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Expert consensus on combination antiviral therapy for high-risk COVID-19 patients: A timely call to action

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SUMMARY: On May 5, 2023, the WHO declared that the COVID-19 pandemic no longer constitutes a public health emergency of international concern (PHEIC), but SARS-CoV-2 continues to spread and evolve on a global scale. The WHO reported that COVID-19 still poses a threat to humanity, and especially in some areas with large numbers of infected people. For some high-risk COVID-19 patients, such as those with underlying conditions, elderly patients, patients who need long-term immunosuppressive therapy after organ transplantation, patients with immunosuppressive diseases, patients who tend not to test negative for SARS-CoV-2 despite standard antiviral therapy, and cancer patients, special attention is still required after infection with SARS-CoV-2. How to clear SARS-CoV-2 in a timely manner is the key to treating such patients. Based on the demands of clinical practice and medical evidence, the National Center for Infectious Diseases of China assembled experts from relevant disciplines to reach the Chinese expert consensus on the combined use of antivirals to treat COVID-19, providing timely suggestions to resolve the medication issues that have been plaguing clinical practice. The consensus suggests that for special patients, combined medication can promptly eliminate the virus without increasing the risk to patient safety.

Keywords: SARS-CoV-2, COVID-19, special patients, antiviral therapy, small-molecule drugs, drug combination, expert consensus

1. Introduction

On May 5, 2023, the WHO declared that the COVID-19 pandemic no longer constitutes a public health emergency of international concern (PHEIC). Significant progress has been made in the global response to the COVID-19 pandemic, but SARS-CoV-2 continues to spread and evolve on a global scale. The continuous mutation of SARS-CoV-2 means that it continues to threaten human health and that it also hampers existing antiviral treatment strategies. The WHO emphasizes that all countries must continue to implement prevention and control measures, including monitoring virus mutations, enhancing vaccination for high-risk groups, and optimizing clinical capabilities, to consolidate the achievements made in the prevention and control of this epidemic.

A large number of COVID-19 patients are still seen in clinical practice. Some high-risk COVID-19 patients, such as those with underlying conditions, elderly patients, patients who need long-term immunosuppressive therapy after organ transplantation, patients with immunosuppressive diseases, patients who tend not to test negative for SARS-CoV-2 despite standard antiviral therapy, and cancer patients, are prone to develop severe or even critical COVID-19, making clinical treatment relatively challenging. This is why the National Center for Infectious Diseases of China assembled experts from relevant disciplines into a team. The team conducted a systematic literature search, identified key issues, put forward relevant recommendations, and reached the Chinese expert consensus on the combined use of antiviral drugs for novel coronavirus infection (*1*), providing timely suggestions to resolve the medication problems that have been plaguing clinical practice.

2. There is still a large number of COVID-19 patients, with some requiring hospitalization and a few deaths related to the virus

According to the official website of the WHO, in the 28 days from June 30 to July 27, 2025, a total of 80,765 new cases were reported in 90 countries across five of the WHO's regions. Overall, new cases from 27 countries in Africa, the Americas, Europe, and Southeast Asia increased by more than 10%. During this period, 171 new ICU hospitalizations were reported in 32 countries

across four of the WHO's regions. Of the 31 countries that have continued to report hospitalized cases over the past 28 days, 10 countries in the Americas, Europe, and the Western Pacific have reported an increase in cases. Of the 24 countries that continued to report ICU admissions during the same period, 4 countries in the Americas and Europe have reported an increase in cases. During that period, 41 countries in five of the WHO's regions reported a total of 846 new COVID-19 deaths. The number of new deaths in 9 countries in the Americas, Europe, and Southeast Asia has increased by more than 10%. In June 2025, 87% of the cases of death reported with information on age occurred involved individuals age 65 and older (*2*).

As of July 27, 2025, a total of 778,457,848 cases of COVID-19 have been reported worldwide. This does not include cases in some countries that have stopped extensive monitoring of COVID-19 cases and cases that have not been reported to the WHO. As of July 27, 2025, a total of 7,099,375 deaths related to COVID-19 have been reported worldwide. In the past week, from July 21 to July 27, 2025, 166 new deaths were reported (*2*). According to a WHO report, COVID-19 still poses a threat to humanity, and especially in some areas (Figures 1 and 2).

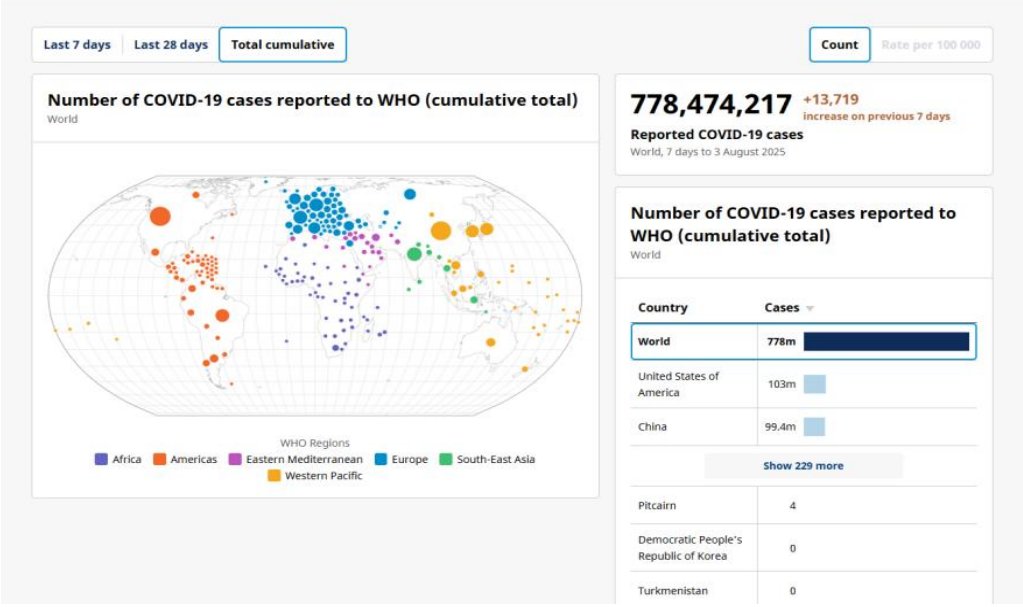

Figure 1. Number of COVID-19 cases reported to the WHO (cumulative total), as of August 3, 2025 (*Source*: World Health Organization, WHO COVID-19 Dashboard).

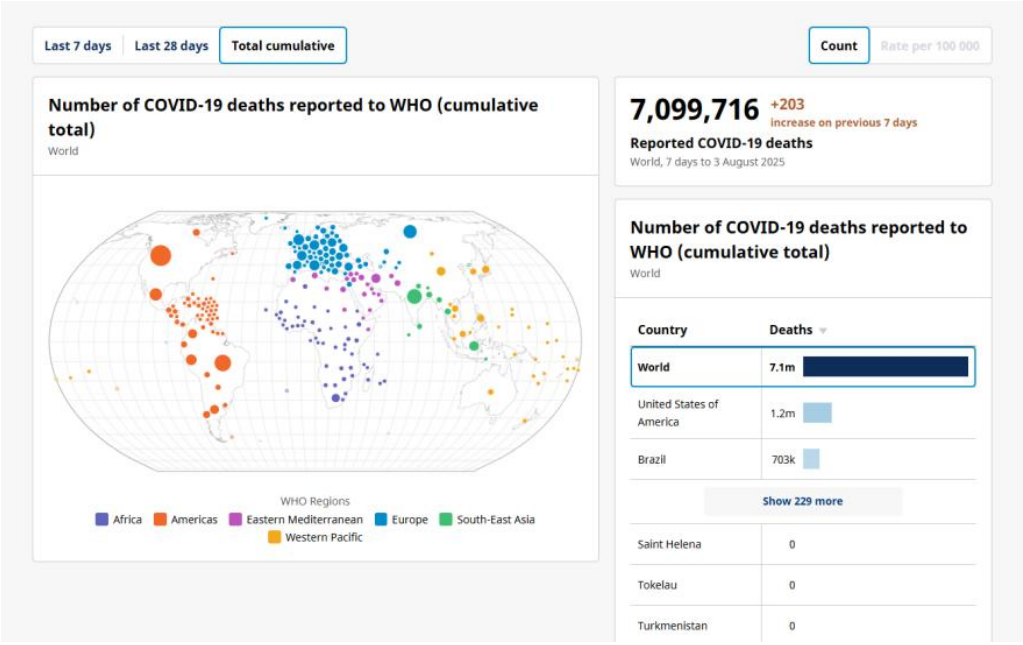

Figure 2. Number of COVID-19 deaths reported to the WHO (cumulative total), as of August 3, 2025 (*Source*: World Health Organization, WHO COVID-19 Dashboard).

According to surveillance data from sentinel hospitals of the Chinese Center for Disease Control and Prevention, the number of COVID-19 patients in China has currently increased significantly compared to before. SARS-CoV-2 is now the most common respiratory pathogen among all common respiratory pathogens. In both southern and northern provinces and cities, COVID-19 is most often detected in outpatient influenza-like cases. The continuous spread of the virus, and especially among high-risk groups with weakened immunity (such as the elderly and those with underlying conditions), makes effective antiviral treatment plans particularly crucial.

From January 10, 2020 to August 18, 2025, Shenzhen Third People's Hospital, which is a designated hospital for COVID-19 treatment, treated 52,478 outpatients and emergency patients with COVID-19 and admitted 14,533 patients. From May 5, 2023 to August 18, 2025, that is, after the WHO declared that the COVID-19 pandemic no longer constituted a public health emergency of international concern, Shenzhen Third People's Hospital treated 15,906 outpatients and emergency patients with COVID-19 and admitted 2,536 patients. Except for a few patients, the vast majority needed to be hospitalized due to underlying conditions such as diabetes, hypertension, cardiovascular and cerebrovascular diseases, chronic kidney disease, AIDS, and post-organ transplantation. However, infection with SARS-CoV-2 has aggravated underlying conditions in a considerable number of patients.

3. In the post-COVID era, attention still needs to be paid to the characteristics of the COVID-19 pandemic, and especially the evolution and mutation of SARS-CoV-2, as well as to research and development of COVID-19 vaccines and new drugs

At present, a considerable amount of experience has been gained in the diagnosis and treatment of COVID-19 patients. However, there are still several aspects that need attention. (*i*) There are many patients with long COVID, which hampers their work and life. According to conservative estimates, at least 400 million people worldwide who have been infected with SARS-CoV-2 also have "long COVID" (*3*). Long COVID symptoms can cover multiple systems, including the respiratory, nervous, urinary, reproductive, motor, digestive, endocrine, and immune systems, and range from mild to severe (*4-7*). However, the mechanism of long COVID remains unclear. (*ii*) Some patients, such as those age 60 or older and HIV patients with other diseases, have a higher risk of death after infection with SARS-CoV-2 (*8*). In addition, patients with underlying conditions, elderly patients, patients who need long-term immunosuppressive therapy after organ transplantation, patients with immunosuppressive diseases, patients who tend not to test negative for SARS-CoV-2 despite standard antiviral therapy, and cancer patients, are prone to develop severe or even critical COVID-19 after infection with SARS-CoV-2, and their clinical treatment is relatively difficult. (*iii*) COVID-19 is significantly associated with an increased risk of developing other diseases, such as diabetes. Close monitoring of blood glucose should be considered after contracting COVID-19, and especially for adult patients who require hospitalization or ICU admission (*9*). (*iv*) Cancer patients are a group that is highly vulnerable to COVID-19. Due to immunosuppression caused by the malignant tumor itself or anti-cancer treatment, cancer patients have a poor prognosis (*10*). (*v*) At present, many countries are at a standstill in the research and development of COVID-19 vaccines and drugs and do not view them as crucial. A study has found that the effectiveness of preventing COVID-19 may increase with an increase in vaccination (*11*). (*vi*) The evolution of SARS-CoV-2 is related to vaccination and other factors. Now that the pandemic has ended, the global vaccination rate has declined, both for the general population and for those most likely to develop severe illness from the virus. This indicates that the efficacy of the vaccine may be diminishing. Detection and sequencing of SARS-CoV-2 is also decreasing. Moreover, the process of tracking viruses has become increasingly complex, thereby providing a selective advantage for SARS-CoV-2 and enabling it to evolve quietly (*12*).

In current clinical practice, however, the resistance of SARS-CoV-2 to antivirals, the rebound of viral load after treatment with certain drugs (such as Paxlovid), and the urgent need for rapid virus clearance in the treatment of critically ill patients all suggest that existing single-agent treatment regimens may have limitations in some cases, and the potency of their inhibition may be insufficient. Therefore, exploring the combined use of antivirals with different mechanisms of action to achieve better efficacy has become an important research direction.

Researchers have discovered multiple SARS-CoV-2 variants, including Alpha, Beta, Gamma, Delta, and Omicron. These variants differ in terms of transmissibility and pathogenicity, with the Omicron variant having greater immune escape ability. The rapid emergence of virus variants not only poses challenges to the efficacy of vaccines and existing prevention and control strategies but may also affect the effectiveness of single-agent antiviral therapy and increase the risk of drug resistance, thereby further highlighting the urgency of exploring more robust treatment strategies such as combination therapy.

4. After conventional single medications treatment and regular courses of treatment, the virus in some special patients still has difficulty turning negative. Combined medication can help eliminate the virus as soon as possible and increase the success rate of rescue

For COVID-19 patients, routine diagnosis and treatment should be based on the COVID-19 Diagnosis and Treatment Protocol (draft Version 10) issued by the National Health Commission of China and relevant prescription information. Nirmatrelvir and ritonavir should be used for 5 days, or molnupiravir for 5 days, or azvudine for up to 14 days. Monotherapy is recommended (*13*). In clinical practice, however, patients who have not tested negative for viral nucleic acids despite treatment with a single antiviral are often encountered, and especially the elderly with multiple underlying conditions, patients who have been using immunosuppressants for a long time after organ transplantation, and patients with immune deficiencies. A clinical study in China examined COVID-19 patients with relatively mild symptoms. A total of 1,571 hospitalized COVID-19 patients were included in this retrospective cohort study. Of them, 272 received nirmatrelvir-ritonavir and 156 received azvudine. Results indicated that the 28-day negative nucleic acid conversion rate was 216/272 (79.41%) in the nirmatrelvir-ritonavir group and 132/156 (84.62) in the azvudine group (*14*). In other words, even among patients with relatively mild COVID-19 symptoms, about 20% who receive single antiviral therapy tend not to test negative for viral nucleic acids.

5. Similar to the cocktail therapy for AIDS, antivirals with different pharmacological mechanisms of action should be used to promptly eliminate the virus without increasing adverse drug reactions

From a pharmacological perspective, the combined use of antivirals with different mechanisms of action, as exemplified by the cocktail therapy used to fight AIDS, is a common strategy to enhance efficacy and reduce the risk of drug resistance. This theory is also applicable to the treatment of COVID-19. At present, the oral small-molecule antivirals mainly used in clinical practice can be roughly classified into two categories: 3CL protease inhibitors as exemplified by nirmatrelvir/ritonavir, simnotelvir/ritonavir (Xiannuoxin), and leritrelvir, and RNA-dependent RNA polymerase (RdRp) inhibitors as exemplified by molnupiravir, azvudine and VV116, which act by inhibiting different stages of viral replication. Cases encountered in clinical practice and various studies have also confirmed the feasibility and potential advantages of this combined strategy, and especially when treating patients with weakened immune function or complex conditions.

6. Combination therapy for some special patients has been successful

A study collected data on nearly 10,000 patients who had received antivirals for COVID-19 in 8 domestic hospitals in China, and it analyzed the changes in viral load after taking the drugs (*15*). Results indicated that combining antivirals with different mechanisms of action was better at inhibiting SARS-CoV-2. Case series studies have also indicated that for some special patients, such as those who are critically ill, immunosuppressed, post-transplant, or who tend not to test negative for viral nucleic acids after conventional treatment, combined use of small-molecule antivirals can, when virus clearance is the main goal, significantly increase the rate at which nucleic acids are detected. It helps to eliminate the virus as soon as possible, improve the success rate of rescue, and does not increase safety risks (*16*).

