGFBP-1, PRL, FoxO1) and IRS-1, IRS-2, GLUT4, GLUT12 during *in vitro* decidualization (Figure 4E).

### 3.6. Quercetin ameliorates the adverse effects of hyperinsulin on decidualization

*In vitro* decidualization experiments were conducted by incubating cells with varying concentrations of insulin (0, 10, 50, 100, 200, 500 nmol/L) for 72 hours. The results demonstrated that treatment with 50 nmol/L insulin significantly decreased the mRNA expression of IGFBP-1 and PRL (Figure 5A) while also significantly reducing IRS1/2 mRNA expression (Figure 5B). However, quercetin treatment was able to significantly restore IGFBP1 and PRL mRNA levels inhibited by 50 nmol/L insulin treatment (Figure 5C), indicating the possible involvement of insulin signaling pathways in decidualization. We also evaluated the effect of quercetin on insulin-mediated GLUT4 translocation and found that high levels of insulin led to reduced GLUT4 mRNA expression in T-HESCs. However, quercetin treatment was able to suppress this effect. Moreover, quercetin was found to inhibit the inhibition of IRS-1 and IRS-2 by hyperinsulin. These findings suggest that quercetin may have potential benefits in regulating insulin signaling pathways.

### 3.7. Quercetin ameliorates the adverse effects of insulin-induced decidualization dysfunction through IRS1/2 receptors

The impact of NT157 on T-HESC cells was explored, and cell viability was found to remain unaffected following treatment with various concentrations of NT157, ranging from 0 to 10 μM (Figure 6A). Molecular docking studies revealed that quercetin exhibited significant affinity for the insulin receptor substrates IRS1 (-8.0 kcal/mol) and IRS2 (-8.2 kcal/mol), indicating that it may contribute to promoting molt by interacting with these proteins as part of the insulin signaling pathway (Figure 6B). RT-qPCR analysis showed that during molt, treatment with 6 μM NT157 led to suppression of *IGFBP-1*, *PRL*, and *FoxO1* expression while reducing *IRS1*, *IRS2*, *GLUT4*, and *GLUT12* gene expression (Figure 6C). Additionally, NT157 inhibited the expression of decidualization-

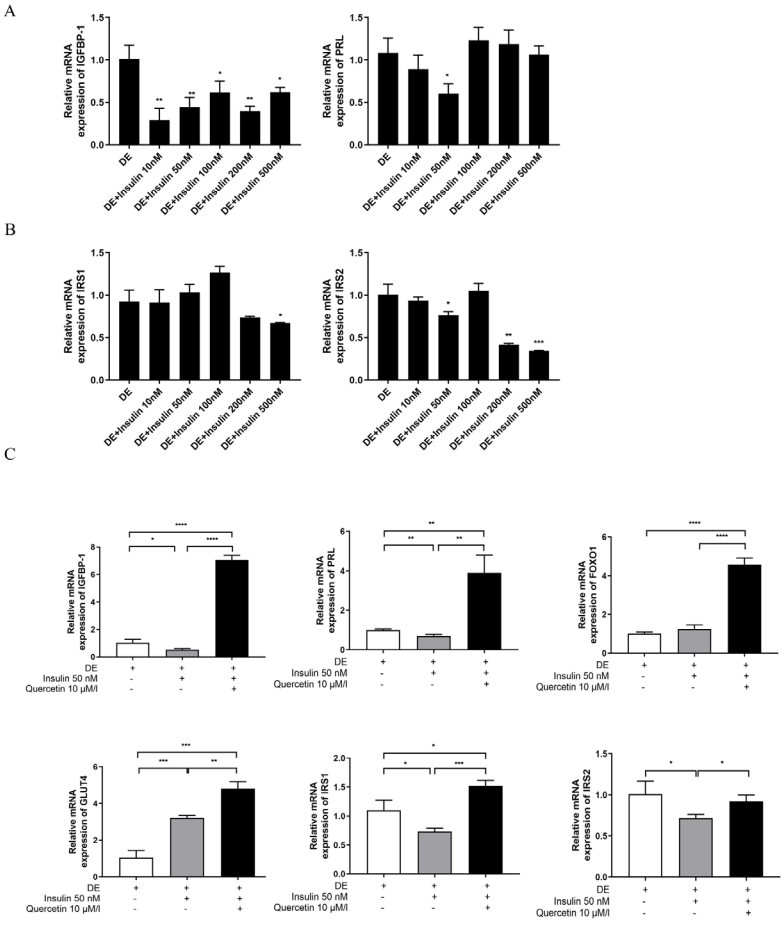



**Figure 5. Quercetin reverses decidualization dysfunction due to high insulin levels. (A)** Results of RT-qPCR conducted to analyze expression patterns of decidualization markers, IGFBP1 and PRL during in vitro decidualization with various concentrations of Insulin. **(B)** The expression of IRS1 and IRS2 during in vitro decidualization of different concentrations of insulin. **(C)** Quercetin reverses reduced decidualization indicators, IRS, GLUT-related gene expression due to high insulin levels. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

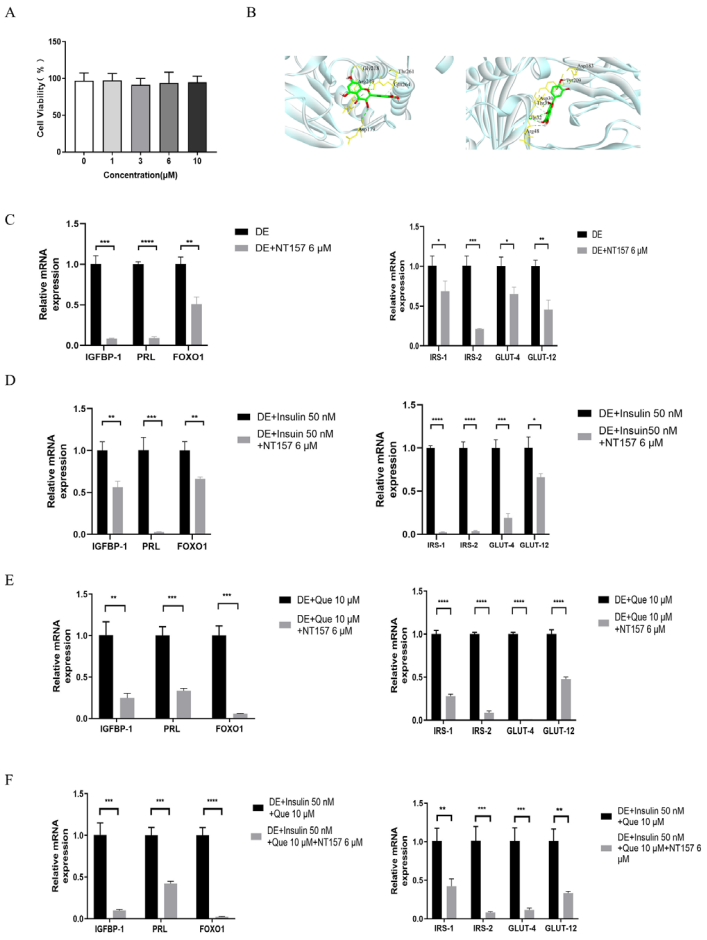



**Figure 6. Quercetin reverses high insulin-induced decidualization dysfunction *via* IRS 1/2 receptors. (A)** Cell viability of T-HESCs was determined by CCK-8 with or without NT 157 treatment (0, 1, 3, 6 and 10 μM) for 72 hours. **(B)** Molecular docking diagram of quercetin with IRS 1 and IRS 2. **(C)** Inhibition of decidualization markers and IRS-related receptors by NT 157. **(D-F)** Quercetin may act through the IRS 1/2 receptor to promote decidualization and alleviate insulin-induced decidualization dysfunction. Bars indicate the mean ± SD of at least three independent experiments and dots indicate the value for each experiment. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. Que, quercetin.

associated genes in high insulin intervention conditions (Figure 6D), while 10 μM quercetin had the opposite effect (Figure 6E), promoting the expression of *IGFBP-1*, *PRL*, *FoxO1*, *IRS*, and GLUT-related genes. The addition of quercetin was not capable of reversing the expression of decidualization-related genes under high insulin conditions. However, it may promote decidualization through the IRS1/2 receptor (Figure 6F).

## 4. Discussion

PCOS is a multifaceted gynecological disorder characterized by hormonal imbalances and metabolic dysfunction (*17-19*). Women with PCOS often experience reproductive disorders, including low pregnancy rates (*20,21*), low live birth rates (*22,23*), and high miscarriage rates (*24,25*). This suggests that many of the symptoms may be related to the endometrial microenvironment and anovulation (*26*). Pregnant women with PCOS have a higher incidence of gestational hypertension, pre-eclampsia, preterm birth, and meconium/placental alterations (*27,28*). Insulin resistance is commonly observed in patients with PCOS, leading to compensatory hyperinsulinemia, which amplifies the bioavailability of androgens and their secretions from ovaries and adrenal glands (*29,30*). This can lead to dysregulation of glucose metabolism in the endometrium and affect endometrial tolerance (*31,32*). High levels of plasma androgens and insulin can also affect the cyclical exfoliation of the endometrium and inhibit the production of IGFBP-1, leading to embryo implantation failure and an increased rate of miscarriage (*24,33*). An increase in insulin levels inhibits the production of IGFBP-1, suggesting that hyperinsulinemia dysregulates the endometrial function, leading to embryo implantation failure and an increased rate of miscarriage (*34,35*). While oral contraceptives (OCs) are a primary therapeutic option for menstrual irregularities in PCOS patients, they are not recommended for use during pregnancy (*36,37*). Insulin sensitizers can address hyperandrogenemia and lower insulin levels in PCOS patients, but they are often associated with gastrointestinal side effects (*38,39*). However, no single pharmaceutical preparation exists to fully manage all PCOS symptoms, and currently

available therapeutic agents can only target specific pathological aspects, presenting limitations (*40*). Given the complexity of PCOS and the challenges it poses, there is an urgent demand for a safe and efficient multi-targeted drug that can offer innovative strategies for treating this condition.

In China, TCM finds common usage as a treatment option for infertility and gynecological problems (*41,42*). TCM can effectively regulate ovarian hemodynamics, serum hormone levels, and menstruation in patients with PCOS, thereby, regulating endocrinology and improving menstrual irregularities, ovulation, and pregnancy rates (*43,44*). The primary pathogenesis of PCOS suggests that Chinese medicine treatment should aim to tonify the kidneys while concurrently addressing the liver, spleen, and heart. The use of Yishen, Huatan, and Huoxue to balance the YHHD formula represents a novel approach to treating PCOS. A preliminary clinical trial has shown that YHHD has demonstrated clinical efficacy in treating PCOS by decreasing LH levels, increasing FSH levels, improving ovarian cysts, promoting follicular development, increasing the number of mature follicles, enhancing ovulation and clinical pregnancy rates, and regulating glucose and lipid metabolism in human subjects. Quercetin as a candidate therapeutic agent for PCOS, which was further studied for its effect on decidualization. The underlying mechanisms through which quercetin and YHHD can provide therapeutic benefits in PCOS could potentially have far-reaching implications for improving maternal-fetal health outcomes and addressing the complexity of this disorder.

While the clinical trials conducted in our study provided substantial insight, they should be interpreted with caution as they were of preliminary and moderate quality. Further research is needed to validate the findings and confirm the effectiveness of quercetin and YHHD in treating PCOS. Specifically, our results indicate that the combined therapy of dydrogesterone and YHHD was associated with a lower rate of early pregnancy loss compared to either therapy used in isolation. This highlights the potential effectiveness of this Chinese and Western combination therapy in reducing the occurrence of early pregnancy loss. The clinical research has established the protective effects of YHHD in early pregnancies of PCOS patients. To further investigate the pharmacological mechanisms underlying YHHD and its primary active component, quercetin, we conducted both *in vitro* and *in vivo* studies to evaluate their effects. By utilizing a combination of network pharmacology, histopathology, and molecular biology analyses, we characterized the underlying mechanisms from a holistic perspective. Our network pharmacological analysis has revealed significant expression of hormone-related signaling pathways, including the cAMP signaling pathway and ovarian steroidogenic pathway. This suggests that YHHD may have a regulatory effect on a range of hormones. The network pharmacology-based predictions suggested that both YHHD and quercetin alleviate decidualization dysfunction by improving insulin resistance and the associated IRS1/2 pathway. Our experimental validation further demonstrated that YHHD and quercetin effectively ameliorate DHEA-induced decidualization dysfunction in PCOS mice and hyperinsulinemia-induced T-HESC.

Quercetin is a naturally occurring flavonoid that can be found in numerous fruits and vegetables (*45,46*). Studies have shown that quercetin has anti-inflammatory and antioxidant properties, as well as its ability to regulate insulin and glucose metabolism (*45,47*). Quercetin has the potential to become a novel treatment for PCOS, given its ability to intervene in multiple pathological pathways through a multi-target approach (*48*). It has been suggested that quercetin may be a promising therapeutic option for the management of PCOS. Consistent with previous research findings (*49,50*), the findings of this study demonstrate that a 20-day treatment with 50 mg/kg of quercetin produces estrogen-like effects, which improve the proportion of ovarian follicles in PCOS mice. Notably, this intervention has been shown to decrease the number of cystic follicles in individuals with PCOS, a marked increase in luteal and normal follicles, improved insulin sensitivity and glucose metabolism and a reduction in the levels of LH and LH/FSH ratios. However, the study didn't observe any significant improvement in the expression of $E_2$, which is inconsistent with previous research (*51*). Based on the pharmacological effects of YHHD and quercetin on the PCOS network, the primary therapeutic target appears to be insulin resistance. Thus, our upcoming research will concentrate on exploring insulin resistance. Our research has demonstrated that quercetin can enhance insulin signaling by increasing the expression of *IRS1* and *IRS2* genes. However, under high insulin conditions, its effects on decidualization-related genes were found to be limited, and NT157 was more effective in suppressing their expression, revealing that quercetin could only promote decidualization and improve insulin sensitivity under normal insulin conditions. Clinical studies have revealed that patients with hyperinsulinemia exhibit reduced embryo implantation capacity, which often leads to high rates of miscarriage after embryo implantation (*52*).