7. An expert consensus provides new ideas and suggestions regarding antiviral therapy for special patients

Based on the demands of clinical practice and medical evidence, the National Center for Infectious Diseases of China assembled experts from disciplines such as infectious diseases, respiratory internal medicine, critical care medicine, and clinical pharmacy to form a team. The team conducted a systematic literature search, identified key issues, and put forward relevant recommendations, reaching the Chinese expert consensus on the combined use of antiviral drugs for novel coronavirus infection (*1*) in order to provide a reference for clinical practice. A point worth noting is that the expert consensus suggests that for patients with confirmed COVID-19, the routine use of a single drug should be considered first. In some high-risk COVID-19 patients where virus clearance is still the main goal, the combined use of small-molecule antivirals can help to clear the virus as soon as possible and improve the success rate of rescue.

8. Conclusion

There are still many patients with COVID-19, and sufficient attention still needs to be paid to the COVID-19 epidemic. Clinical practice and numerous sources have confirmed that for special patients, combined medication can promptly eliminate the virus without increasing the risk of drug safety. The publication of the Chinese expert consensus on the combined use of antivirals to treat COVID-19 provides timely suggestions to resolve the medication problems that have long plagued clinical practice. Continuous monitoring should be enhanced and the SARS-CoV-2 genome should be analyzed and determined in light of complex and volatile trends in current viral epidemics to enable the issuance of more timely warnings, targeted response measures should be implemented, and relevant drug and vaccine research and development should be carried out.

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

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Consensus

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Chinese expert consensus on the combined use of antiviral drugs for novel coronavirus infection

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SUMMARY: The persistent mutation of the novel coronavirus (SARS-CoV-2) not only remains a threat to human health but also continues to challenge existing antiviral therapeutic strategies. In current clinical practice, the resistance of novel coronavirus to antivirals, the rebound of viral load after treatment with drugs such as nirmatrelvir/ritonavir (NTV/r), and the urgent need for rapid clearance of the virus in the management of critically and emergently ill patients suggest that the existing single-drug regimens may have limitations and that the intensity of suppression may be insufficient in some cases. In clinical practice, we have observed that a combination of antivirals with different mechanisms of action can result in better efficacy and not significantly increase adverse drug reactions (ADRs). For some immunosuppressed, post-transplantation, or other special patients in particular, such as those in whom COVID-19 nucleic acids tended not to be negative after conventional treatment, when virus clearance is still the main goal, the combination of small-molecule antivirals can help to clear the virus as early as possible and attempt to improve the success rate of salvage. Based on evidence-based medicine and in light of the current situation of China, we assembled experts from disciplines such as infectious diseases, respiratory medicine, critical care medicine, and clinical pharmacy into a group to carry out a systematic literature search and identify key issues and to put forward relevant recommendations to reach an Expert Consensus on Combined Use of Oral Small-molecule Antivirals to Treat COVID-19, which is intended to serve as a reference for clinical practice.

Keywords: novel coronavirus infection, COVID-19, antiviral therapy, small molecule drugs, drug combination, expert consensus

1. Introduction

Despite significant progress in the global response to the COVID-19 pandemic, the continued mutation of SARS-CoV-2 not only makes it a continuing threat to human health but also continues to challenge existing antiviral therapeutic strategies. The World Health Organization (WHO) emphasizes the need for countries to continue to implement preventive and control measures, including monitoring virus mutation, enhancing vaccination of high-risk groups, and optimizing clinical capacity in order to consolidate the gains made in the prevention and control of the epidemic.

Sustained viral transmission, and especially in at-risk populations with weakened immunity such as elderly patients and patients with an underlying condition, makes effective antiviral treatment programs particularly critical. In current clinical practice, however, the resistance of novel coronavirus to antivirals, the rebound of viral load after treatment with drugs such as nirmatrelvir/ritonavir (NTV/r), and the urgent need for rapid clearance of the virus in the management of critically and emergently ill patients suggest that existing single-drug regimens may have limitations and that the intensity of suppression may be insufficient in some cases. Therefore, an important research direction is to explore the combination of oral small-molecule antivirals with different mechanisms of action in order to achieve better efficacy. The currently approved oral small-molecule antivirals for COVID-19 can be mainly divided into two categories: 3CL proteolytic enzyme inhibitors (exemplified by NTV/r) and RNA replicase inhibitors (exemplified by molnupiravir).

A retrospective analysis of data from nearly 10,000 patients who had been treated with oral small-molecule antivirals to analyze the changes in viral load after treatment found that combining antivirals with different mechanisms of action resulted in better suppression of SARS-CoV-2 (*1*). According to the COVID-19 Diagnosis and Treatment Program (draft 10th edition) and related prescribing information issued by the National Health and Wellness Commission of China, the use of NTV/r for 5 days or azvudine for a maximum of 14 days is recommended for single-agent treatment (*2*). Previous case summaries have also shown that sequential or concomitant therapy with NTV/r and azvudine can increase the nucleic acid negative conversion rate and accelerate recovery without an increase in adverse drug reactions (ADRs), and all the ADRs observed were mild and low-grade. One study concluded that NTV/r and azvudine are safe and effective, whether administered sequentially or concomitantly, in patients with COVID-19 due to the Omicron variant (*3*).

The long-term symptoms associated with SARS-CoV-2 infection, which are referred to here as "long COVID (LC)" are also a conspicuous global public health concern (*4*). The variants of SARS-CoV-2 differ in their transmissibility and pathogenicity, with the Omicron variant having greater immune escape. One study even observed that despite the production of neutralizing antibodies in COVID-19 patients, they may still excrete infectious SARS-CoV-2 for more than 3 months (*5*). In addition, the rapid emergence of viral variants not only challenges vaccine efficacy and existing prevention and control strategies but may also affect the effectiveness of single-agent antiviral therapy and increase the risk of drug resistance, thus further highlighting the urgency of exploring more robust therapeutic strategies, such as drug combinations (*6*).

2. Methods

Based on the needs of clinical practice and according to evidence-based medicine and in light of the current situation in China, we assembled experts from disciplines such as infection diseases, respiratory internal medicine, critical care medicine, and clinical pharmacy to form a group to carry out a systematic literature search and identify key issues and to put forward relevant recommendations to reach an Expert Consensus on the Combined Use of Oral Small-molecule Antivirals to Treat COVID-19. It is intended for reference in clinical practice. The strength of the recommendations and the grades of evidence are shown in Tables 1 and 2 and are based on the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) (*7*).

Consensus Text

3. Theoretical basis for the combined use of antivirals

Although vaccination has significantly reduced the severe case rate and the mortality rate of COVID-19, viral mutations that allow the virus to break through the immune barrier and cause human infections, which are referred to as breakthrough infections, are still seen, and especially when new variants are prevalent or individuals with poor immune responses such as decreasing levels of antibodies over time or inadequate cellular immune responses; rapid and effective control of viral replication is essential to prevent disease progression (*8*). Cellular immunity plays an important role in combating SARS-CoV-2, which is synergistic with humoral immunity (*9*). Theoretically, the combined use of antivirals targeting different targets can gain the time for the host's immune system (including humoral immunity and cellular immunity) to clear the virus through more potent or broad-spectrum viral suppression and may reduce the generation and selection pressure of immune escape variants by enhancing the intensity of viral suppression. This constitutes the theoretical basis for the combined use of antivirals.

Viral resistance has been associated with SARS-CoV-2 viral spiking protein mutations, and single antiviral therapy increases the emergence of drug-resistant mutations, some of which are found in strains that can disrupt antibody-binding sites and thus evade antibody neutralization (*10*). Some antivirals can induce resistance to SARS-CoV-2, which may lead to increased morbidity and mortality. Combined drug administration may improve efficiency and reduce the need for healthcare resources through the selection of drug combinations to maximize antiviral efficacy and reduce adverse drug interactions (*11*). Combination therapy can enhance the inhibitory effect on the virus, reduce the possibility of drug-resistant mutations, increase the virus clearance rate, and decrease the occurrence of virus rebound (*12*).

The presence of multiple SARS-CoV-2 antibody escape variants in patients with an immune deficiency may be one of the reasons why the nucleic acids of the novel coronavirus tend not to be negative in such patients. Similar corroboration is provided by a case reported in the United States, where a male COVID-19 patient in his 50s on long-term immunosuppressant therapy received convalescent plasma therapy, but the virus from samples on day 21 and 27 had developed new mutations (*13*). The use of strategies such as combinations of drugs that can more potently and durably inhibit viral replication can help to reduce the chances of viral mutation and immune escape.

Table 1. Recommended clinical classification

Strength of Recommendation	Grade	Grade Interpretation and Clinical Recommendations
A	Strong	Evidence is positive or good (Grade I - II); evidence is fair (Grade III - IV), but it is clearly recommended in domestic and international guidelines; it is able to improve health outcomes, and the benefits outweigh the disadvantages.
B	Medium	Evidence is fair (level III-IV), can improve health outcomes
C	Weak	Evidence is insufficient or contradictory. Its pros and cons cannot be identified, but it may improve health outcomes

Table 2. Level of evidence

Level of evidence	Grading Interpretation
I	Meta-analysis or systematic evaluation based on multiple randomized controlled trials; large randomized controlled trials
II	Based on at least 1 high-quality randomized controlled trial; observational or cross-sectional studies with standardized design and clear outcomes; prospective cohort studies
III	Based on well-designed non-randomized case-control studies; observational studies; non-prospective cohort studies
IV	Based on non-randomized retrospective studies; case reports; expert consensus

4. Pharmacologic basis for the combined use of antivirals

From a pharmacological point of view, similar to combination antiviral therapy for AIDS, a combination of antivirals with different mechanisms of action is a common strategy to improve efficacy and reduce the risk of drug resistance, and this theory is also applicable to the treatment of COVID-19. Currently, the main oral small-molecule antivirals used in clinical practice can be divided into two categories: 3CL protease inhibitors and RNA polymerase inhibitors, which work by inhibiting different aspects of viral replication. A 59-year-old female patient with follicular lymphoma, who had received anti-tumor treatment but had not been vaccinated against COVID-19, had persistent COVID-19 and her condition repeatedly worsened. After the initiation of combination antiviral therapy with NTV/r for 5 days and remdesivir for 10 days, the patient recovered (*14*). Another study of immunocompromised patients treated at the Infectious Diseases Clinic of the University of Turin, Italy between March 2022 and February 2023 noted good results in some patients who were treated with NTV/r in combination with either molnupiravir or remdesivir (*15*). Evidence from clinical practice is mounting daily in support of the combination of antivirals with different mechanisms of action as a promising treatment option, and especially when dealing with refractory or high-risk COVID-19 cases.

Recommendations

Recommendation 1: Drug combination should strictly follow the principle of selecting drugs with different pharmacological mechanisms of action with a view to achieving synergistic efficacy and reducing the risk of drug resistance. [Evidence Level: III, Recommendation Grade: A]

Recommended combination regimen: A 3CL protease inhibitor should be selected for combination with an RNA polymerase (RdRp) inhibitor. Available 3CL protease inhibitors ("Drug I") include NTV/r, simnotrelvir/ritonavir, leritrelvir, and atilotrelvir/ritonavir. Optional RNA polymerase (RdRp) inhibitors ("Drug II") include molnupiravir, azvudine, or mindeudesivir (VV116). Co-administration means selecting a drug from the "Drug I" category and combining it with one from the "Drug II" category. If ritonavir-containing drugs are selected, due consideration should be given to their interaction with other drugs used.

Unrecommended combination regimens: Two antivirals with the same mechanism of action should not be used in combination. In other words, use of the drugs listed in "Drug I" above in combination is not recommended, nor is use of the drugs listed in "Drug II" above in combination recommended.

Recommendation 2: During the process of combining medications, the patient's condition needs to be dynamically monitored, The cycle threshold value (Ct value) of nucleic acids for COVID-19, ADRs, adverse drug interactions, *etc*., need to be monitored and the drugs used need to be dynamically adjusted. If, for example, a ritonavir-containing 3CL protease inhibitor is currently in use, and if the condition requires the addition of a drug that may cause adverse interactions with ritonavir, the drug should be adjusted to a 3CL protease inhibitor without ritonavir, such as leritrelvir. After the combination, if the nucleic acid Ct value is greater than 30, or if clinical symptoms or imaging improve, or if an ADR is probably, very probably, or definitely associated, or if there is an adverse drug interaction, then consideration should be given to discontinuing the combination and, if necessary, to discontinuing all antivirals for SARS-CoV-2 altogether. [Evidence Level: III, Recommendation Grade: A]

Successful cases of salvage of refractory COVID-19 through use of small-molecule anti-SARS-CoV-2 drugs with different pharmacologic effects are reported in the literature. A 47-year-old male patient with non-Hodgkin's lymphoma and an immune deficiency whose condition was refractory to COVID-19 was successfully treated with NTV/r in combination with remdesivir (*16*). These cases offer potential remedies for refractory COVID-19. A 73-year-old lymphoma patient with COVID-19 who was treated with rituximab started early antiviral treatment with NTV/r (300/100 mg every 12 hours for 5 days) as a monotherapy while positive for SARS-CoV-2 nucleic acids (day 1), but the patient's symptoms recurred and the COVID-19 nucleic acids remained positive. On day 64 of the positive detection of SARS-CoV-2 nucleic acids, NTV/r (300/100 mg every 12 hours) combined with molnupiravir (800 mg every 12 hours) was administered for a total of 9 days and was successful, resulting in no ADRs (*17*).

A 64-year-old male patient with asthma and chronic lymphocytic leukemia (CLL) was initially treated with a conventional 5-day course of NTV/r , but his symptoms recurred, and he was subsequently treated with a combination of NTV/r and remdesivir (*18*). Three days afterwards, the patient's temperature returned to normal, and his physical pain, coughing, and breathing difficulties were significantly alleviated; after 9 days of treatment, his SARS-CoV- 2 nucleic acid test was negative and he was discharged. The combination of medications was discontinued after 20 days of treatment and the patient's symptoms were completely relieved; chest CT showed significant alleviation of ground glass opacity. At follow-up two months later, the patient's symptoms completely disappeared, and the SARS-CoV- 2 nucleic acid test was consistently negative. In a retrospective study in China, 3,647 patients were treated with NTV/r, 379 were treated with simnotrelvir/ritonavir, and 34 were treated with a combination of simnotrelvir/ritonavir and

mindeudesivir (VV116) (*1*). The combination therapy had significant superiority compared to single-agent use in patients with an initial Ct value of < 30. The patients in the combination therapy group had the fastest virus clearance rate, and no increase in liver or kidney toxicity related to the increase in medication was observed

An *in vitro* experimental study reported that drug combinations similar to nirmatrelvir and molnupiravir, as well as the combination of camostat and molnupiravir, have inhibitory effects on SARS-CoV-2 variants (beta and delta), further enhancing their antiviral efficacy (*19*). That study provides strong evidence for the development of combined drug regimens against SARS-CoV-2, suggesting that combinations of drugs are more effective than single drugs, and they are expected to provide more options in controlling SARS-CoV-2 infections and preventing serious diseases. In animal experiments, NTV/r combined with molnupiravir administration was found to reduce the viral mutation-inducing effects of molnupiravir (*20*). In a macaque model of COVID-19, use of molnupiravir and nirmatrelvir in combination improved the individual inhibitory effect of both drugs, resulting in milder disease progression, greater reduction of virus shedding from mucosal tissues of the upper respiratory tract, greater reduction of viral replication in the lower respiratory tract, and reduced lung pathology; these findings indicate the superiority of molnupiravir and nirmatrelvir in the combined treatment of SARS-CoV-2 infections (*21*). These experimental results support the combination of drugs.