This study validates the potential therapeutic use of YHHD for PCOS treatment and uncovers its mechanism of action in regulating hormone levels, reversing insulin resistance, and promoting pregnancy, which have been validated in subsequent clinical trials and animal experiments. Additionally, quercetin, as a major compound in YHHD, has shown promising effects in reversing the negative impact of high insulin levels and restoring the expression of key proteins involved in decidualization dysfunction. These findings support the potential use of quercetin as a therapeutic intervention for PCOS-related decidualization dysfunction. Nonetheless,

further rigorous pharmacological and clinical studies are essential to confirm its therapeutic efficacy and safety.

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[§]These authors contributed equally to this work.

*Address correspondence to:
Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: dr.wangling@fudan.edu.cn

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Correspondence

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# The prospects of automation in drug discovery research using silkworms

**Atsushi Miyashita*, Masanobu Miyauchi, Fumiaki Tabuchi**

Teikyo University Institute of Medical Mycology, Tokyo, Japan.

**SUMMARY** We have established several models of infectious diseases in silkworms to explore disease-causing mechanisms and identify new antimicrobial substances. These models involve injecting laboratory-cultured pathogens into silkworms and monitoring their survival over a period of days. The use of silkworms is advantageous because they are cost-effective and raise fewer ethical concerns than mammalian subjects, allowing for larger experimental group sizes. To capitalize on these benefits, there is a growing importance in mechanizing and automating the experimental processes that currently require manual labor. This paper discusses the future of laboratory automation, specifically through the mechanization and automation of silkworm-based experimental procedures.

***Keywords*** silkworm, experimental infection, alternative animals, laboratory automation

## 1. Importance of alternative experimental models

Modern medicine and health science have significantly contributed to our well-being, owing much to the invaluable sacrifices of laboratory animals. Research conducted with mammals, especially mice, has been pivotal in advancing from our fundamental science to practical clinical applications. Nonetheless, increasing animal welfare awareness has made it difficult to conduct experiments using mammals in the field of research and development in non-medical fields such as food and cosmetics.

In the pursuit of alternatives to using mammals in research, the concept of employing silkworms in health science has been explored. This idea has been put forward by various research teams, including ours, for approximately two decades (*1-17*). Such studies have shown that *Staphylococcus aureus* infects and kills silkworms and that the therapeutic efficacy of antibiotics can be evaluated in the same model, and have searched for compounds that are effective in treating infections from soil bacteria libraries. As a result, a new antibiotic, Lysocin E, was identified using the silkworm assays, and was also found to be therapeutic in mice. Traditionally, life science research involving animal testing has relied on mammals, like mice. The ethical dilemmas and the high costs associated with mouse experiments present considerable challenges in this field of research. We propose that these problems can be overcome by using invertebrate alternatives, such as the silkworm (*5,10,14,16-22*).

Silkworms facilitate easy administration of precise sample doses, enabling detailed evaluation of drug effects on an individual basis. Additionally, the low cost of silkworm-based experiments presents a stark contrast to the expenses incurred with mouse models. A typical research laboratory in an academic or government setting is capable of assessing up to 100 chemical compounds daily with silkworms, using a standard dose for three trials per compound, totaling the use of 300 silkworms.

## 2. Limitations of silkworm models

While the experimental system using silkworms has the above advantages, there are also technical challenges to be overcome before it can be more widely used as a research and development platform in the future. For example, there is a lack of standardization of the method to rear the silkworms in university laboratories and other environments (Figure 1). Indeed, some of our collaborator groups sometimes experience problems with silkworm rearing (the process of hatching the eggs to make them into 5-instar larvae). There is a lack of documentation that summarizes a unified view on what specifications of equipment are required to rear silkworms stably using facilities such as universities (*e.g.*, volume of incubators, types of containers used for rearing silkworms). Furthermore, in infection experiments using silkworms,

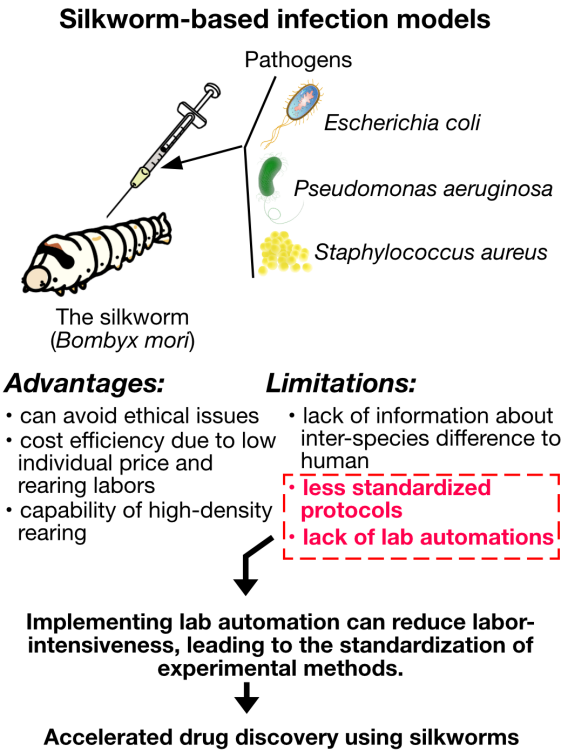


**Figure 1. Prospects of silkworm-based infection models.** By implementing lab automation, we can reduce labor-intensiveness, leading to the standardization of experimental methods. This will accelerate drug discovery research using silkworms.

a syringe with a needle for tuberculin (typically 1 mL) is used to inoculate a sample such as a bacterial solution directly into the hemolymph. Not only does learning this technique require a relatively long period of training, but there are also risks such as needlestick accidents. Due to the nature of the silkworm-based experimental system, a large number of individuals (several hundred or more) are used in a single experimentation, resulting in frequent injection operations and a high risk of needlestick accidents. The authors' laboratory currently rears more than 6,000-10,000 silkworms for experiments every month (*i.e.*, on the scale of 100,000 silkworms per year), so if the probability of a needlestick accident is about 1 in 100,000 (*i.e.*, very rare), this means that about one needlestick accident would occur every year. So far, no severe accidents have occurred, but this is a serious issue from the perspective of promoting the wider use of experimental systems using silkworms in the future.

The observation of silkworms after inoculation with the microorganisms (monitoring survival numbers) is also a major burden for the experimenter. With 240 silkworms at a time and a sample size of 4 silkworms per group, this gives an experimental design of 60 groups. In addition, to date, no criteria have been established for determining whether a silkworm infected with pathogenic bacteria is alive or dead, which has led to variations in judgement between individuals. Therefore, when observing a large number of silkworms several times a day, errors or misses may occur in the silkworm observation records. Furthermore, if observation is dependent on human labor, it is impossible to conduct observations at nights or at other times when the experimenter is not present (*e.g.* Sundays). Therefore, issues also remain in terms of the comprehensiveness of the observation records.

## 3. Perspectives of laboratory automation

Overcoming the above-mentioned challenges necessitates a push towards maximizing the mechanization and automation of silkworm-based experiments. Beyond automating the rearing and injection processes, leveraging the rapidly advancing technologies in object recognition and detection can facilitate the automation of the observation process as well.

Efforts are underway to automate various processes in insect research, including studies on silkworms. This includes advancements in the automation of rearing, experimental procedures, and result monitoring. There's a growing trend in utilizing automated systems for insect rearing to improve the efficiency of producing populations for experiments. With the aid of image processing and embedded systems, it is now possible to continuously monitor and adjust the temperature and humidity levels crucial for the growth of silkworm larvae. Moreover, with the application of deep learning, systems are being crafted that can accurately differentiate between healthy and unhealthy silkworms, which is further streamlining and automating tasks within the sericulture industry (*23*).

In addition, there are many manual processes involved in measuring nematode longevity, and the separation of adults and larvae for experiments has been done manually. However, Felker, *et al*. reported the automation of this process (*24*). Automation has also been implemented in the injection of samples into insects and nematodes. Cornell *et al*. created a system that automatically injects nucleic acids (DNA and RNA) and drugs into *Drosophila* embryos aligned on sheets for transgenic operations. Embryos aligned by the novel device are imaged and recognized by the software, and samples are automatically injected (*25*).

By using specialized camera technology on nematodes, the system can automatically recognize and locate the nematode, determine the success of the injection, and microinject the sample into the nematode at a rate of one every 10 seconds (*26*). The use of automated systems for monitoring insect behavior and ecology is also being studied. Insect monitoring of wing-beat harmonics, melanization and direction of flight are conducted by sensing near-infrared light scattered from behind the insect (*27*).

In addition, the use of smartphones to automatically quantify the number of *Drosophila* offspring and the measurement of adult parameters is being attempted

(*28*). Xu *et al*. used a mobile phone and a macro lens to measure the body length and head width of the larvae of the fall armyworm, and analysed and collected these data using the random forest technique, which resulted in a 92.22% accuracy in identifying the number of larval instars, ranging from 1 to 6 instars. (*29*).

Furthermore, recently, it has become easier for personal computers to perform various computational processes based on images acquired from cameras and other sources by using open-source image recognition toolkits such as OpenCV. By utilizing machine learning libraries such as PyTorch, which runs on the Python programming language, based on the obtained image information, it is expected to be possible to recognize silkworms in real time and obtain numerical information on their appearance and behavior, thereby providing a development platform for the automation of survival count observation.

## 4. Concluding remark

By advancing mechanization and automation efforts, a framework can be developed that allows researchers to carry out only the essential tasks in the lab, enabling them to automatically gather experimental data without being physically present. Our research team is focused on pioneering the automation of silkworm experimental processes to facilitate an environment where researchers can devote more time to intellectual tasks by minimizing the labor and time spent on manual lab work. Automating the processes involved in rearing, experimenting, and observing is anticipated to become a critical component for broadening the application of silkworm-based research platforms in various fields.

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**Address correspondence to:*
Atsushi Miyashita, Institute of Medical Mycology, Teikyo University. 359 Otsuka, Hachioji, Tokyo 192-0352, Japan.
E-mail: atmiyashita@main.teikyo-u.ac.jp

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# Rapamycin *vs* TORin-1 or Gleevec *vs* Nilotinib: Simple chemical evolution that converts PAK1-blockers to TOR-blockers or *vice versa*?

**Hiroshi Maruta[1,*], Hong He[2]**

[1] PAK Research Center, Melbourne, Australia;
[2] Melbourne University Hospital (Austin Health), Melbourne, Australia.

**SUMMARY** Both PAK1 (RAC/CDC42-activating kinase 1) and TOR (Target of Rapamycin) are among the major oncogenic/ageing kinases. However, they play the opposite role in our immune system, namely immune system is suppressed by PAK1, while it requires TOR. Thus, PAK1-blockers, would be more effective for therapy of cancers, than TOR-blockers. Since 2015 when we discovered genetically that PDGF-induced melanogenesis depends on "PAK1", we are able to screening a series of PAK1-blockers as melanogenesis-inhibitors which could eventually promote longevity. Interestingly, rapamycin, the first TOR-inhibitor, promotes melanogenesis, clearly indicating that TOR suppresses melanogenesis. However, a new TOR-inhibitor called TORin-1 no longer suppresses immune system, and blocks melanogenesis in cell culture. These observations strongly indicate that TORin-1 acts as PAK1-blockers, instead of TOR-blockers, *in vivo*. Thus, it is most likely that melanogenesis in cell culture could enable us to discriminate PAK1-blockers from TOR-blockers.

***Keywords*** PAK1, TOR, melanogenesis, immune system, rapamycin, TORin-1, Gleevec, nilotinib

## 1. Introduction

In mid-1994, a new Ser/Thr-kinase called "TOR" was identified in both yeast and mammals, by four groups independently, among the direct "targets" of rapamycin, an antibiotic found in Easter Island around 1970s (*1-4*). Around the very beginning of 1994, the first mammalian Ser/Thr-kinase coined "p21-activated kinase 1" (PAK1) was cloned by Ed Manser's team at Singapore National University (*5*), which is activated by two small G proteins called RAC and CDC42 (p21). Very interestingly, PAK1 is highly homologous to an amoeba kinase called "myosin I heavy chain kinase" (MIHCK), which we discovered in a soil amoeba in 1977 (*6,7*). MIHCK is essential for F-actin-dependent activation of a single headed myosin (myosin I) ATPase. This amoeba kinase phosphorylates the regulatory light chain of smooth muscle myosin II (double-headed) ATPase as well, which becomes F-actin-activatable upon the phosphorylation (*8*). This finding suggests an interesting possibility that PAK1 might be involved in hypertension. Around the turn of this century, both PAK1 and TOR were first identified among major "oncogenic" kinases (*9-12*). Later both kinases were recognized as the major "ageing" kinases as well, dysfunction of which would lead to longevity (*13-15*).