Recommendation 3: For critical COVID-19 according to the COVID-19 Diagnosis and Treatment Program (draft 10th edition) issued by the National Health Commission of China are met, that is, any of the following conditions are met: 1. Respiratory failure and need for mechanical ventilation; 2. Shock; and 3. The presence of other organ failure requiring ICU supervision and treatment. Regardless of previous antiviral use, as long as the disease duration is within 5 days or the viral nucleic acid test is positive and the Ct value is less than 30, combining medication with oral small-molecule drugs against COVID-19 is recommended (refer to *Recommendation 1* for drug selection), as long as there are no contraindications to the use of those drugs. For patients with combined hepatic and renal insufficiency, however, *Recommendations 11* and *12* need to be referred to, hepatic and renal function need to be fully determined, and caution should be exercised when combining drugs if conditions permit. [Evidence Level: III, Recommendation Grade: A]

Most of the patients with critical COVID-19 have an impaired immune system and prolonged detoxification of SARS-CoV-2, and that prolonged detoxification may induce the emergence of mutant strains or induce vaccine immune escape, potentially increasing the difficulty of treatment (*22*). *In vitro* characterization and sequencing revealed mutations in SARS-CoV-2, suggesting that chronic infection with SARS-CoV-2 leads to viral evolution and reduces susceptibility to neutralizing antibodies in immunosuppressed individuals treated with plasma during convalescence (*23*). Several animal studies have confirmed that molnupiravir has a synergistic effect with nirmatrelvir, which more effectively inhibits SARS-CoV-2 virus replication and reduces morbidity and mortality in animals infected with COVID-19 (*24,25*), and extended antiviral regimens or combinations of antivirals with different pharmacological mechanisms may be considered in critically ill patients with prolonged viral retention.

The general view is that SARS-CoV-2 is the initiating factor of COVID-19, and virus clearance is fundamental. The Third People's Hospital of Shenzhen used combined NTV/r and azvudine treatment in 12 cases of critical patients with COVID-19 and achieved better results (*3*). Sequential or concomitant therapy with NTV/r and azvudine may increase the nucleic acid negative conversion (NANC) rate and accelerate recovery without an increase in ADRs. All of the ADRs observed in that study were mild and low-grade. It concluded that NTV/r and azvudine are safe and effective in patients with COVID-19 caused by the Omicron variant, whether administered sequentially or concomitantly. As an example, a critical patient with COVID-19 and asthma and acute lymphoblastic leukocytes responded poorly to a routine course of NTV/r and was then switched to molnupiravir combined with NTV/r, which resulted in a better outcome (*18*).

Fifteen critical patients were studied, of whom 11 suffered from blood disorders and 4 were diagnosed with HIV/AIDS; of the 15 critical patients, 6 received a single antiviral regimen, 4 received antivirals and monoclonal antibodies sequentially, 2 received three antivirals (remdesivir, NTV/r, or molnupiravir) or two drugs, and 3 were given two antivirals or one antiviral plus a monoclonal antibody (*15*). Results indicated that the COVID-19 nucleic acid test was negative within 16 days after the end of treatment, and the median time to viral conversion was 2.5 days, confirming that the combination regimen displayed better efficacy and safety in immunosuppressed high-risk populations in that study.

Recommendation 4: For severe COVID-19 according to the COVID-19 Diagnosis and Treatment Program (draft 10th edition) issued by the National Health and Health Commission of China, if the antiviral has been used for one course of treatment but the COVID-19 nucleic acid test is still positive with a Ct value of less than 30, and there are no contraindications to the use of medication, a combination of oral small-molecule antivirals should be used, and the antiviral can continue to be used as previously, along with another antiviral with different pharmacologic effects, or a switch can be made to a new group of antivirals in line with the

provisions of *Recommendation 1*. [Evidence Level: III, Recommendation Grade: A]

Recommendation 5: For severe COVID-19 according to the COVID-19 Diagnosis and Treatment Program (draft 10th edition) issued by the National Health and Health Commission of China, if antivirals have previously been used but for less than one full course of treatment, the COVID-19 nucleic acid test is still positive with a Ct value of less than 30, disease tends to progress or worsen, such as the persistence of a fever, and lung imaging shows that the infection has not improved or has progressed, then as long as there are no contraindications to the use of these drugs, a combination of oral small-molecule antivirals should be used, and the antiviral can continue to be used as previously, along with another antiviral with different pharmacologic effects in line with the provisions of *Recommendation 1*. [Evidence Level: III, Recommendation Grade: A]

Recommendation 6: For severe or moderate COVID-19 according to the COVID-19 Diagnosis Treatment Program (draft 10th edition) issued by the National Health and Wellness Commission of China, if antivirals have previously been used but not for a full course of treatment, the viral nucleic acid test is still positive, the patient's condition tends to improve, and patients are non-critical and not in high-risk groups, then they can be closely observed, and antivirals need not be combined for the time being. [Evidence Level: III, Recommendation Grade: A]

Recommendation 7: For moderate COVID-19 according to the COVID-19 Diagnosis and Treatment Protocol (draft 10th edition) issued by the National Health and Wellness Commission of China, if a course of antiviral medication has been used, the COVID-19 nucleic acid test is still positive, and the Ct value is less than 30, then as long as there are no contraindications to the use of these drugs, oral small-molecule antivirals should be used, and the antiviral can continue to be used as previously, along with another antiviral with different pharmacologic effects, or a switch can be made to a new group of antivirals in line with the provisions of *Recommendation 1*. [Evidence Level: III, Recommendation Grade: A]

Case-cohort studies have shown that prolonged use of antivirals leads to better results. In an American cohort study, a mathematical model was developed to analyze the viral load dynamics of 51 patients infected with SARS-CoV-2 who were treated with a regular 5-day course of NTV/r, 20 of whom experienced a viral rebound (*26*). Dividing the population into a group that experienced viral rebound (20 individuals) and a group that did not experience viral rebound (31 individuals) showed that there were significant differences between the two groups in terms of target cell protection parameters and mortality of infected cells and that these differences resulted in better maintenance of target cells in the group that did not experience viral rebound. Extending the NTV/r regimen to 10 days significantly reduced the risk of viral rebound. There was an average delay of 1.23 days in the initiation of the adaptive immune response in the NTV/r-treated group compared to the untreated group. Target cell preservation and incomplete viral clearance were the main causes of viral rebound after NTV/r treatment. Extending the NTV/r treatment regimen to 10 days can significantly reduce the risk of viral rebound. The Third People's Hospital of Shenzhen used a combination of NTV/r and azvudine treatment for 8 critical patients with COVID-19 and achieved better results; the drug combination did not increase the risk of safety and significantly improved the nucleic acid conversion rate, especially for patients in whom a single drug was ineffective (*3*).

Another study reported that 67 patients treated with a combination of at least two direct antivirals (protease inhibitor + polymerase inhibitor) had a viral clearance rate of 79% and a relapse rate of 16% (*27*). In immunocompromised patients with persistent SARS-CoV-2 infection, combination therapy is beneficial in achieving SARS-CoV-2 clearance and reducing the risk of relapse. In clinical trials, combination regimens have been found to enhance antiviral efficacy, reduce the emergence of drug-resistant variants, and lower the dose of each component of combination therapy while targeting viral invasion and viral replication, providing opportunities for synergistic drug combinations (*19*).

In refractory cases with recurrent symptoms, the combination of 3CL protease inhibitors and RNA polymerase inhibitors has been successful. A patient first tested positive for SARS-CoV-2 in October 2022 and was subsequently treated with NTV/r but had recurrent symptoms (*17*). The patient received a combination of molnupiravir and NTV/r on day 64, which resulted in a negative antigen test and rapid symptomatic relief 5 days later (day 69). *In vitro* studies have shown that molnupiravir exhibits enhanced antiviral activity when combined with other antivirals (*17*).

Nine patients with hematologic malignancies in a hospital in central Italy who had received unsuccessful SARS-CoV-2 therapy were treated with a combination of antivirals for persistent infection (*28*). The combination therapy consisted of NTV/r plus molnupiravir (n = 4), NTV/r plus remdesivir (n = 4), or remdesivir plus molnupiravir (n = 1) over a 10-day course of treatment, with 8 of them having clinical and virologic success confirmed by radiologic follow-up, and all the patients receiving the combination tolerated the drug well.

Recommendation 8: In patients > 65 years of age and with underlying conditions such as cardiovascular disease (including hypertension), chronic lung disease, diabetes, chronic liver, renal disease, and neoplasms,

as well as in patients on maintenance dialysis, the presence of early warning indicators of severe/critical illness, including 1. Progressive exacerbation of hypoxemia or respiratory distress; 2. Deterioration of tissue oxygenation indices (*e.g.,* pulse oximetry or the oxygenation index) or progressive elevation of lactate; 3. Progressive decrease according to the peripheral blood lymphocyte count or a progressive increase in inflammatory factors such as interleukin 6 (IL-6), C-reactive protein (CRP), and ferritin; 4. Significantly elevated coagulation-related markers, such as D-dimer; and 5. Significantly progressive lung lesions on chest imaging. Regardless of the previous use of antivirals, as long as the COVID-19 nucleic acid test is positive and the Ct value is less than 30 and there are no contraindications to the use of drugs, a combination of oral small-molecule antivirals is recommended (for drug selection, refer to the provisions of *Recommendation 1*). For patients with combined hepatic or renal insufficiency, however, *Recommendations 11* and *12* need to be referred to, hepatic and renal function need to be fully determined, and caution needs to be exercised when combining drugs if conditions permit. [Evidence Level: III, Recommendation Grade: A]

Elderly patients and some patients with COVID-19 and underlying conditions have a higher mortality rate. In a retrospective cohort study at Rasoul Akram Hospital, Tehran, Iran, results indicated that mortality was higher in patients who were male, older than 55 years of age, and who suffered from comorbidities such as renal disease, cancer, and Alzheimer's disease (*29*).

One study proposed a comprehensive predictive model based on multiple factors. Being older, being male, and being of a certain race were associated with a higher risk of serious illness and death. Fever, shortness of breath, dyspnea, and gastrointestinal symptoms are early warning signs of exacerbation. Conditions such as hypertension, diabetes, obesity, chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), chronic liver disease (CLD), chronic kidney disease (CKD), and cancer significantly increase the risk of serious illness. Immunodeficiencies (*e.g.*, HIV infection or congenital immunodeficiencies) may increase the risk of serious illness, and especially those associated with type I interferon (IFN-I). Acute kidney injury (AKI), coagulation disorders, and thromboembolism (such as pulmonary embolism) are hallmarks of deterioration. Administration of an anticoagulant may reduce mortality. Leukocytosis, lymphocytopenia, eosinophilia, elevated D-dimer, and elevated indicators such as lactate dehydrogenase (LDH), CRP, procalcitonin (PCT), IL-6, IL-1, and ferritin are associated with the severity of the disease. Impaired interferon type I (IFN-I) activity or autoantibodies may lead to severe disease. The extent of pneumonic lesions on chest CT correlates with disease severity. Vitamins C and D, a high-fiber diet, a Mediterranean diet, intermittent fasting, and a ketogenic diet may be beneficial in improving prognosis. Smoking significantly increases the risk of severe disease. Healthcare workers have a high risk of exposure (*30*). An active combination of oral small-molecule antivirals is recommended for this particular group of patients, similar to critically and severely ill patients.

Recommendation 9: In patients with an immunodeficiency (*e.g.*, patients with AIDS and those using corticosteroids or other immunosuppressive drugs for a prolonged period leading to compromised immunity) or patients using immunosuppressants for a prolonged period for organ transplantation who present with early warning signs as described in *Recommendation 8*, regardless of previous antiviral use, then as long as COVID-19 nucleic acid testing is positive with a Ct value of less than 30 and there are no contraindications to the use of drugs, the use of a combination of oral small-molecule antivirals is recommended (for drug selection, refer to the provisions of *Recommendation 1*). [Evidence Level: IV, Recommendation Grade: A]

Recommendation 10: For patients with COVID-19 who are undergoing organ transplantation or who have a blood disease or tumor, selection of a ritonavir-free drug is recommended when selecting a 3CL protease inhibitor, such as leritrelvir due to the adverse interactions between ritonavir and commonly used immunosuppressants such as tacrolimus, methylprednisolone, and cyclosporine, the blood concentration of which can be increased by ritonavir. [Evidence Level: IV, Recommendation Grade: A]

The literature points to the generation of SARS-CoV-2 variants in immunosuppressed patients and its public health implications and calls for comprehensive measures to reduce the risk of variants and to protect this high-risk group and public health. Immunosuppressed patients (*e.g.,* cancer patients, organ transplant recipients, or HIV-infected individuals) may experience prolonged SARS-CoV-2 infections due to compromised immunity. This persistent infection provides an environment for the SARS-CoV-2 to evolve rapidly, leading to the emergence of multiple mutations and variations, which are related to globally concerned variants such as Alpha, Beta, Gamma, Delta and Omicron. Viral evolution is characterized by (i) Adaptive evolution: viruses gain transmission or immune escape advantages through mutation, (ii) Convergent evolution: different viral lineages independently produce the same key mutations, or (iii) Jump evolution: rapid accumulation of multiple mutations may occur in immunosuppressed patients, resulting in highly mutated viral variants. Immunosuppressed patients may become a source of new mutations, and enhanced protective measures need to be taken to reduce the spread of COVID-19. Vaccination should be given priority to this group of people, and their immune responses

should be monitored. Household contacts should also be vaccinated to break the chain of community transmission. Monoclonal antibodies and antivirals such as molnupiravir can be used for prophylaxis or treatment, but the potential for mutation-induced drug resistance needs to be borne in mind. A new generation of vaccines against variants needs to be developed. Further studies on the relationship between specific immunosuppressive conditions and viral variants need to be conducted in the future, and targeted prevention and control guidelines need to be developed (*22*).

In a retrospective cohort study, for 11 immunocompromised patients (7 in the early stage of COVID-19 and 4 in the late stage), treatment lasted 10 days, with intravenous remdesivir plus 5 days of oral NTV/r for all patients. Patients in the early treatment group had 100% virologic clearance 30 days after the end of treatment and all survived at follow-up: 50% and 75% in the late treatment group, respectively (*31*). The results of that study provide new combination therapy options and support the early treatment of COVID-19 in immunocompromised patients, suggesting that early combination therapy may be helpful in achieving complete and durable viral clearance and preventing the development of severe disease.

A hospital in New Jersey in the US studied a man in his 50s who was hospitalized for COVID - 19, had received a kidney transplant, and was on long-term immunosuppressive therapy (*13*). The study analyzed the viral genome by sequencing nasopharyngeal swabs and tracheal aspirate samples collected on multiple occasions. In immunosuppressed patients receiving convalescent plasma therapy, there is a risk of their becoming immune to escape mutant strains that may possess greater antibody resistance. The study's results indicated the risk of viral evolution that may be triggered by convalescent plasma therapy in immunosuppressed hosts, and the possible impact of the patient's immune status on viral evolution needs to be considered when formulating antiviral treatment strategies, which may benefit from combining antivirals.

Due to the use of immunosuppressants, the risk of SARS-CoV-2 infection increases in solid organ transplant recipients (SOTRs), and the hospitalization rate, severe case rate, ICU hospitalization rate, and mortality rate are all higher than those for non-organ transplant patients (*32*). Being older, being male, and having multiple underlying conditions are associated with a higher risk of death in SOTRs. The cited study emphasized the high risk of COVID-19 in SOTRs, and targeted measures need to be taken to improve their prognosis. Another study has suggested that SOTRs infected with SARS-CoV-2 have a higher risk of AKI than non-transplant patients.(*33*). Age, multi-organ failure and mechanical ventilation are the main predictors of mortality in organ transplant recipients. Studies have highlighted the need for individualized management strategies for SOTRs. Another study has found that patients who have undergone solid organ transplantation (SOT) face a higher hospitalization rate and risk of adverse renal events after being infected with the SARS-CoV-2, highlighting the importance of enhanced protection and early intervention (*34*).