## 2. PAK1 versus TOR in our immune system and melanogenesis

Since then, a series of PAK1-blockers (as well as TOR-inhibitors) were identified or developed (*16-20*) for possible therapy of cancers (and all solid tumours) as well as many other PAK1/TOR-dependent diseases including hypertension (high blood pressure) and inflammation. However, there is a great problem (concern) around TOR-inhibitors, in particular rapamycin which was found to suppress/damage our "immune system" (for a review, *19*), while PAK1-blockers such as FK228 and curcumin activate our immune system (depending on both B- and T-cells) (*21,22*). Finally in 2017 we proved genetically that these B/T cell-dependent immune systems are normally suppressed by PAK1 (*23*, for a summary of PAK1 *vs*. TOR, see Figure 1). Naturally, since then, at least for cancer therapy, we focused our effort mainly on PAK1-blockers. However, screening PAK1-blockers by animal experiments would cost both money and time. Thus, during 2015-16, we finally

managed to device a "simple" cell culture system using melanoma cell line B16F10 in which the serum (PDGF)-dependent melanogenesis is inhibited by silencing PAK1 gene (*24,25*, for summary see Figure 1). Since this melanogenesis in cell culture is fortunately boosted by rapamycin or other TOR-inhibitors (*26,27*, for summary see Figure 1), we could easily exclude TOR-inhibitors, which suppress our immune system as well (see Figure 1).

Interestingly, however, regarding the so-called anti-cancer immune "check-point", rapamycin and PAK1-blockers share the "same" boat, namely both inhibiting PD-L1 expression in cancer cells (*28,29*), indicating that both TOR and PAK1 are essential for PD-L1 expression (*30,31*).

## 3. PAK1-blockers or TOR-inhibitors? That is the question

Rather ironically, however, we recently noticed that a few chemicals called "TOR-inhibitors" apparently inhibit melanogenesis as if they were PAK1-blockers (*32*, see Figure 1), while a new Gleevec derivative, called "nilotinib", which is supposed to block PAK1, promotes melanogenesis as if it were a TOR-inhibitor (*33,34*, see Figure 2).

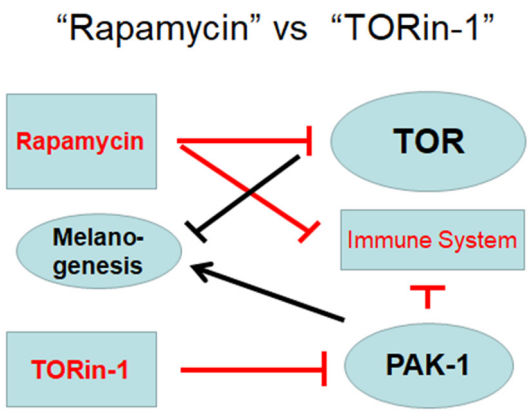


**Figure 1. "TORin-1" blocks PAK1 instead of TOR in cells.**

### 3.1. "TORin-1" developed as a TOR-inhibitor, blocks "PAK1"

As a so-called "side effect", rapamycin is known to inhibit the symptom of MS (multiple sclerosis), an auto-immune disease, as well as organ rejection, and therefore it has been marketed only as an "immune suppressor" since 1999. Although rapamycin is anti-oncogenic as well, it has never been recommended for the therapy of cancers or a rare benign tumour called TSC (tuberous sclerosis complex) which are mainly caused by abnormal activation of TOR.

Fortunately, a few other TOR-inhibitors have been developed in this century, which no longer cause this "side effect" of TOR, namely the immune-suppression. Among them is TORin-1 which was developed in 2010 by a group led by David Sabatini at Dana Farber Cancer Institute and MIT. This drug is an ATP-antagonist (*20*, see Figure 2), and directly inhibits the phosphorylation by both TORC1 and TORC2 with $IC_{50}$ between 2 and 10 nM *in vitro*. In cell culture, however, its $IC_{50}$ is around 250 nM, indicating that its "cell-permeability" might be rather poor (and clearly water-insoluble). Nevertheless, TORin-1 was effective at a dose of 20 mg/kg against the growth of a human U87MG (glioma) xenograft in mice. However, for its future clinical use, both cell-permeability and water-solubility of this chemical should be boosted somehow.

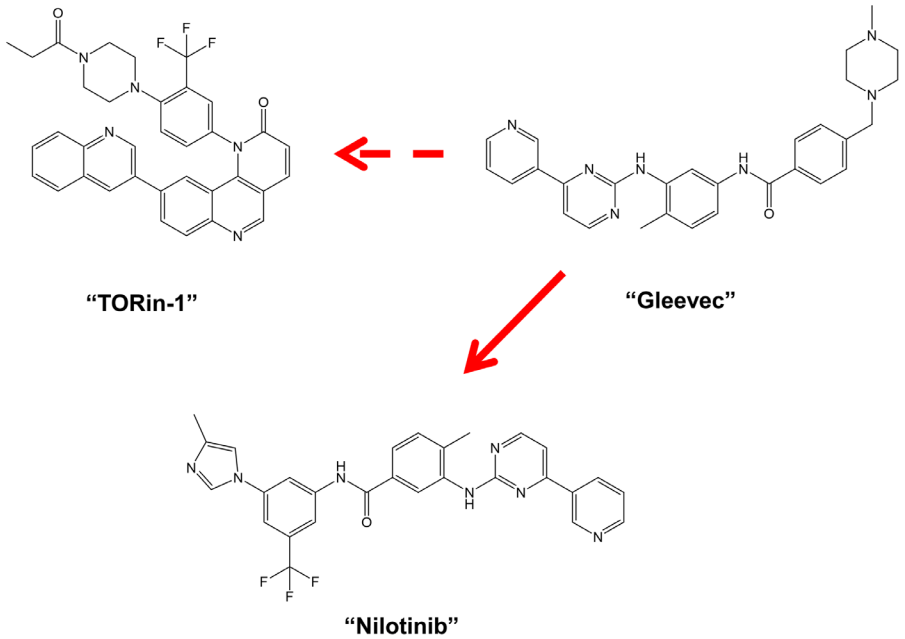


**Figure 2. "TOR"-inhibitor or "PAK1"-blocker? That is the question.**

The tumour-suppressor gene product called "Merlin" inhibits not only PAK1 directly (*35*), but also appears to inhibit TOR as well (directly or indirectly) (*36,37*), and the "loss-of-function" mutation of Merlin causes a rare benign tumour called NF2 (neurofibromatosis type 2), and shortens the lifespan of mice by 40% (from 2.5 years to 1.5 years). However, silencing PAK1 gene alone is sufficient to suppress the growth of NF2 tumours, and normalize the lifespan in mice (*15*).

Likewise, the tumour-suppressor complex of TSC1 and TSC2 appears to block not only TOR, but also PAK1 somehow (*38*), and the "loss-of-function" mutation of TSC1 or TSC2 causes a rare benign tumour called TSC. Rather surprisingly, the major target of TORin-1 in cells appears to be PAK1, instead of TORC1 or 2, mainly because it blocks "PAK1-dependent" melanogenesis (*32*), and shows no effect on immune system *in vivo*. Furthermore, according to 2014 article by US Team in Boston, prostaglandin synthesis by COX-2 whose gene expression depends on PAK1, was not affected by rapamycin, but significantly blocked by TORin-1, clearly indicating that (*i*) in addition to TOR, PAK1 is also abnormally activated somehow in TSC-deficient tumor cells, and (*ii*) TORin-1 blocks PAK1 at least in these TSC tumors (*38*). Interestingly, Gleevec and TORin-1 are chemically very similar (see Figure 2), but in terms of PAK1-blocking activity in cells, TORin-1 is still 40 times more potent than Gleevec in cells. Thus, TORin-1 could be potentially useful for all PAK1-dependent cancers or solid tumours including NF and TSC tumours for which no "effective" (FDA-approved) therapeutic has been available in the market so far.

### 3.2. "Nilotinib", developed as a "Gleevec" derivative, inhibits TOR *in vivo*

It also appears to be true in the "exactly" opposite direction. Gleevec was originally developed by Novartis team around mid-1990s mainly for the treatment of CML (Chronic Myeloid Leukemia), caused by abnormal activation of a Tyr-kinase called ABL. However, it was later found that Gleevec blocks PAK1 by inhibiting a few other Tyr-kinases such as PDGFR and c-KIT with $IC_{50}$ around 10 μM in cells (for review, *39*, see Figure 2). Interestingly Gleevec treatment of melanocytes as well as CML patients causes a significant reduction of melanogenesis in cells or whitening of skins (*40*), clearly indicating the suppression of PAK1-dependent melanogenesis. Meanwhile, Gleevec treatment of CML patients also causes Gleevec resistance. Thus, around 2005, Novartis developed a new Gleevec derivative called "nilotinib" (see Figure 2), which works even on Gleevec-resistant CML. Nilotinib is a fluoride derivative of Gleevec (see Figure 2, *39*). However, just like TORin-1 which is also a fluoride compound, nilotinib appears to change its "major" target *in vivo*. Although nilotinib directly inhibits a series of Tyr-kinases including ABL and PDGFR *in vitro*, and eventually blocking PAK1 *in vivo*, nilotinib even at 5 μM appears to inhibit TOR as well *in vivo*, namely promoting melanogenesis in cell culture just like rapamycin (*33,34*). This "stunning" observation clearly indicates that nilotinib serves dominantly as a TOR-inhibitor in cells, instead of a PAK1-blocker. Thus, nilotinib could be potentially useful for both organ transplantation and MS therapy as well, just like rapamycin. Furthermore, the "main" reason why nilotinib is effective to "Gleevec-resistant" CML cells might not be due to its "*direct*" effect on a (Gleevec-resistant) ABL "mutant" in CML patients, but a "*side*" effect against TOR instead. In fact, according to the 2023 article (*https://www.mdpi.com/1422-0067/24/2/1234*) by an Italian team, TOR is abnormally activated in CML cells/*in vivo*, in particular under "hypoxia".

### 3.3. Pomalidomide, developed as a thalidomide analog

Thalidomide was best known as a "teratogenic" drug, which eventually caused the birth of so-called "thalidomide-children", after it is taken by pregnant women who suffer from "morning sickness".

Thereafter it was totally banned by FDA since the beginning of 1970s. However, ironically, it was found later (around 1990s) that if it is taken by "non-pregnant" people, it wouldn't cause any harmful "side effect". Instead, it was re-discovered as a "miracle" drug which cures a series of inflammatory or infectious diseases such as leprosy (*41*). However, thalidomide is known to suppress our immune system, just like rapamycin, strongly suggesting, if not proven as yet, that it inhibits TOR somehow (*42*). Yes, thalidomide stimulates "melanogenesis" (re-pigmentation) in hairs when old ladies (ca 75 years old) with "white" hairs are used for therapy of MM (Multiple Myeloma) or MS (Multiple Sclerosis) (*43*). Its rather complicated derivative called "lenalidomide" is also still melanogenic clinically (*44*, see Figure 3). Finally, a simple "amino" derivative of thalidomide called "pomalidomide" was developed (see Figure 3). So far pomalidomide showed neither melanogenic or immune-suppressive effect *in vivo*. Most

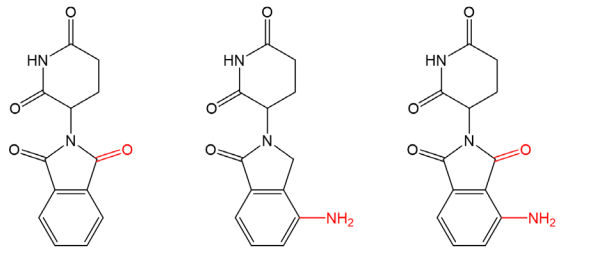


**Figure 3. "Thalidomide" and its derivatives.**

importantly, pomalidomide is no longer teratogenic even if it is taken by chick or fish embryos or pregnant persons (for a review, *45*).

According to the most recent reviews by experts on thalidomide and its derivatives, among their direct targets is a brain protein called "cereblon" (CRBN), and their binding to CRBN, a substrate recognition receptor for an E3 ubiquitin ligase (called "CRL4"), induces the recruitment of non-native substrates to CRL4CRBN and eventually their proteolytic degradation (*46*). In other words, thalidomide is among "PROTACs" which bind both E3 ubiquitin ligase(s) and their specific targets.

However, non-teratogenic pomalidomide still shows both "anti-angiogenic" and anti-cancer activities by ubiquitination-induced "proteolytic" degradation (so-called "PROTAC" action) of another target, a Tyr-kinase called "JAK", eventually blocking PAK1, just like thalidomide (*47*). Thus, it is now crystal-clear that the "teratogenesis" by thalidomide has nothing to do with its "anti-angiogenesis".

Finally we dug out a "possible" link of thalidomide to TOR (*48*): This drug activates somehow (but not through its direct action on CRBN) an anti-oncogenic kinase called "AMPK" (AMP-activated kinase), which activates the anti-oncogenic "TSC" complex, that eventually inhibits TOR.