Recommendation 11: In patients with renal insufficiency, and especially severe renal insufficiency, combined medications are not recommended. For severe renal insufficiency, molnupiravir can be used in regular doses; for patients with mild or moderate renal impairment, NTV/r can be used and the dose used can be adjusted according to the glomerular filtration rate (eGFR); for mild renal impairment, azvudine can also be used. Relevant trials and studies on VV116 and leritrelvir have not been conducted, so there are no clinical data on patients with renal impairment and there are no reliable references. Thus, those drugs are not recommended for patients with renal insufficiency. Regardless of whether a patient has mild or moderate renal insufficiency, a combination of drugs should be used with extreme caution, fully weighing the pros and cons. [Evidence Level: IV, Recommendation Grade: A]

Recommendation 12: Combining medications is not recommended in patients with hepatic insufficiency, and especially in patients with severe hepatic impairment. For patients with severe hepatic impairment, molnupiravir at regular doses may be used; for patients with mild (Child-Pugh A) or moderate (Child-Pugh B) hepatic impairment, NTV/r, simnotrelvir/ritonavir, leritrelvir, azvudine, and VV116 may be used with caution. In patients with either mild or moderate hepatic insufficiency, a combination of drugs should be used with extreme caution, fully weighing the pros and cons. [Evidence Level: IV, Recommendation Grade: A]

Molnupiravir does not require dose adjustment in patients with renal impairment. NTV/r does not require dose adjustment in patients with mild renal impairment ($60 \leq eGFR < 90$). In patients with moderate renal impairment ($30 \leq eGFR < 60$), the dose of NTV/r should be reduced to 150 mg/100 mg once/12 h for 5 d. NTV/r should not be used in patients with severe renal impairment ($eGFR < 30$), including those with end-stage renal disease on hemodialysis. An important point worth noting that the concepts of the eGFR and creatinine clearance (CrCl) differ, and CrCl should not be used as a substitute for the eGFR. When the CrCl is very low, the eGFR may still be >30. The relevant formula for calculating the eGFR, which can be found at *https://www.23bei.com/tool/603.html*, is used for reference.

Recommendation 13: According to the COVID-19 Diagnosis and Treatment Program (draft 10th edition) issued by the National Health and Wellness Commission of China, the combination of drugs is not recommended

for patients diagnosed with mild COVID-19, in order to avoid unnecessary drug exposure and potential adverse effects and in light of the self-limiting course of the disease. [Evidence Level: III, Recommendation Grade: A]

Co-administration is not recommended when not necessary, taking into account the adverse effects of the drugs. Although the ADRs to NTV/r are generally considered to not ne serious, a total of 8,098 reports of ADRs to it were identified from January-June 2022, with the most common symptoms being dysphagia, diarrhea, cough, fatigue, and headaches; serious cases were also reported, with cardiac arrest, tremor, sedation, and death reported in 1,3, 67, and 5 cases, respectively (*35*). ADRs have also been reported in relation to molnupiravir, with the most commonly reported being related to gastrointestinal disorders and skin and subcutaneous tissue disorders. In addition, individuals 65 years of age and older are at higher risk for heart disease, hepatobiliary disease, renal and urinary tract disease, and vascular disease. In patients younger than 65 years of age, molnupiravir demonstrated a lower risk of serious ADRs compared to other RNA antivirals such as remdesivir. However, its safety still needs to be closely monitored in elderly patients 65 years of age and older. As the use of molnupiravir increases, and especially in high-risk populations, further studies need to be conducted to continuously assess its safety (*36*). Therefore, in patients with mild COVID-19, monotherapy is recommended as the mainstay, and combinations are not recommended for non-essential use.

Recommendation 14: For pediatric patients, drug combinations are not recommended. For COVID-19 in children (<18 years of age), oral small-molecule antivirals should not be used unless necessary, as indicated by the current Chinese Drug Formulary and the COVID-19 Diagnosis and Treatment Program (draft 10th edition). For adolescent patients ages 12-17 years with a body mass ≥ 40 kg, high-risk factors (as defined in the National Diagnosis and Treatment Program (10th edition)), and a SARS-CoV-2 infection, off-label medication should be considered when necessary, and NTV/r should be given at the adult dosage. [Evidence Level: III, Recommendation Grade: A]

NTV/r is not recommended for use in children under 12 years of age or those weighing less than 40 kg because its safety and effectiveness have not been demonstrated in pediatric patients. Monoravir is not recommended for use in persons under 18 years of age, and animal studies have shown that it can cause impaired conversion of cartilage into new bone (*37*). When using NTV/r, an important point to note is that ritonavir is a potent CYP3A4 inhibitor, which may significantly increase the risk of ADRs in children when combined with other drugs metabolized by CYP3A4 (*e.g.*, immunosuppressants and anticoagulants) (*38*).

Recommendation 15: In pregnant women, drug combinations are not recommended. NTV/r may be used with caution at routine doses with the patient's informed consent when the potential benefit to the mother outweighs the potential risk to the fetus, as assessed for the specific combination. [Evidence Level: III, Recommendation Grade: A]

The higher odds of a cesarean delivery, low birth weight, and preterm delivery in pregnant women with COVID-19 suggests a possible association between development of COVID-19 and pregnancy complications. Although the risk of vertical transmission is low, SARS-CoV-2 can be detected in the placenta.

The Chinese package inserts for simnotrelvir/ritonavir, leritrelvir, atilotrelvir/ritonavir, molnupiravir, azvudine, and VV116 state that all of these drugs are contraindicated for use in pregnant women, are not recommended for use, or have been shown to be toxic to the fetus in animal studies. When choosing oral small-molecule antivirals, these factors need to be taken into account and the safety of the drugs should be weighed for both the mother and the fetus (*39*). According to the current Chinese package inserts, in addition to considering NTV/r in pregnant women with COVID-19, leritrelvir and atilotrelvir/ritonavir may be used with caution when the benefits outweigh the risks, with the informed consent of the patient. In principle, drug combinations are not recommended for pregnant woman.

Booster vaccination is advocated for pregnant women, due to the higher risk of severe disease after COVID-19 infection in pregnant women and the significantly increased risk of severe disease and adverse pregnancy outcomes after infection in unvaccinated individuals. Priority needs to be given to increasing vaccination rates in pregnant women (and especially those in younger and low-income groups) to protect the health of mothers and infants (*40*). The literature suggests that the benefits of using NTV/r in pregnant women with COVID-19 may outweigh the associated risks. However, data from animal studies have indicated that molnupiravir may pose a risk of fetal harm when administered during pregnancy (*37*). A single-arm meta-analysis of 427 pregnant patients receiving NTV/r was performed to comprehensively assess outcomes in the maternal, delivery, and neonatal phases, and results indicated that it was safe and effective in pregnant women with mild or moderate COVID-19, with low rates of hospitalization and low adverse maternal outcomes (*41*). Subsequent dosing for pregnant women with COVID-19 requires relevant dosing adjustments based on additional clinical studies and updated package inserts.

5. Conclusion

A point worth noting is that in clinical practice, the conventional use of a single drug for the treatment

of COVID-19 should be considered first. Our suggestion is that in special cases, such as critically ill, immunosuppressed, post-transplant, or other special patients, or patients in whom COVID-19 nucleic acids tend not to be negative after conventional treatment, when virus clearance is still the main goal the combined use of small-molecule antivirals, after full consideration and discussion, can help clear the virus as soon as possible and improve the success rate of rescue.

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Consensus

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Chinese expert consensus on the combined use of antiviral drugs for influenza

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SUMMARY: Influenza is an acute respiratory infectious disease caused by influenza viruses, and it poses a serious threat to global public health. High-risk groups include the elderly, infants and young children, pregnant women, and patients with chronic underlying diseases. These groups are prone to developing severe illness after infection, which can lead to serious complications and even death. Early antiviral treatment is key to reducing the rate of severe illness and death. Currently, authoritative guidelines at home and abroad recommend early, single-agent antiviral therapy as the standard regimen. However, anti-influenza virus monotherapy has problems such as drug resistance and poor therapeutic effect. To address these problems, this consensus was developed by organizing experts from the departments of Infectious Diseases, Respiratory Medicine, Critical Care Medicine, and Pharmacy. These experts systematically sorted out domestic and international evidence on combined antiviral therapy for influenza and formulated expert recommendations on combined antiviral therapy for influenza in specific populations.

Keywords: influenza viruses, influenza antiviral therapy, resistance, combined therapy

1. Introduction

Influenza viruses cause seasonal influenza and influenza pandemics, posing a serious threat to human health and public health. According to the 2017 Global Burden of Disease Study (GBD 2017), up to 145,000 people worldwide die each year due to influenza-associated lower respiratory tract infection (LRTI) (*1*). A study showed that during the period 2010–2015, there were an average of 88,100 influenza-related respiratory disease deaths per year in mainland China, equivalent to 8.2% of all respiratory disease deaths (*2*). Dr Tedros Adhanom Ghebreyesus, Director-General of the World Health Organization(WHO), has warned that the threat of a pandemic flu remains a constant concern. Risk of new influenza viruses crossing from animals to humans and causing a real pandemic is ongoing. We must remain vigilant and be well-prepared (*3*).

Antiviral drugs play a crucial role in controlling influenza outbreaks and epidemics. Up to now, the primary antiviral medications approved for treating influenza virus infections include three classes: transmembrane protein M2 ion channel inhibitors, neuraminidase inhibitors (NAIs), and RNA-dependent RNA polymerase (RdRp) inhibitors (*4*). Recently, new anti-influenza virus drugs such as ZSP1273 have been launched. Meanwhile, more anti-influenza virus drugs with new targets and completely new mechanisms of action are under development. However, the types of currently available antiviral drugs for influenza remain relatively limited. Moreover, influenza viruses are highly prone to mutations, and the number of drug-resistant virus strains is constantly increasing. Drug resistance caused by viral mutations and drug abuse remains a serious issue, and monotherapy for influenza viruses is facing the challenge of drug resistance. For instance, the detection rate of neuraminidase inhibitor-resistant strains is on the rise among immunocompromised patients. Typical cases include the H275Y mutation in Influenza A virus (IAV) subtype H1N1 and the I221T/V mutation in Influenza B virus (IBV). In addition, the polymerase inhibitor baloxavir can induce the PA/I38T mutation. Furthermore, antiviral treatment of severe influenza still faces many challenges and uncertainties at present. According to relevant data, the diagnosis time of patients with severe influenza in China is relatively late, which may lead to delays in treatment timing. As a result, most patients miss the optimal time window for antiviral treatment (*5*). Patients with severe influenza may experience prolonged replication and shedding of the virus in the upper and lower respiratory tracts. The virus excretion time of severe patients is prolonged, and the duration of antiviral treatment may need to be extended (*6,7*).

Combination drug therapy has emerged as a key strategy to address drug resistance and enhance therapeutic efficacy in severe influenza cases. By leveraging synergistic effects to inhibit viral replication through multiple targets, this approach offers distinct clinical advantages (*8*). Specifically, combination drug therapy can reduce emergence of drug-resistant viral strains and mitigate treatment-related adverse effects, which may in turn lower incidence of severe influenza and improve the success rate of treating severe cases. Han J *et al* (*9*) pointed out a critical issue: currently circulating IAV strains (such as H1N1 and H3N2) have developed resistance to neuraminidase inhibitors. What is worse, they are almost completely resistant to M2 ion channel inhibitors. This growing resistance problem is further exacerbated by the use of subtherapeutic doses in both clinical treatment and chemoprophylaxis. Novel therapies targeting host components and new strategies for combination therapy show potential for maximizing the reduction of viral resistance.

Currently, anti-influenza virus therapy recommended in national and international expert consensus statements and clinical practice guidelines is typically based on monotherapy. However, in specific clinical scenarios (such as severe infections, patients at risk of drug resistance, or patients with immunosuppression), combination therapy strategies should be considered. This approach is also expected to become one of the future development trends in influenza treatment. By using drugs that act on different targets, we can not only reduce the development of viral drug resistance and minimize adverse reactions caused by the dosage of a single drug but also formulate individualized treatment plans based on the severity of the patient's condition. Especially for patients with severe influenza, special attention should be paid to host immune regulation therapy (*10*).Therefore, the combination regimens proposed in this consensus are expert recommendations. They apply to populations that are critically ill, immunosuppressed, or suspected of having drug resistance.

These recommendations are intended to inform clinical decision-making, and they are not routine first-line recommendations. Any off-guideline medication must undergo individualized risk-benefit assessment and be fully communicated with the patient or their family.

2. Methods

To ensure this consensus has a solid evidence-based foundation, we conducted systematic searches in multiple well-known medical databases (including PubMed and Web of Science) by September 25, 2025, using the search formula "Combination Therapy" AND "Influenza Virus". We aimed to collect key studies in all relevant fields through comprehensive literature searches, to ensure this consensus was developed based on the best available evidence. The evidence-based medicine (EBM) evidence of this expert consensus adopts the 2011 Oxford Centre for Evidence-Based Medicine (OCEBM) Levels of Evidence and Grades of Recommendations (*11*) (Table 1).

Table 1. Level of evidence

Recommendation Strength	Evidence Level	Description
A	1a	System review of randomized controlled trials (RCTs))
	1b	RCTs with small confidence intervals for results
	1c	Any evidence showing an "all or nothing effect"
B	2a	Systematic evaluation of cohort studies
	2b	Individual cohort studies (including low-quality RCTs, *e.g.*, those with >20% loss-to-follow-up rates)
	2c	Studies based on patient outcomes
	3a	Systematic evaluation of case-control studies
	3b	Single case-control study
C	4	Case series reports, low-quality cohort studies and low-quality case-control studies
D	5	Expert opinion (*i.e.*, speculation based solely on basic research or clinical experience that is not supported by clinical studies)

3. Current primary antiviral drugs for influenza

3.1. Life cycle of influenza viruses and targets and mechanisms of antiviral drug action

The replication process of influenza virus comprises six core steps, including viral entry, viral uncoating, viral genome replication and transcription, viral protein translation, viral assembly, and viral budding (*12*). This series of highly ordered steps provides clear targets for antiviral drug development. First, during the viral invasion stage, hemagglutinin (HA) inhibitors block fusion of the virus with the host cell membrane to prevent infection. After virus entry, during the uncoating stage, M2 ion channel inhibitors and HA inhibitors suppress acidification inside the virus to prevent the release of the virus's genetic material. Subsequently, in the core process of viral genome replication and transcription in the cell nucleus, RdRp inhibitors can directly inhibit the replication of viral genetic information. RdRp inhibitors include three categories: RNA polymerase acidic protein inhibitors (PA), RNA polymerase basic protein 1 inhibitors (PB1), and RNA polymerase basic protein 2 inhibitors (PB2). When newly formed vRNPs need to be transported from the nucleus to the cytoplasm for assembly, vRNP export inhibitors can interrupt this process. During the viral assembly stage, NAIs, M2 ion channel inhibitors, and HA maturation inhibitors interfere with the correct processing of viral proteins. Finally, when progeny virus particles bud on the cell surface, NAIs prevent the virus from detaching from the host cell surface (Figure 1).

3.2. Anti-influenza virus treatment drugs

The current major anti-influenza virus therapies are listed in Table 2.

4. The necessity of combination antiviral therapy

4.1. Antiviral drug resistance in influenza viruses

Influenza virus is a pathogen with rapid mutation ability, and its genome can evolve through multiple mechanisms such as point mutations (such as variations in PB2, PA, and NA genes), segmental recombination and genomic recombination. At present, multiple key drug resistance sites have been identified: the S31N mutation of the M2 protein confers resistance to amantadine drugs; the H274Y and R292K mutations in the NA gene significantly reduce sensitivity to neuraminidase inhibitors (*e.g.*, oseltamivir); and the I38T mutation in the PA protein significantly diminishes the antiviral activity of baloxavir. In addition, novel mutations such as the K229R mutation in the PB1 gene and the P653L mutation in the PA gene also indicate a potential risk of resistance to favipiravir (*13*).

Between 2018 and 2020, the resistance rate of Influenza A (H1N1)pdm09 strains to NAIs reached 1.3% worldwide. Meanwhile, IBV also exhibited a resistance rate of approximately 1% (*14*). Children, patients receiving prophylactic drug treatment, and individuals with impaired immune function (such as hematopoietic stem cell transplant recipients or immunocompromised patients) have become high-risk groups for NAI resistance (*15-17*). More notably, among the more than 30 newly identified drug-resistant mutations from 2016 to 2024, approximately 80% are distributed in IBV.These mutations can lead to a drastic reduction in drug sensitivity: a single mutation can reduce the inhibitory effect by 10 to 1,000 times, while multi-site synergistic mutations (such as the combination of H274Y and I222R) can even increase drug resistance by more than 10,000 times. In addition, some mutation combinations can also cause cross-resistance or multidrug resistance phenotypes. Particularly, after some drug-resistant strains acquire compensatory mutations (such as the H274Y variant accompanied by D354G), their replication fitness and transmission ability are restored.

Drug resistance of influenza viruses to antiviral agents has become a major challenge in clinical practice. Faced with the constantly evolving drug resistance of influenza viruses, there is an urgent need to optimize

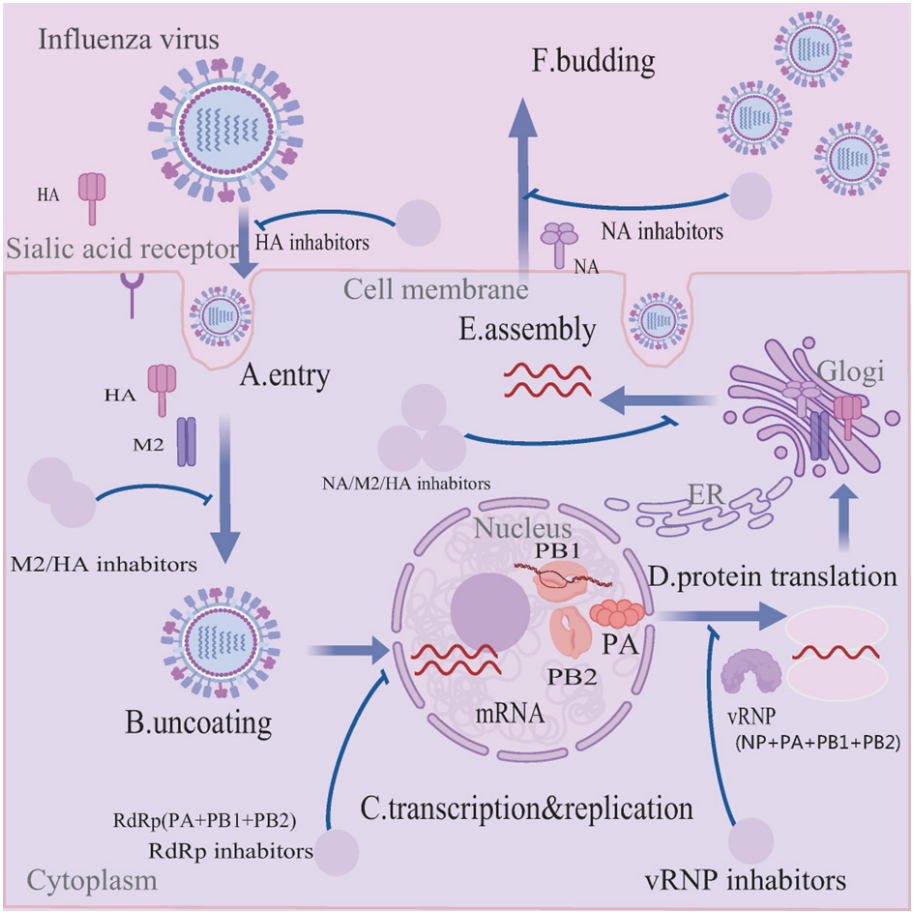

Figure 1. Replication cycle of influenza virus and crucial steps targeted by virus-directed antiviral compounds. Replication cycle of influenza virus encompassing six core steps (viral entry, uncoating, replication and transcription, protein translation, assembly, and budding) and the key stages targeted by virus-directed antiviral compounds. Approved drugs for influenza treatment are indicated in bold. (Figure created with MedPeer)

Table 2. The main anti-influenza virus treatment drugs

Category	Mechanism	Representative drugs
M2 ion channel inhibitors	Inhibits M2 ion channel function and interferes with viral capsidization.	Amantadine, Rimantadine
Neuraminidase inhibitors	Inhibits neuraminidase activity and blocks virus budding	Oseltamivir, Zanamivir, Peramivir, Laninamivir
RNA polymerase acid protein inhibitors	Inhibits viral RNA polymerase activity and prevents viral synthesis	Baloxavir marboxil, Baloxavir, Suraxavir Marboxil
RNA polymerase basic protein 1 inhibitors	Targeting the catalytic function of PB1 to block RNA chain synthesis	Favipiravir, Ribavirin
RNA polymerase basic protein 2 inhibitors	Targeting the cap-binding domain of PB2 and blocking the transcription of viral mRNAs	ZSP1273, Pimodivir
Hemagglutinin inhibitors	Prevents viral release by selectively inhibiting steps such as HA maturation, intracellular trafficking, and embedding in the host cell membrane	Arbidol, Monoclonal antibodies (CR6261, VIS410,MHAA4549A,MEDI8852,CT-P27)

existing influenza antiviral treatment strategies, explore combined treatment regimens, and develop drugs targeting novel targets.These measures will help effectively address the challenges posed by influenza virus drug resistance.

Recommendation 1: Influenza viruses are prone to developing drug-resistant strains. Monotherapy is likely to induce the selection of drug-resistant strains. Particular attention should be paid to influenza virus resistance in patients with severe influenza, children, and immunocompromised patients (Evidence Level:4, Recommendation Strength: C).

4.2. The influence of drug resistance

4.2.1. Enhancement of virus spread ability

Some drug-resistant mutations of influenza virus may enhance the virus's transmission capacity. This enables the virus to spread rapidly in populations, especially among immunosuppressed patients. Seibert CW *et al.* (*18*) conducted a study using a guinea pig transmission model. They found that influenza viruses carrying S247N and H275Y mutations had high resistance to oseltamivir. These viruses also showed enhanced effective transmission ability. Hickerson *et al.* (*19*) noted that influenza viruses may develop drug-resistant mutations against baloxavir. These mutations include I38L and I38T. Additionally, IAV has emerged with the E199D mutation, and IBV has emerged with the I38T mutation. These initial mutations slightly impaired the virus's replication capacity. However, during continuous viral passage, IAV acquired the compensatory mutation D394N, while IBV evolved the E329G mutation. These subsequent mutations can enhance replication capacity of drug-resistant viruses. They also promote fixation of antiviral resistance in viral populations. Moreover, they facilitate further spread of such resistance. This poses a potential public health threat.

4.2.2. Increased risk of drug resistance gene spread

Influenza viruses (*e.g.*, avian Influenza H5N1) may infect humans *via* cross-species transmission if they accumulate drug-resistant mutations in animal hosts. From 2003 to 2024, the WHO recorded 954 confirmed cases of human infection with highly pathogenic avian Influenza A(H5N1) virus across 24 countries. These cases resulted in 464 deaths, corresponding to a mortality rate of 48.64% (*20*). The highly pathogenic avian Influenza A(H5N1) virus of the 2.3.4.4b evolutionary branch was isolated from severe human cases in Chile. This virus showed high-titer replication ability in the respiratory and extrapulmonary tissues of ferrets (*21*). After the emergence of the self-evolved 2.3.4.4b branch, the highly pathogenic avian Influenza A(H5N1) virus has become a new recombinant virus with stronger cross-species transmission ability, capable of spreading widely among multiple mammalian species, including dairy cows, cats, and raccoons (*22*). The H5N1 virus of the 2.3.4.4b evolutionary branch has developed a new mutation through reassortment events. It possesses dual-receptor binding ability, enabling it to bind to both avian and human receptors. Through molecular adaptation, the H5N1 virus has enhanced its cross-species spread ability. This has led to its transmission in cattle, humans, and other mammals. It is recommended to adopt a multi-target antiviral regimen to reduce the risk of drug resistance (*23*).

4.2.3. Increased risk of clinical treatment failure and death

With development of drug resistance in influenza viruses, amantane drugs (such as amantadine and rimantadine) have become ineffective against most prevalent strains. Neuraminidase inhibitors (such as oseltamivir and zanamivir) were once the main treatment options. However, under drug selection pressure, influenza strains have also developed resistance to these drugs. Influenza drug-resistant strains can reduce the efficacy of monotherapy for influenza and may even lead to the failure of treatment (*24*).

4.2.4. Increased burden of public health

Patients infected with drug-resistant strains require longer hospital stays and more intensive supportive care (such as mechanical ventilation and Extracorporeal Membrane Oxygenation (ECMO)), which increases the pressure on the healthcare system. Patients with drug-resistant infections need to use high-cost alternative drugs (such as new polymerase inhibitors or combination therapies). The per capita treatment cost can increase by 5 to 10 times, leading to a squeeze of medical resources. Drug resistance reduces the effectiveness of antiviral drugs such as neuraminidase inhibitors; Large-scale drug reserves prepared in the early stage may fail to play their expected role, resulting in the waste of drug reserves.

Recommendation 2: Drug resistance of influenza viruses may lead to increased viral transmission ability and a high risk of drug-resistant gene spread (Level of evidence: 4); it may also result in the failure of clinical antiviral treatment, elevate the mortality rate among patients with severe influenza, and increase the burden on public health (Evidence Level: 4, Recommendation Strength: C).

4.3. The theoretical basis of combined antiviral therapy

Influenza virus has high genetic variability, making it prone to resistance to single drugs. To prevent this, strategies of combination therapy or drug enhancement are optional (*25*). Antiviral combination therapy acts on different viral replication links and exerts synergistic effects, reducing single-drug pressure to boost efficacy and lower resistance risk. The WHO's 2024 guidelines (*26*) do not recommend routine combination therapy; single drugs (*e.g.*, oseltamivir, baloxavir marboxil) remain preferred. However, for severe influenza patients (*e.g.*, requiring mechanical ventilation/ECMO) or immunodeficient patients (*e.g.*, post-hematopoietic stem cell transplantation), if viral load does not drop significantly 48 hours after monotherapy, combination therapy (*e.g.*, oseltamivir + baloxavir marboxil) may be considered, subject to individual evaluation. According to *the Diagnosis and Treatment Protocol for Influenza*

(2020 Edition) (*27*) and *(2025 Edition)* (*28*), severe/critical cases may have extended treatment courses based on etiological results. Combining drugs with the same mechanism or increasing dosages is not recommended, but combining those with different mechanisms is not ruled out.

Recommendation 3: For patients with severe influenza or suspected drug-resistant strain infection, after individualized assessment, combined antiviral treatment with drugs of different mechanisms of action and different targets can be considered (Evidence Level: 5, Recommendation Strength:D).

5. Combination antiviral therapy regimen

To date, compared with single-drug treatment, combination therapy with virus-targeted drugs and host-targeted drugs has achieved more positive clinical outcomes. These outcomes include reducing viral shedding, shortening the duration of influenza-related symptoms, and decreasing the selection of drug-resistant variants. Notably, the combination of a virus-targeted drug with anti-inflammatory/immunomodulatory agents has become one of the most promising treatment approaches. A brief introduction to the combination therapy regimens is provided below.

5.1. Sequential monotherapy

Early studies have identified the possibility of single-drug sequential therapy for immunocompromised patients (*29*). Five patients who received allogeneic hematopoietic stem cell transplantation still had symptoms and shed influenza viruses after one or more oseltamivir courses, and were then given sequential baloxavir therapy. Among the three patients with wild-type influenza virus infection, two achieved viral clearance after baloxavir treatment, while another developed a baloxavir-resistant polymerase variant (I38T). Subsequent studies further validated this approach (*30*) : in severe Influenza A (H5N6) cases where oseltamivir was ineffective, baloxavir marboxil rapidly reduced patients' viral load and cytokine levels. In recent years, clinicians observed that some immunocompromised patients or elderly patients with chronic comorbidities still had high influenza virus nucleic acid load 5 days after single oseltamivir treatment or single baloxavir marboxil treatment, with no obvious improvement in pneumonia. Sequential use of these two drugs promoted viral nucleic acid negativity and prevented disease progression. Additionally, sequential therapy is safe with no drug-drug interactions, though verification *via* multicenter large-cohort studies is still required. It should be noted that for sequential treatment with baloxavir marboxil, the single adult dose must not exceed 40 mg. For elderly patients (≥65 years old), adjust the dose based on renal function, and use with caution if creatinine clearance is < 30 mL/min.

Recommendation 4: For immunocompromised patients or elderly patients with chronic underlying diseases, if their condition shows no significant improvement after standard antiviral treatment and they remain persistently positive for Influenza virus nucleic acid, sequential antiviral therapy is recommended. The recommended regimens include oseltamivir followed by baloxavir marboxil, or baloxavir marboxil followed by oseltamivir (Evidence Level: 4, Recommendation Strength:C).

5.2. The combination of virus-targeted drugs with different mechanisms

5.2.1. The combination of different NAIs

In mouse models, for A(H3N2) and wild-type A(H1N1)pdm09 viruses, zanamivir monotherapy was more effective than oseltamivir monotherapy or the oseltamivir-zanamivir combination; however, for the oseltamivir-resistant A(H1N1)pdm09 H275Y virus variant, combination therapy was comparable to zanamivir monotherapy, and both were superior to oseltamivir monotherapy (*31*). In the hollow fiber infection model (HFIM) system, combined treatment with oseltamivir (75 mg Q12h, $t_{1/2}$: 8 h) and zanamivir (600 mg Q12h, $t_{1/2}$: 2.5 h) remained effective against viruses resistant to both agents (*32*). In randomized controlled trials of adult seasonal influenza (mainly H3N2), oseltamivir-zanamivir combination therapy was not more effective than oseltamivir monotherapy, nor was it significantly better than zanamivir monotherapy (*33*); retrospective studies on adult H7N9 infection also showed that oseltamivir-peramivir combination therapy was not superior to oseltamivir monotherapy (*34*). A case report (*35*) indicated that in critically ill Influenza A patients receiving invasive ventilation and ECMO support, the combined regimen of oral oseltamivir and inhaled zanamivir failed to prevent disease deterioration. Existing evidence shows that for wild-type influenza viruses, NAIs combination therapy does not consistently outperform monotherapy. Its potential value may be limited to specific scenarios, such as when NAI-resistant strain infection is confirmed or highly suspected (*e.g.*, in areas with prevalent resistant strains) and no better alternatives (such as baloxavir) are available, and it can be used as a tentative strategy.

Recommendation 5: Routine use of NAIs combination therapy is not recommended for treating seasonal influenza or avian influenza (such as H7N9) infections. Examples of such combination therapy include oseltamivir combined with zanamivir or peramivir (Evidence Level: 1b, Recommendation Strength: A).

5.2.2. The combination of NAIs and RNA polymerase acid protein inhibitors

An *in vitro* study found that combining baloxavir with neuraminidase inhibitors (*e.g.*, oseltamivir, laninamivir) exerted a significant synergistic effect. This effect enhanced the inhibitory activity against influenza virus (*36*). In ferret model experiments, therapeutic effects of baloxavir and oseltamivir were tested separately and in combination. Results showed that compared with monotherapy, combination therapy significantly reduced the upper respiratory tract virus titer in ferrets. It also significantly lowered the rate of drug-resistant virus generation. In ferrets treated with oseltamivir alone, a new oseltamivir-resistant mutation (NA/H275Y) was observed. This phenomenon was not detected in the combination therapy group (*37*). Clinically, combination of baloxavir and oseltamivir has shown relatively favorable effects in treating two patients after hematopoietic stem cell transplantation. For one patient, flu symptoms were rapidly relieved after receiving this combination therapy, and the virus test result turned negative. The other patient also showed a good early response to the same treatment but experienced virus recurrence in the later stage (*38*). Subsequently, international reports indicated that a 10-day regimen of zanamivir combined with baloxavir could effectively control the persistent replication of influenza virus in patients after hematopoietic stem cell transplantation (*39*).

5.2.3. The combination of NAIs and RNA polymerase basic protein inhibitors

A prospective study on adult influenza (*40*) showed that combining favipiravir with oseltamivir accelerated clinical recovery in patients with severe influenza. This effect was more significant than that of oseltamivir monotherapy. This treatment strategy deserves further evaluation in randomized controlled trials. A randomized double-blind trial (*41*) compared the pharmacokinetics and efficacy of pimodivir combined with oseltamivir. The trial included elderly and non-elderly hospitalized patients. Results showed the combination therapy group was safe and effective: its viral load was significantly lower than the placebo group, and symptom relief time was shorter (72.45 hours *vs* 94.15 hours). The incidence of influenza-related complications was also lower (7.9% *vs* 15.6%). Finberg RW *et al.* (*42*) found that compared with the placebo group, the pimodivir-oseltamivir combination group had a significantly lower viral load titer over time. The symptom relief time of the combination group also tended to be shorter than that of the placebo group. The early Phase II study showed positive results. Two subsequent key Phase III clinical trials were conducted in inpatients and high-risk outpatients. These trials failed to reach the primary endpoint. The research and development of this drug has been terminated. It should no longer be considered for clinical treatment.