In other words, pomalidomide is simply a PAK1-blocker, while thalidomide is a "CRBN/TOR" inhibitor as well as a PAK1-blocker. Very recently (2023), a Chinese group developed a potent TOR-specific PROTAC (protein-targeting chimera) by combining "POM" and a TOR-binding ligand called "MLN0128" (*49*), perhaps for their great fun, or at least proving that pomalidomide alone never inhibits (or binds) TOR. By the way, POM is 10 times more potent than thalidomide and lenalidomide for therapy of a hematological cancer called MM clinically, most likely because pomalidomide is no longer immune-suppressive (*50*).

## 4. Closing remarks

Although both PAK1 and TOR share the major roles in our life, namely "oncogenic" and "ageing", they play the "exactly opposite" roles in at least two other physiological functions, namely B/T-cell based immune system and PDGF-dependent melanogenesis. Thus, based on these differences, in particular their opposite roles in the melanogenesis of B16F10 melanoma cells, we are able to distinguish easily between PAK1-blockers and TOR-inhibitors. At least in three examples shown above (TORin-1, nilotinib, and pomalidomide), a "simple" modification of TOR-inhibitors (or PAK1-blockers) could result in their functional conversion to PAK1-blockers (or TOR-inhibitors) for so-called "chemical evolution", just like "penguins", which cannot fly in sky suddenly started swimming in sea with their "unique" feathers (called "flippers"), in order to reach their "new" home called "South Pole" (Antarctica) from their "old" homes (New Zealand or Australia) through the great ocean as soon as "vicious" cats were unfortunately introduced there (latter) by *Homo sapiens*.

Back in 2020, we briefly review three distinct PAK1-blockers whose anti-oncogenicity was potentiated 500-3,000 times for clinical application by a rather simple chemical modification (*51*). One of them is 1, 2, 3-triazolyl ester of ketorolac called "15K". This ester is more than 500 times "cell-permeable" than ketorolac which bears a COOH, and its anti-PAK1 activity also has been proven to be 500 times more potent than ketorolac in cell culture (*52*), and still remains to be "anti-melanogenic" (unpublished observation). However, although a CYP24-resistant derivative of vitamin D3 called "MART-10" was proven to be 1,000 times more potent than D3 as an anti-cancer agent in cell culture and *in vivo*, nobody knows whether MART-10 still remains as a PAK1-blocker (or TOR-inhibitor). Although D3 has been proven to block the RAC-PAK1 interaction (clearly being a PAK1-blocker) as is Ketorolac, to our surprise D3 has been recently reported to restore pigmentation in "vitiligo", and promote melanogenesis of B16F10 melanocytes, indicating the possibility that D3 might inhibit TOR as well (*53*). Thus, we are planning to test the effect of MART-10 on melano-genesis, determining if it blocks mainly PAK1 or TOR. By the way, according to 2018 clinical trial report from US and UK team (54), D3 (4,400 IU/d) stimulates both B-and T-cell based neonatal immunity of maternal supplementation, strongly suggesting that D3 is an immune promoter (mainly being a PAK1-blocker).

If we could give a "last" brief word (s) to readers, the whole "kinase" research world, hopefully for a further evolution, we would like to encourage them to do a cross-talk, or "horizontal"-thinking, instead of "vertical" thinking (as if they were mathematicians), breaking so-called "sectionalism". For an instance, it has been firmly established since 2010 that "AMPK-activators are PAK1-blockers", since the anti-oncogenic LKB1 was found to inactivate the oncogenic PAK1 by phosphorylating Thr at position 109, while it activates the anti-oncogenic AMPK by phosphorylating Thr at position 172 as well (*55*). Penguins learned how to swim by watching fishes, while flying fishes learned how to fly, by watching birds

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**Address correspondence to:*
Hiroshi Maruta, PAK Research Center, Melbourne, Australia.
E-mail: mogumaruta123@gmail.com

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Letter to the Editor

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# Metastasis to hypopharynx from epidermotropic metastatic malignant melanoma

**Satoru Mizuhashi[1], Azusa Miyashita[1,*], Haruka Kuriyama[1], Toshihiro Kimura[1], Hisashi Kanemaru[1], Satoru Miyamaru[2], Sho Saeki[3], Satoshi Fukushima[1]**

[1] Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan;
[2] Department of Otolaryngology-Head and Neck Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan;
[3] Department of Respiratory Medicine, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.

**SUMMARY** Previous reports proposed the concept and criteria of epidermotropic metastatic malignant melanoma (EMMM): (a) dermal involvement equal to or broader than the epidermal involvement, (b) atypical melanocytes within the dermis, (c) thinning of the epidermis, (d) widening of the papillary dermis with an epithelial collarette, and (e) vascular invasion of atypical melanocytes. However, it remains unclear whether EMMM also involves the mucosal epithelium. In this case, the patient was diagnosed with EMMM based on the histopathological findings of the patient's multiple skin lesions and clinical course. The patient also developed metastasis to the hypopharynx. Although histopathological findings of the lesion suggested the possibility of melanoma in situ, as the lesion included atypical melanocytes in the mucosal epithelium, the clinical course supported the diagnosis of hypopharyngeal metastasis from EMMM. This case suggests that EMMM may have epitheliotropic features not only in the skin but also in the mucosa.

***Keywords*** metastasis to mucous membranes, epidermotropism, hypopharyngeal metastasis

Letter to the Editor,

Melanoma metastasis usually involved the dermis and subcutaneous tissue and less frequently involves the overlying epidermis (*1*). A previous report proposed the concept of epidermotropic metastatic malignant melanoma (EMMM) (*2*). It is often challenging to recognize EMMM because its histopathological appearance can mimic primary melanoma (*3*). Some reports have described the histopathological criteria for defining EMMM (*3,4*). However, it remains unclear whether EMMM also involves the mucosal epithelium. We herein report a case with an important finding of hypopharyngeal metastasis from EMMM.

A 50-year-old man presented with an upper lip melanoma at our hospital. The melanoma lesion was resected and reconstructed using a cross-lip flap. Right cervical lymph node dissection was performed, because metastasis was found in the sentinel lymph node. A BRAF mutation was not detected in the primary tumor sample. Two months after operation, a small pigmented macule developed in the hypopharynx (Figure 1a). Biopsy of the lesion revealed localized melanoma within the mucosal epithelium and in the subepithelial layer (Figures 1b and 1c). It was difficult to determine whether it was a new primary lesion or a melanoma metastatic to the mucosa at this point. Three months later, multiple pigmented macules were observed on the face and genital area of the patient (Figures 2a and 2b). At the same time, computed tomography (CT) revealed multiple frosted glassy shadows in the lungs (Figure 2c).

Skin biopsies were performed for several pigmented macules. Histopathologically, the patient's skin lesions exhibited some characteristic features of EMMM (Figures 2e-2h): the epidermal component was limited to the area above the dermal component; the epidermal and dermal components contained large atypical melanocytes; and angiotropic spread of atypical melanocytes was observed around the dermal blood vessels. Regarding pulmonary lesions, CT-guided biopsy revealed metastatic melanoma with lepidic growth (Figure 2d).