5.2.4. The combination of NAIs and Envelope glycoprotein hemagglutinin inhibitors

MEDI8852 is a novel monoclonal antibody. In mouse and ferret models, the combination of MEDI8852 and oseltamivir significantly enhances therapeutic efficacy when treatment is delayed. Additionally, combining MEDI8852 with oseltamivir shows notable effects in preventing and treating Influenza A virus (H5N1 and H7N9) infections (*43*). A randomized, double-blind, placebo-controlled clinical trial found that the combination of oseltamivir and MEDI8852 is similar to oseltamivir monotherapy in reducing viral shedding. The combination treatment does not induce viral drug resistance changes and demonstrates good safety (*44*). Yi *et al.* (*45*) developed a new antibody mixture named CT-P27. In mouse models of influenza virus infection, CT-P27 exhibits *in vivo* therapeutic efficacy and preventive potential. It also shows a synergistic effect when used in combination with oseltamivir. In immunodeficient nude mouse models, researchers evaluated the triple therapy of favipiravir combined with two monoclonal antibodies (targeting the HA stem and HA receptor-binding sites). They found that single-drug or dual-drug combinations could inhibit viral replication but not completely eliminate the virus. However, the triple combination therapy successfully cleared the virus, enabling nude mice to survive for 188 days without any recurrence signs. No drug-resistant mutations were detected in this study, and the virus's adaptability was not affected either. This triple combination therapy includes favipiravir, anti-HA stem monoclonal antibody and anti-HA receptor-binding site monoclonal antibody. It provides the possibility of eradicating influenza virus in immunodeficient hosts, thereby offering a new treatment strategy for patients with severe influenza or immunodeficiency (*46*). Gaisina I *et al.* (*47*) found that combining the small-molecule IAV entry inhibitor ING-1466 with oseltamivir or baloxavir marboxil can synergistically enhance therapeutic efficacy.

Recommendation 6: Oseltamivir combined with baloxavir can effectively control influenza virus replication (Evidence Level: 2c, Recommendation Strength:B); Oseltamivir combined with favipiravir is superior to monotherapy in reducing influenza virus load for influenza treatment (Evidence Level: 2a, Recommendation Strength:B); Oseltamivir combined with hemagglutinin inhibitors (e.g., MEDI8852) helps reduce viral shedding and enhance therapeutic efficacy (Evidence Level: 1b, Recommendation Strength: A).

5.3. The combination of host-targeted drugs and antiviral drugs

5.3.1. The combination of nonsteroidal anti-inflammatory drug and Oseltamivir phosphate

In Phase III clinical trials, combining oseltamivir with celecoxib significantly reduced patient mortality compared with oseltamivir monotherapy (*48*). Similarly, in Phase IIB/III clinical trials, the combination of clarithromycin-naproxen and oseltamivir produced two key effects: it significantly reduced the 30-day and 90-day mortality of hospitalized patients infected with H3N2 influenza, and shortened the overall hospital stay (*49*). In addition, a study on hospitalized children with influenza showed results: children treated with the combination of clarithromycin, naproxen and oseltamivir had a shorter fever resolution time than those treated with oseltamivir alone. Their influenza virus titer also decreased significantly faster(*50*). These studies suggest that the treatment regimen of non-steroidal anti-inflammatory drugs combined with oseltamivir has greater potential for influenza treatment.

5.3.2. The combination of immunomodulatory drugs and Oseltamivir phosphate

Long JS *et al.* (*51*) evaluated the effect of oseltamivir combined with human interferon λ on the drug resistance barrier of pandemic H1N1 virus strains A/Netherlands/602/2009 (H1N1pdm09) *via* an *in vitro* infection model. Results showed oseltamivir monotherapy led to rapid viral drug resistance *via* a single neuraminidase gene mutation, while combining with interferon λ significantly delayed the emergence of drug-resistant variants. Some literature has explored new drug development strategies, including targeting viral polymerase complexes (*e.g.*, PB1, PB2, PA) and leveraging host factors such as combining NAIs, polymerase inhibitors and immunomodulators like interferon λ. However, these strategies still need more clinical data to verify their broad applicability and safety (*52*).

Allotern is an immunomodulatory drug with antiviral activity against multiple viruses, including influenza virus. According to studies, when Allotern is used in combination with zanamivir, it can inhibit the production of inflammatory mediators and the migration of inflammatory cells to lung tissue. This effect effectively alleviates progression of lung inflammation induced by H1N1 Influenza virus (*53*).

Nitazoxanide (NTZ) belongs to the class of thiazole antibiotics, and it enhances the host's antiviral resistance by regulating the host's immune response. *In vitro* experiments have demonstrated that compared with oseltamivir or nitazoxanide monotherapy, the combination of these two drugs shows greater efficacy in preventing infection and shortening duration of viral shedding. Moreover, in animal models, this combined regimen not only significantly boosts the antiviral effect of oseltamivir but also successfully blocks the virus from spreading to the lower respiratory tract (*54*).

5.3.3. The combination of host-targeted drugs and baloxavir or Oseltamivir phosphate

The antiviral activity of the MEK inhibitor ATR-002 was evaluated in A549 cells. Both its monotherapy and combination with baloxavir marboxil against wild-type influenza strains and drug-resistant strains (with PA-I38T mutation) were tested *via* virus titer reduction assay and co-analysis. Results showed that ATR-002 exerted significant inhibitory effects on both wild-type and PA-I38T mutant strains. When used in combination with baloxavir marboxil, it exhibited a synergistic effect: combination therapy reduced viral load more effectively, especially when targeting drug-resistant strains, and its inhibitory effect was significantly better than that of either single drug used alone. The combination of ATR-002 and baloxavir marboxil provides a new therapeutic strategy for overcoming baloxavir marboxil resistance. It is also expected to open up new avenues for the treatment of drug-resistant influenza (*55*).

The combination of four MEK inhibitors (PD-0325901, AZD-6244, AZD-8330 and RDEA-119) with oseltamivir significantly enhanced oseltamivir's antiviral activity (*56*). This combination therapy demonstrates the potential of MEK inhibitors and deserves further verification through preclinical *in vitro* and *in vivo* experiments.

Combination treatment with CXCR2 antagonists and antiviral drugs can significantly reduce the incidence and mortality of toxic and sublethal influenza infections (*57,58*). Hanlon *et al.* (*59*) demonstrated that M85, a novel antiviral compound, effectively inhibits influenza virus entry in mouse influenza models. It exerts this effect by targeting the host kinase PIK3C2β. In addition, M85 shows a synergistic effect when combined with oseltamivir.

Recommendation 7: Combining host-targeted drugs with baloxavir marboxil and/or oseltamivir can exert a synergistic effect. It can rapidly reduce viral load and the incidence of drug-resistant influenza virus strains (Evidence Level: 2b, Recommendation Strength: B). The mentioned host-targeted drugs include non-steroidal anti-inflammatory drugs (e.g., celecoxib, naproxen), immunomodulatory drugs (e.g., interferon λ, nitazoxanide), and other host-targeted drugs (e.g., MEK inhibitors).

6. Population applicable for combined antiviral therapy

Combination antiviral therapy may be required for patients with resistance to current anti-influenza viral agents/poor efficacy of antiviral therapy, as well as patients with severe influenza, immunocompromised patients, and other critically ill high-risk patients who may require combination antiviral therapy due to persistent viral replication.

6.1. Patients infected with ineffective/resistant strains of antiviral therapy

The poor efficacy of anti-influenza virus treatment is defined as follows: after standardized use of anti-influenza virus drugs, the patient's symptoms do not improve as expected (*e.g.*, fever [≥38℃] lasting more than three days, and persistent or aggravated symptoms such as cough). Another sign is the continuous replication of the virus, which can be observed through positive nucleic acid testing indicating active viral replication. The poor efficacy of anti-Influenza virus treatment is often closely associated with the "drug resistance" of influenza viruses and infection with "drug-resistant strains". Drug resistance is defined as a functional state. In this state, influenza viruses lose or weaken their sensitivity to drugs through genetic mutations and other means under drug pressure. Drug-resistant strains, by contrast, refer to individual viruses or virus populations. They carry specific drug-resistant mutations and can stably exhibit this "drug-resistant state", serving as specific carriers of the drug-resistant state. According to the WHO definition, for IAV, a strain is determined to be drug-resistant if the concentration (IC_{50}) required for a drug to inhibit 50% of viral replication is more than 100 times higher than the normal value. If the IC_{50} is 10 to 100 times higher than the normal value, it indicates reduced sensitivity, which may affect therapeutic efficacy (*60*). To accurately identify drug resistance, current methods for detecting influenza virus drug resistance mainly include phenotypic analysis and genotypic analysis. Phenotypic analysis includes plaque reduction experiments, chemiluminescence methods, and fluorescence methods. Genotypic analysis includes real-time fluorescence quantitative PCR, digital PCR and other techniques. Technologies such as gene chips, CRISPR detection, and next-generation sequencing are still in the research stage (*61*). In clinical practice, the more common types of drug-resistant strains mainly include oseltamivir-resistant strains and baloxavir-resistant strains. The existence of such drug-resistant strains often directly leads to reduced efficacy or failure of the corresponding drugs. In clinical settings, these factors are of paramount importance when modifying treatment plans.

6.1.1. Oseltamivir resistant strains

For oseltamivir-resistant strains, combining favipiravir can restore the sensitivity of resistant viruses to antiviral drugs (*62*). Favipiravir can effectively inhibit the activity of the PB1 subunit of influenza viruses. It has inhibitory activity against influenza strains resistant to neuraminidase inhibitors and amantadine drugs. Meanwhile, it almost does not inhibit human DNA synthesis and has good safety(*63,64*). *In vitro* experiments have shown that the PB2 inhibitor pimodivir has a synergistic antiviral effect with oseltamivir (*65*). This drug is effective against IAV, including neuraminidase inhibitor-resistant and amantadine-resistant strains. However, it is ineffective against IBV (*66*). In terms of clinical research, results from the TOPAZ trial (*42*) indicated that when treating patients with acute, uncomplicated seasonal influenza A, pimodivir monotherapy could reduce viral load in a dose-dependent manner. The efficacy was more significant when pimodivir was used in combination with oseltamivir. Another set of clinical research data shows that in high-risk outpatients, the combined treatment of pimodivir and oseltamivir can also shorten the duration of influenza symptoms (*67*).

6.1.2. Baloxavir resistant strains

For baloxavir-resistant strains (with PA/I38T or PA/E23K mutation), combination therapy has shown potential to delay the occurrence of drug resistance. Koszalka P *et al.* (*37*) reported that in ferret models, the combination of baloxavir and oseltamivir could reduce the selection pressure on viruses with reduced drug sensitivity. This in turn lowers the risk of drug resistance. Park *et al.* (*68*) further verified the effect of baloxavir marboxil/oseltamivir monotherapy or combination therapy on the drug-resistant substitution of A(H1N1) pdm09 virus during continuous passage in mice. Deep sequencing analysis showed that the PA-I38X amino acid substitution variant emerged in 67% (n=4/6) of the mouse virus populations treated with baloxavir marboxil monotherapy. The combination of baloxavir marboxil and oseltamivir could inhibit the production of this variant, providing a therapeutic strategy to reduce influenza virus drug resistance. Guo X *et al.* (*36*) evaluated the antiviral effect of combining baloxavir with neuraminidase inhibitors on wild-type influenza viruses and drug-resistant mutant influenza viruses. The results showed that this combination had a significant synergistic effect. Given the rapid emergence of baloxavir resistance, these results are believed to provide a useful reference for influenza combination therapy.

Recommendation 8: For influenza patients infected with oseltamivir-resistant strains or with poor antiviral response to oseltamivir, combination of favipiravir and pimodivir is recommended for influenza virus antiviral treatment (Evidence Level: 2b, Recommendation Strength: B); For influenza patients infected with baloxavir-resistant strains or with poor antiviral response to baloxavir, combination with oseltamivir is recommended for influenza virus antiviral treatment (Evidence Level: 3a, Recommendation Strength: B); It is also recommended to select an individualized combination treatment regimen of antiviral drugs and host-targeted drugs.

6.2. Severe/critical influenza patients

According to *the Diagnosis and Treatment Plan for Influenza (2025)* (*28*), adult influenza is defined as severe if any of the following criteria are met: 1.The respiratory rate is ≥30 breaths per minute; 2. Oxygen saturation is ≤93% during resting room air inhalation; 3. The ratio of arterial partial pressure of oxygen to fractional inspired oxygen (PaO_2/FiO_2) is ≤300 mmHg; 4. Lung imaging shows that lesions progress by more than 50% within 24–48 hours. Childhood nfluenza is defined as severe if any of the following criteria are met: 1. Persistent high fever lasting more than 3 days; 2. Shortness of breath (≥30–60 breaths per minute, depending on age); 3. Oxygen saturation is ≤93%; 4. Presence of symptoms such as drowsiness or convulsions; 5. Severe dehydration; 6. Exacerbation of underlying diseases. On the basis of severe influenza, a case is considered critical if any life-threatening manifestation occurs, including respiratory failure requiring mechanical ventilation, septic shock requiring vasoactive drugs, and organ failure (*e.g.*, acute kidney injury or acute necrotizing encephalopathy).

Combination therapy may shorten the course of illness in patients with severe influenza, but the supporting evidence is limited (*69*). A prospective study on adults found that the combination of favipiravir and oseltamivir promotes clinical recovery in patients with severe influenza more quickly than oseltamivir monotherapy (*40*). Fukao K *et al.* (*70*) compared the efficacy of baloxavir marboxil monotherapy, oseltamivir monotherapy, and the combination of the two in mouse models infected with influenza virus. *In vitro* experiments showed that baloxavir marboxil and neuraminidase inhibitors could synergistically inhibit viral replication. In animal experiments, combination therapy was superior to monotherapy in reducing viral titers, mortality, and inflammatory responses. A post hoc analysis was conducted on the FLAGSTONE study (*71*). A total of 143 patients with severe nfluenza were included in the efficacy analysis, including those with immunosuppression, diabetes, or chronic lung disease. Among them, 92 patients received baloxavir combined with neuraminidase inhibitors (dual antiviral group), and 51 patients received neuraminidase inhibitors alone (single antiviral group). Compared with neuraminidase inhibitor monotherapy, the combination of baloxavir and neuraminidase inhibitors showed a better effect in reducing mortality. In the future, multicenter prospective cohort studies and randomized controlled trials need to be conducted to clarify the efficacy and safety of combination therapy in various critically ill patient groups.

Recommendation 9: For patients with severe or critical influenza, antiviral treatment is recommended as follows: one option is oseltamivir combined with favipiravir (Evidence Level: 2b, Recommendation Strength: B). The other option is oseltamivir combined with baloxavir (Evidence Level: 1b, Recommendation Strength: A).

6.3. Immunosuppressed/compromised patients

For influenza patients with weakened immune systems, their immune systems cannot effectively clear the virus, so they often need long-term treatment with neuraminidase inhibitors. Such patients excrete the virus for a long time, which is highly likely to induce drug-resistant mutations—these mutations seriously affect antiviral efficacy and prolong infections. Thus, there is an urgent clinical need for strategies to rapidly and strongly inhibit viral replication, and sequential or combined antiviral therapy is particularly important. Mhamdi Z *et al.* (*72*) studied immunosuppressed mice infected with H3N2 virus, which received 10-day monotherapy or combination therapy with oseltamivir, favipiravir, or baloxavir. Results showed oseltamivir and favipiravir monotherapy only delayed mortality (average death days: 21.4, 24 *vs.* 11.4 in the untreated group). The combination of oseltamivir and favipiravir increased the survival rate to 80% and reduced lung viral titer; the combination of oseltamivir and baloxavir provided complete protection (100% survival) and significantly lowered lung viral titer. Oseltamivir and baloxavir monotherapy induced E119V (NA) and I38T (PA) substitutions respectively, but no resistance mutations were detected in the combination group—indicating combination therapy reduces drug resistance.

Clinical research on combined anti-influenza virus therapy in immunosuppressed populations has also made certain progress. Two patients with severe influenza pneumonia on immunosuppressive drugs had no virus clearance after 18 and 5 days of oseltamivir monotherapy respectively. After adding zanamivir and amantadine to their regimens, their throat swabs and plasma samples turned PCR-negative in 3 and 4 days respectively (*73*). In conclusion, existing evidence shows that for influenza patients with weakened immune function, although combined antiviral therapy can prolong survival or reduce viral load to some extent, a more potent, multi-target combination (*e.g.*, triple combination of polymerase inhibitors, neuraminidase inhibitors, and monoclonal antibodies) may be needed to achieve complete clearance and avoid drug resistance. Future priority should be given to prospective clinical studies to verify the effectiveness and safety of such strategies in severely immunosuppressed populations.

Recommendation 10: For patients with suppressed or weakened immune function, sequential or combined treatment with oseltamivir, baloxavir, favipiravir, etc. can be selected (Evidence Level: 2c, Recommendation Strength: B).

6.4. Elderly patients aged ≥65 years

Elderly individuals, especially those over 65 years old, often have multiple underlying diseases. This makes them prone to worsening existing conditions

after influenza infection and may even trigger severe complications, that threaten their lives. For the elderly, the harm of influenza goes far beyond respiratory symptoms. Within a few weeks after acute infection, it may also induce extrapulmonary complications such as myocardial infarction or stroke, further increasing the medical burden. Studies have shown that elderly patients over 75 years old stay in the hospital for about two more days on average due to flu-related complications than those aged 50 to 64 (*74*). In a Phase 2b clinical study (*41*), O'Neil B *et al.* compared the antiviral effects of pimodivir combined with oseltamivir versus oseltamivir monotherapy in non-elderly adults (18–64 years old) and elderly patients (65–85 years old). The results showed that the combination group significantly shortened the time to relief of influenza symptoms: 72.45 hours for elderly patients and 94.15 hours for non-elderly patients. Moreover, the incidence of influenza-related complications in the combination group was also significantly lower than that in the monotherapy group, at 7.9% and 15.6% respectively. However, this evidence only comes from a single exploratory study and has not been fully validated in large-scale elderly populations. In addition, there is a lack of systematic assessment on the safety of combination treatment for this population.

Recommendation 11: For elderly patients aged ≥65 years, individualized combined antiviral treatment can be formulated based on the patient's immune status, underlying diseases, and condition severity. Relevant recommendations for severe or critical cases and patients with suppressed or weakened immune function can be referred to (Evidence Level: 4, Recommendation Strength: C).

6.5. Pregnant women

Pregnant women and those within two weeks after childbirth are at high risk of influenza. Influenza may cause a series of complications in pregnant women, such as increased risks of premature birth, miscarriage, cesarean section, maternal respiratory failure, and death. Therefore, during the influenza season, it is recommended that pregnant women and women within two weeks after childbirth undergo influenza testing. Once diagnosed or suspected of having influenza, antiviral treatment should be initiated as soon as possible (*75*). For pregnant women, the preferred antiviral drug is oseltamivir. Small cohort studies have shown that inhaled zanamivir is safe for both pregnant women and exposed infants. However, relevant research data on baloxavir and peramivir in pregnant women are still insufficient. Favipiravir is contraindicated for pregnant women due to its reproductive toxicity (*74*). Therefore, the safety of antiviral drugs for pregnant women with influenza still requires further research, and combination antiviral therapy is not recommended at present.

Recommendation 12: For pregnant women with influenza, their condition changes should be closely monitored. Routine combined antiviral treatment for influenza is not recommended. (Evidence Level: 2a, Recommendation Strength: B).

6.6. Children

According to *Practical Guidelines for Influenza Vaccination and Antiviral Drug Use in Children (2024 Edition)* (*76*), for children with severe influenza and those infected with drug-resistant mutant strains, if their condition does not improve and continues to deteriorate 48 hours after initiating antiviral drug treatment, combination therapy of neuraminidase inhibitors and baloxavir or sequential therapy can be considered. The dosage and administration method should refer to the single-drug treatment standards. Baloxavir is only used for children aged 5 years and above; it is not recommended for children under 5 years old due to insufficient safety data. In addition, the course of combination treatment should not exceed 7 days.

Recommendation 13: Routine combination of antiviral drugs for the treatment of childhood influenza is not recommended. However, for children over 5 years old with severe conditions and weakened immune function, if the viral nucleic acid level is ≤30, sequential use or combination of baloxavir and oseltamivir is recommended (Evidence Level: 1a, Recommendation Strength: A).

7. Considerations for combined antiviral therapy for influenza

7.1. Drug interactions

In studies on drug interactions in influenza antiviral combination therapy, the *in vitro* and *in vivo* safety as well as synergistic effects of different drug combinations have been preliminarily verified. *In vitro* studies have shown that the synergistic index and antagonistic index of ZSP1273 combined with oseltamivir are 852.41 and -0.19 respectively. This indicates a strong synergistic effect between the two drugs (*77*). At present, research on drug interactions in special populations is still relatively limited. In the future, further targeted studies are needed to improve medication guidance for different populations.

7.2. Overlapping risk of drug resistance

MU *et al.* (*62*)demonstrated that the combination of famciclovir and oseltamivir may promote the emergence of oseltamivir-resistant variants, thereby accelerating the evolution of resistance mutations. For instance, this combination could lead to the occurrence of PA/I38T + H274Y double mutant strains. Thus, this antiviral combination regimen is not recommended for all

influenza patients. Instead, it should be customized for specific populations, and the use of combination therapy requires individual assessment before decision-making. This approach enables more effective management of therapeutic risks and optimization of treatment outcomes.

7.3. Adverse effects and safety

Combination therapy of naproxen with oseltamivir was associated with a low incidence of adverse events (AEs) in one study (*49*). Another study demonstrated no statistically significant differences in AEs (including vomiting, diarrhea, and abdominal pain) when compared with oseltamivir monotherapy (*50*). In subjects receiving combination therapy with the monoclonal antibody MEDI8852 and oseltamivir, the incidence of AEs was relatively elevated. Nevertheless, the majority of these events were classified as mild or moderate, with bronchitis identified as the most frequent adverse event (*44*).By contrast, the combination of ZSP1273 and oseltamivir exhibited a favorable safety and tolerability profile, and no clinically significant drug-drug interactions were detected (*78*). A randomized controlled trial (RCT) observed that co-administration of baloxavir with a neuraminidase inhibitor did not increase the risk of AEs (*71*).

These studies indicate that although antiviral combination regimens are linked to AEs, their overall safety profile remains acceptable. This finding provides a scientific basis for clinicians to select appropriate drug combinations according to patients' specific conditions in clinical practice. Future studies could further explore the safety and efficacy of these regimens across different disease stages and drug doses, with the aim of continuously optimizing treatment strategies.

7.4. Cost-effectiveness

Jiang Y *et al*. constructed a linked dynamic communication economic evaluation model to assess the cost-effectiveness of oseltamivir combined with baloxavir marboxil in the context of an influenza pandemic in China. The study results showed that adding baloxavir marboxil to the treatment regimen could reduce the cumulative incidence of influenza infection from 49.49% to 43.26% and increase quality-adjusted life years (QALYs). Compared with oseltamivir monotherapy, the intervention regimen combined with baloxavir marboxil achieved a net monetary benefit of 77.85 yuan per person, with a willingness-to-pay (WTP) threshold of 80,976 yuan per QALY (*79*).

From the perspective of Japanese healthcare costs, baloxavir (used for post-exposure prophylaxis) and ranimivir (used for treatment) showed high cost-effectiveness. This means combining these two drugs for influenza prevention and treatment can achieve better therapeutic effects at lower costs. When using the EuroQol-5 Dimension-5 Level (EQ-5D-5L) to measure health-related quality of life, the combination of baloxavir and ranimivir also significantly improved patients' quality of life (*80*).

Baloxavir and ranimivir are not only cost-effective in healthcare but also have significant value in social and public health dimensions. Thus, this drug combination deserves further promotion and application in future influenza pandemic preparedness and response strategies.

7.5. Monitor

7.5.1. Monitoring of antiviral efficacy

Based on pharmacokinetic (PK) and pharmacodynamic (PD) data, anti-influenza drugs typically reach a plasma concentration plateau after 5–6 half-lives. Specifically, oseltamivir has a half-life of 6–10 hours, while zanamivir has a half-life of 2.5–5.1 hours. The efficacy of anti-influenza drugs can be assessed 48 hours after initiating the recommended treatment (*81*). It is recommended to recheck influenza nucleic acid 5 days after treatment initiation. For combined antiviral therapy, influenza virus nucleic acid changes can also be monitored at the above time points. For certain patients, the interval for influenza nucleic acid monitoring can be shortened appropriately, including those with risk factors for severe influenza, influenza patients with immunosuppression or impaired immune function, patients with severe or critical influenza, and patients whose condition worsens during treatment.

7.5.2. Monitoring of antiviral efficacy

From global surveillance data, the resistance rate of influenza viruses to neuraminidase inhibitors (NAIs, *e.g.*, oseltamivir, peramivir) showed a slight upward trend (0.09%–0.23%) globally from 2020 to 2023. The incidence of baloxavir susceptibility reduction events also remained relatively low (0.07%–0.12%) (*82*). Although the current overall resistance rate is low, vigilance is needed regarding the epidemiological changes of drug-resistant strains in long-term monitoring. These changes directly determine clinical treatment efficacy and the success of public health prevention and control strategies, so risks must be managed through systematic monitoring and targeted responses.

According to *the China Influenza Surveillance Weekly Report*, the Influenza A (H1N1)pdm09 strain in China has remained generally sensitive to oseltamivir since 2009. However, local transmission of the H275Y mutant strain has been detected in some regions, and the resistance of this subtype to NAIs still requires close attention (*83*). In clinical practice, regular monitoring of drug-resistant mutations is essential, especially for severe patients with persistent viral replication. If the main cause of disease progression is confirmed to be persistent viral positivity

leading to elevated inflammatory factors, sequential or combined antiviral therapy should be adopted. Alternatively, antiviral drugs can be combined with host-targeting monoclonal antibodies or immunomodulators. Meanwhile, the turnaround time for drug resistance testing should be optimized, and rapid PCR technology should be used to detect specific mutations, facilitating timely adjustment of treatment strategies. Additionally, when evaluating new drugs or formulating combination strategies for immunosuppressed or critically ill patients, emphasis should be placed on analyzing drug resistance. Medical institutions with appropriate testing capabilities are recommended to perform drug resistance gene testing before initiating combined antiviral therapy. They should also conduct such testing if the influenza virus nucleic acid CT value is <30 five days after the start of antiviral treatment (*74*).

Recommendation 14: The overall safety of combined anti-influenza virus therapy is favorable. However, attention should still be paid to adverse drug reactions and drug interactions (Evidence Level: 2a, Recommendation Strength: B). During combined therapy, monitoring of influenza virus nucleic acid load is recommended. Medical institutions with testing capabilities may conduct drug resistance gene testing (Evidence Level: 5, Recommendation Strength: D).

8. Future research directions

The rapid evolution of influenza viruses and the spread of drug resistance have become major threats to global public health. To address this challenge, researchers are conducting in-depth explorations from multiple dimensions, including molecular mechanisms, model prediction, and immune mechanisms, with the aim of innovating existing prevention, control, and treatment strategies. Researchers identified novel allosteric drug-resistant mutations of neuraminidase *via* deep mutational scanning. This study revealed a new mechanism: these mutations do not directly act on the drug-binding pocket but cause resistance by affecting protein tetramerization (*84*). A site-dynamics-based evolutionary model and a machine learning method were developed in two studies, respectively. These tools can more accurately predict the antigenic evolution of influenza viruses, especially H3N2, and provide powerful computational support for vaccine strain selection (*85,86*). Yang B *et al.* found that the breadth of influenza antibody cross-reactivity is affected by viral subtypes and exposure history. Repeated exposure to H3N2 virus can shape and expand the scope of human antibody responses, which lays a framework for understanding immune imprinting and vaccine design (*87*).

Looking ahead, future research directions mainly include the following: developing novel broad-spectrum antiviral drugs and new influenza vaccines; establishing a national influenza virus drug resistance monitoring network that integrates real-time sequencing data and develops an artificial intelligence (AI)-based model for predicting viral evolution and drug resistance to provide early warning of drug-resistant mutations; developing individualized precision treatment strategies based on machine learning and AI algorithms to automatically recommend optimal single-drug or combination therapy regimens; and conducting multicenter RCTs to evaluate the safety and long-term efficacy of combination therapy in preventing severe cases and in populations such as immunocompromised patients and children.

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Review

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Deciphering the nexus of aging and pan-cancer: Single-cell sequencing reveals microenvironmental remodeling and cellular drivers

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SUMMARY: Aging constitutes a major risk factor for pan-cancer development, with epidemiological studies indicating that 60% of new malignancies occur in adults age 65 and older. This review synthesizes cutting-edge insights from single-cell sequencing databases (*e.g.*, TCGA and GEO) that decipher how aging reprograms the tumor microenvironment (TME) to fuel carcinogenesis. Single-cell RNA sequencing (scRNA-seq) has revealed that senescent cell subpopulations (*e.g.*, CDKN2A$^+$/LMNB1$^-$ cells) accumulate in aged tissues at frequencies up to 15%, driving genomic instability and secrete pro-tumorigenic senescence-associated secretory phenotype (SASP) factors (IL-6 and TGF-β). These factors remodel the TME by inducing fibroblast activation and extracellular matrix degradation, accelerating metastasis by 40-70% in murine models. Crucially, immunosenescence diminishes anti-tumor immunity, with scRNA-seq profiling showing 40-60% increases in exhausted PD-1$^+$ T cells and immunosuppressive myeloid cells in aged TMEs. Pan-cancer analyses have identified conserved aging gene signatures (*e.g.*, p16INK4a upregulation in 12+ cancer types) that correlate with 30-50% poorer survival. While technical challenges persist — including batch effects in scRNA-seq data and low senescent cell abundance (< 5%) — emerging solutions like deep learning can enhance detection sensitivity. Therapeutically, senolytic strategies deplete senescent cells, improving drug response by 3.5-fold in preclinical trials. Future research must integrate multi-omics and AI to examine aging-related targets, advancing personalized interventions for aging-associated malignancies.

Keywords: single-cell sequencing, aging, pan-cancer, tumor microenvironment, biomarkers

1. Introduction

Aging is a fundamental biological process characterized by a gradual decline in physiological function that significantly correlates with the onset and progression of various diseases, and particularly cancer (*1*-*3*). The aging process is not merely a passive accumulation of cellular damage; rather, it is a complex interplay of genetic, epigenetic, and environmental factors that culminate in cellular senescence, altered tissue homeostasis, and changes in the tumor microenvironment (TME) (*4*,*5*). The TME is integral to cancer biology, influencing tumor initiation, progression, and response to therapy. As individuals age, the TME undergoes significant transformations that can foster a pro-tumorigenic environment, thereby increasing the risk of cancer development (*6*-*8*). The mechanisms by which aging influences cancer are multifaceted and involve alterations in cellular signaling pathways, immune responses, and metabolic processes (*9*-*11*). Despite the established link between aging and cancer, the underlying molecular mechanisms remain poorly understood, necessitating further exploration.

Recent advances in single-cell RNA sequencing (scRNA-seq) technologies have revolutionized our understanding of the intricate relationship between aging and cancer (*12*,*13*). scRNA-seq allows for the detailed characterization of cellular heterogeneity within tumors and the TME, providing insights into the differential expression of aging-related genes across various cell populations (*14*,*15*). This technology enables researchers to investigate the unique transcriptional profiles of individual cells, thereby elucidating how aging affects cellular function and contributes to tumorigenesis (*16*-*18*). Studies utilizing scRNA-seq have quantified these associations, showing that senescent cell subpopulations

(*e.g.*, CDKN2A^{+}/LMNB1^{-} cells) accumulate in aging tissues at frequencies up to 15% (*19-22*). These cells drive genomic instability and secrete pro-tumorigenic senescence-associated secretory phenotype (SASP) factors, fundamentally altering the TME. Using single-cell sequencing data, researchers can identify specific aging-related pathways and gene signatures that may serve as potential biomarkers for cancer prognosis and therapeutic targets.