We diagnosed the patient with EMMM based on the histopathological findings of the patient's skin lesions and clinical course. Additionally, the clinical course supported the diagnosis of hypopharyngeal metastasis from EMMM. The lesions included atypical melanocytes in the mucosal epithelium, suggesting epidermotropism.

We observed an important clinical characteristic of EMMM, which is epitheliotropic features in the hypopharynx. To our knowledge, this is the first report on the histopathological finding of hypopharyngeal

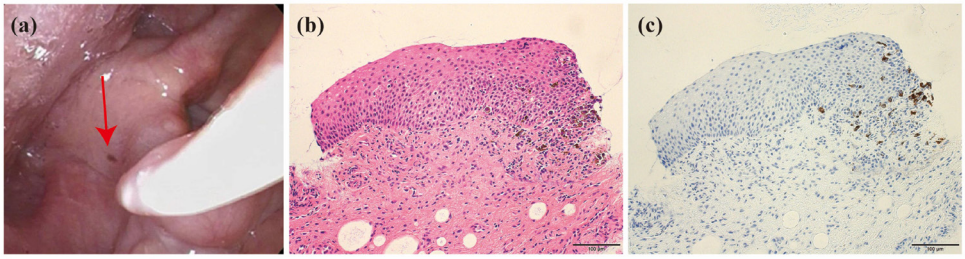


**Figure 1. Histopathological findings of hypopharyngeal metastasis from epidermotropic metastatic malignant melanoma. (a)** Clinical findings of the pigmented macule in hypopharynx. **(b)** Histopathological findings of the hypopharyngeal lesion (hematoxylin-eosin). Scale bar = 100 μm. **(c)** Immunohistochemical analysis with HMB-45 antibody (#M063401, Agilent, Santa Clara, CA, USA) and with Giemsa as a counterstain. Scale bar = 100 μm.

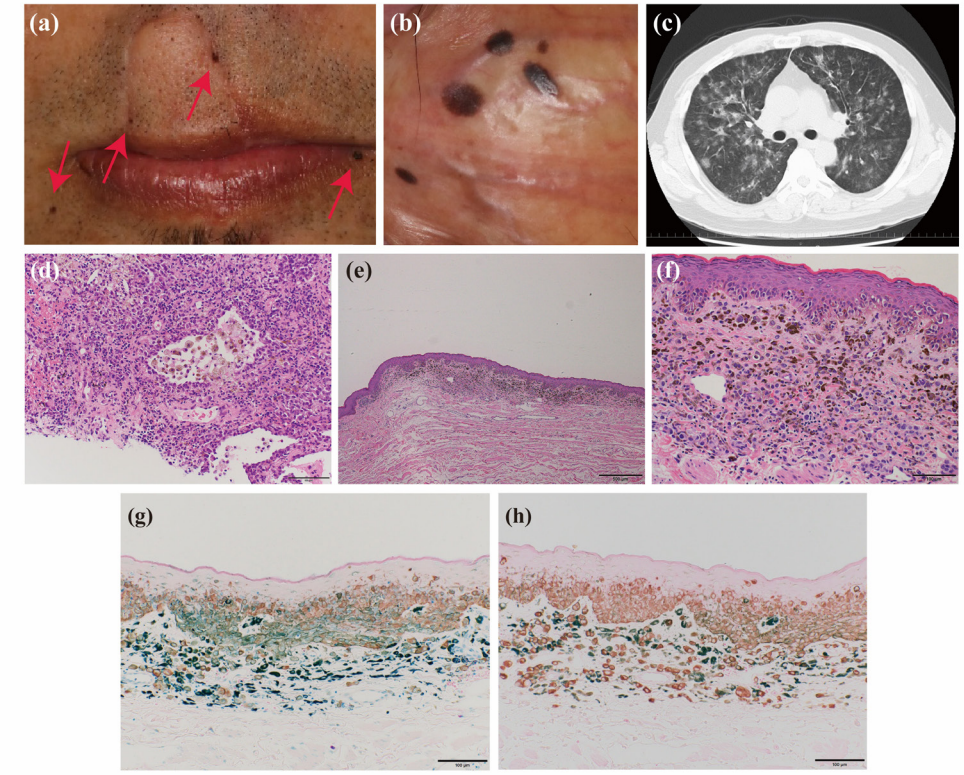


**Figure 2. Clinical features and histopathology. (a,b)** Clinical findings of multiple pigmented macules in the face and genital area. **(c)** Computed tomography findings of multiple frosted glassy shadows in the lung. **(d)** Histopathological findings of lung lesions (hematoxylin-eosin). Scale bar = 100 μm. **(e,f)** Histopathological findings of the skin lesion (hematoxylin-eosin). **(e)** Scale bar = 500 μm. **(f)** Scale bar = 100 μm. **(g)** Immunohistochemical analysis of the skin lesion with HMB-45 antibody (#M063401, Agilent, Santa Clara, CA, USA) and with Giemsa as a counterstain. Scale bar = 100 μm. **(h)** Immunohistochemical analysis of the skin lesion with MART-1 antibody (#413381, Nichirei Bioscience Inc, Tokyo, Japan) and with Giemsa as a counterstain. Scale bar = 100 μm.

metastasis from EMMM.

In 1978, Kornberg *et al*. (*2*) proposed the EMMM concept and criteria: (a) dermal involvement equal to or broader than the epidermal involvement, (b) atypical melanocytes within the dermis, (c) thinning of the epidermis, (d) widening of the papillary dermis with an epithelial collarette, and (e) vascular invasion of atypical melanocytes. Furthermore, some reports showed that the presence of angiotropism is suggestive of EMMM (*5-9*). In this case, the patient's multiple skin lesions exhibited some characteristic histological features of EMMM. The patient was diagnosed with EMMM based on the histopathological findings of patient's multiple skin lesions and clinical course.

A previous study indicated that gastric metastasis from EMMM may include atypical melanocytes in the superficial mucosa (*10*). However, little is known about the histopathological findings of hypopharyngeal metastasis of EMMM. In this case, atypical melanocytes were localized to the mucosal epithelium and in the subepithelial layer of the hypopharyngeal lesion. Although it was difficult to determine whether that is

a new primary lesion or a melanoma metastatic to the mucosa, the clinical course supported the diagnosis of hypopharyngeal metastasis from EMMM. This finding is similar to that of a previous report on gastric metastasis (*10*). These suggest that EMMM may have epitheliotropic features not only in the skin but also in the mucosa. However, further investigations are required to confirm the histopathological findings of mucosal metastasis from EMMM.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Azusa Miyashita, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan.
E-mail: azusa-miyashita@kumamoto-u.ac.jp

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**Drug Discoveries & Therapeutics**
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@ddtjournal.com