Moreover, the use of scRNA-seq in cancer research has uncovered the TME's role in mediating the effects of aging on tumor development (*23*). The TME consists of various cell types, including immune cells, stromal cells, and cancer-associated fibroblasts, each of which can influence tumor behavior (*24*). Aging alters the composition and functionality of these cells, leading to a TME that supports tumor growth and immune evasion (*25*,*26*). Age-related immunosenescence leads to a quantifiable shift in immune cell populations within the TME. scRNA-seq profiling reveals 40-60% increases in exhausted PD-1^{+} T cells and immunosuppressive myeloid cells in aged compared to young TMEs (*27-29*), severely diminishing immune surveillance and anti-tumor efficacy. Understanding these interactions at the single-cell level is crucial to developing targeted therapies that can effectively modulate the TME and enhance the efficacy of cancer treatments in older patients.

In summary, the interplay between aging and cancer is a complex and multifaceted relationship that warrants further investigation. The emergence of scRNA-seq technologies provides a powerful tool to elucidate the molecular and cellular mechanisms underlying this relationship. By determining the contributions of aging-related changes in the TME and cellular heterogeneity, researchers can identify novel therapeutic strategies in order to improve outcomes for aging cancer patients. This review aims to summarize the latest advances in scRNA-seq research focused on the correlation between aging and pan-cancer, exploring potential mechanisms and clinical uses that may emerge based on this understanding.

2. The basic relationship between aging and cancer

2.1. Aging as a risk factor for cancer

Aging is increasingly recognized as a significant risk factor for the development of cancer, primarily due to the cumulative effects of DNA damage and epigenetic alterations that occur over time. As individuals age, their cells experience a variety of intrinsic and extrinsic stressors that contribute to the accumulation of DNA damage, including oxidative stress, inflammation, and exposure to environmental carcinogens. This accumulation can lead to genomic instability, a hallmark of cancer, which increases the likelihood of malignant transformation. Notably, the aging process is also associated with changes in the epigenome, such as DNA methylation and histone modifications, which can silence tumor suppressor genes and activate oncogenes, further increasing the risk of cancer (*30*). Additionally, the SASP plays a crucial role in promoting a tumor-friendly microenvironment. Senescent cells secrete various pro-inflammatory cytokines, growth factors, and proteases that can alter the surrounding tissue architecture and promote tumorigenesis (*31*). This interplay between aging, DNA damage, and the SASP underscores the complexity of cancer development in older adults, highlighting the need for targeted strategies to mitigate these risks.

2.2. Molecular mechanisms linking aging and cancer

The molecular interplay between aging and cancer involves multiple interconnected pathways that drive genomic instability and create a permissive microenvironment for carcinogenesis:1) Telomere dysfunction: Critically shortened telomeres trigger the DNA damage response (DDR), activating p53/p21-mediated senescence while simultaneously increasing genomic instability through catastrophic events such as chromothripsis (chromosome shattering) and kataegis (localized hypermutation) (*2*). This dual role explains why telomere attrition not only limits cellular lifespan but also promotes oncogenic mutations. 2) Mitochondrial dysfunction: Age-related accumulation of mtDNA mutations promotes ROS overproduction, which activates NF-κB-driven inflammation and stabilizes HIF-1α to enhance tumor glycolysis (*18*). This metabolic shift fuels tumor growth while creating an oxidative microenvironment that damages neighboring cells. 3) Epigenetic drift: Global hypomethylation coupled with CpG island hypermethylation silences tumor suppressors (*e.g.*, RASSF1A) while activating oncogenic retrotransposons (*9,32*). This "epigenetic noise" disrupts transcriptional fidelity, allowing pre-malignant clones to evade growth control. 4) SASP amplification: Senescent cells secrete IL-6/IL-8 *via* NF-κB signaling, inducing STAT3 phosphorylation in premalignant cells to promote stemness (*10*). Crucially, SASP factors remodel the extracellular matrix (ECM) through MMP-9/12 upregulation, facilitating metastatic niche formation (*33*). Paradoxically, while persistent p53 activation in aged tissues induces senescence as a tumor-suppressive mechanism, the resulting SASP fuels inflammation-driven malignancy (*34*).

These mechanisms synergistically create a self-reinforcing loop: Telomere dysfunction and mitochondrial ROS accelerate epigenetic alterations, which in turn stabilize the senescent phenotype and amplify the SASP. Age-related immunosenescence further compromises surveillance of these evolving malignant clones (*35*). Targeting these intersections — such as combining senolytics with DDR inhibitors —

represents a promising therapeutic strategy for aging-associated cancers (Figure 1).

3. Use of single-cell sequencing technology in aging and pan-cancer research

3.1. Overview of single-cell sequencing technology

Single-cell sequencing technologies, and particularly scRNA-seq and single-cell ATAC sequencing (scATAC-seq), have revolutionized our understanding of cellular heterogeneity and dynamics in various biological contexts, including aging and cancer. scRNA-seq allows for the quantification of gene expression at the single-cell level, enabling researchers to determine the transcriptomic landscape of individual cells within complex tissues. This high-resolution approach reveals the diverse cellular states and identities that contribute to tissue function and pathology. In contrast, scATAC-seq focuses on the accessibility of chromatin, providing insights into regulatory elements that govern gene expression. By assessing the open chromatin regions, researchers can infer the active regulatory networks that control cellular responses to environmental cues and developmental signals. The combination of these techniques offers a comprehensive view of cellular behavior, allowing for the identification of distinct cell populations and their functional roles in aging and tumorigenesis. Moreover, the ability to analyze thousands of individual cells simultaneously enhances the detection of rare cell types and transient states, which are often missed in bulk sequencing approaches. This granularity is particularly advantageous in the context of aging and cancer, where cellular diversity plays a critical role in disease progression and therapeutic responses (*36,37*).

3.2. Identification of aging-related cell subpopulations

The use of scRNA-seq technologies has profoundly advanced our understanding of aging-related cellular changes by revealing distinct pro-tumorigenic subpopulations that drive microenvironmental remodeling in aged tissues. These studies have not only identified and quantified key senescence markers — such as upregulation of CDKN2A and downregulation of LMNB1 — within specific aging cell subpopulations present at frequencies up to 15% in aged tissues (*21,22*), but they have also delineated functionally relevant cell states contributing to tumor progression.

Notably, several specific pro-tumorigenic subpopulations have been characterized through scRNA-seq. For instance, senescent fibroblasts ($p16^{+}/FAP^{+}/CAV1^{-}$) constitute 8–15% of stromal cells in aged tumors and secrete HGF and Wnt5a to activate β-catenin signaling in carcinoma cells, thereby promoting epithelial-to-mesenchymal transition (EMT) (*38*).

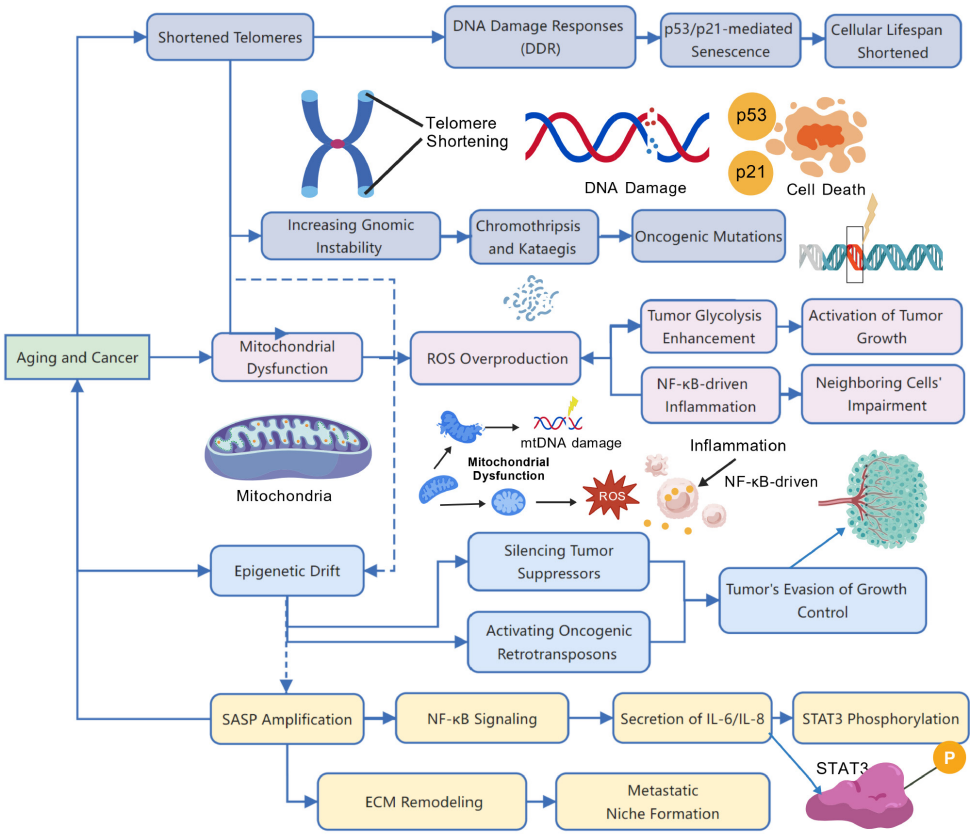

Figure 1. Flowchart of molecular and microenvironmental mechanisms of aging driven cancer.

Spatial transcriptomic mapping further indicates their preferential localization at tumor–stroma interfaces. Similarly, age-associated T cells ($CD8^+GZMB^-PD\text{-}1^+$) exhibit impaired cytotoxicity due to TOX-mediated exhaustion programs and are enriched in ovarian and colorectal cancers. Pseudotime trajectory analysis has revealed that their differentiation from naïve T cells is driven by TCF7 downregulation (*39*).

Another salient population includes lipofuscin-laden macrophages ($CD163^+TREM2^+$), which amass oxidized lipids from aged tissue environments and secrete TGF-β to induce $FoxP3^+$ regulatory T cell differentiation, fostering immunosuppressive niches (*29*). Integrated lipidomics and scRNA-seq analyses have confirmed the accumulation of peroxidized phospholipids in these cells. Additionally, prefibrotic pericytes ($PDGFR\beta^+/NG2^+/\alpha\text{-}SMA^-$) contribute to vascular leakiness *via* Angpt2 secretion, accelerating hematogenous dissemination by 3.2-fold in murine models of metastasis. Their transcriptomes exhibit NOTCH3 downregulation, indicating disrupted endothelial–pericyte crosstalk (*21*).

These subpopulations engage in synergistic crosstalk: senescent fibroblasts recruit lipofuscin-laden macrophages *via* CCL2 secretion, while age-associated T cells exhibit impaired clearance of pre-malignant clones due to macrophage-derived TGF-β. Therapeutically, targeting their unique surface markers — such as using FAP-directed CAR-T cells against senescent fibroblasts — has been found to reduce the tumor burden by 40–60% in preclinical models, highlighting the translational potential of these findings (Figure 2).

4. The impact of the aging microenvironment on tumorigenesis

4.1. The role of the SASP

The SASP is a critical factor in the TME, and particularly in the context of aging. SASP factors (*e.g.*, IL-6, TGF-β) secreted by senescent cells exert potent pro-tumorigenic effects. IL-6 is known to activate various signaling pathways that lead to increased cell proliferation and survival, while TGF-β can induce EMT in tumor cells, facilitating metastasis (*38*,*39*). Crucially, they remodel the TME by inducing fibroblast activation and ECM degradation, which are processes that accelerate metastasis by 40-70% (*38-41*). scRNA-seq technologies have revealed intricate communication mechanisms between SASP factors and both tumor cells and stromal cells within the TME. These studies have demonstrated that SASP factors can influence the behavior of neighboring cells, promoting a more aggressive tumor phenotype. scRNA-seq has precisely mapped the spatial distribution and transcriptional profiles of senescent fibroblasts within the TME, confirming their secretion of factors that significantly enhance the invasive properties of adjacent cancer cells, indicating a potent paracrine signaling mechanism (*40*,*41*). This interplay not only highlights the role of SASP in tumorigenesis but also suggests potential therapeutic targets aimed at modulating the SASP to inhibit tumor progression and improve patient outcomes.

4.2. Immunosenescence and tumor immune evasion

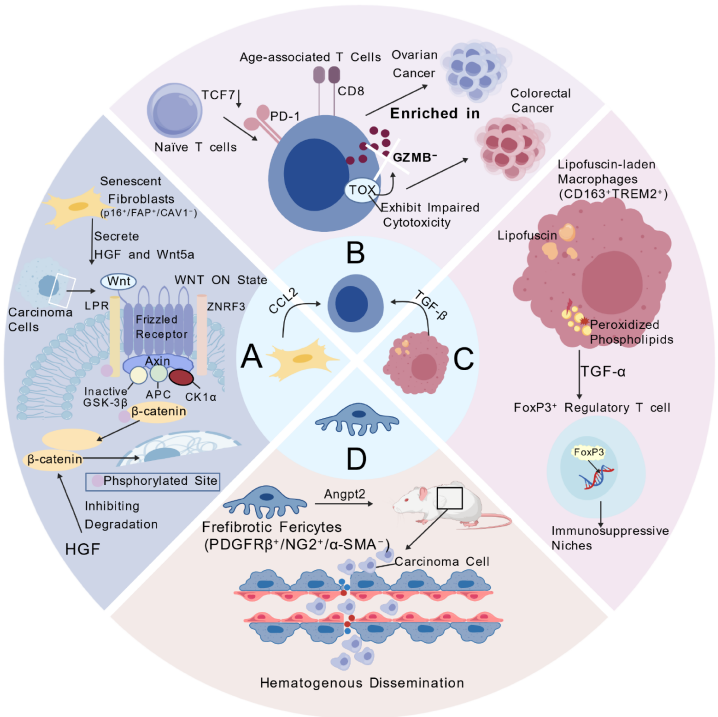

Figure 2. Identification of aging-related cell subpopulations.

Immunosenescence, the age-related functional decline of the immune system, profoundly reshapes the TME and facilitates tumor immune evasion. This process involves multiple cellular and structural networks that collectively impair anti-tumor immunity.

With aging, T cell populations undergo significant alterations: $CD8^+$ T cells exhibit clonal expansion of terminally exhausted subsets (*e.g.*, $TIM\text{-}3^+LAG\text{-}3^+$), while $CD4^+$ T cells shift toward Th17 polarization driven by RORγt upregulation (*23*). These changes are quantifiable *via* scRNA-seq, which reveals a 40–60% increase in exhausted $PD\text{-}1^+$ T cells within aged TMEs compared to younger counterparts (*28,42*).

Concurrently, myeloid-derived suppressor cells (MDSCs; $CD11b^+Gr\text{-}1^+$) expand substantially — by approximately 2.3-fold in aged murine models — and contribute to immune suppression through arginase-1 (ARG1)-mediated arginine depletion. This metabolic impairment inhibits T cell receptor (TCR) signaling and attenuates cytotoxic responses (*27*). In parallel, dendritic cells (DCs) experience functional decline; conventional type 1 DCs (cDC1) downregulate the costimulatory molecules CD80 and CD86 by nearly 60%, markedly reducing their capacity to prime naïve T cells (*26*).

Aged lymphoid tissues also exhibit structural degeneration, particularly within tertiary lymphoid structures (TLS). Loss of fibroblastic reticular cells disrupts CXCL13 secretion, impairing B cell recruitment and functional organization in lymph nodes (*13*). Moreover, scRNA-seq studies consistently demonstrate that aged TMEs are enriched with immunosuppressive myeloid cells — such as M2 macrophages and MDSCs — by 40–60%, actively suppressing T cell activation and fostering a pro-tumorigenic milieu (*29,43*).

The cumulative impact of these changes — ranging from cellular exhaustion and functional dysregulation to structural decay — severely compromises immune surveillance and promotes tumor immune evasion (Figure 3). Consequently, targeting these age-specific alterations in the immune landscape represents a promising avenue for restoring anti-tumor immunity in older cancer patients.

5. Patterns of aging-related gene expression in pan-cancer

5.1. Pan-cancer analysis of aging-related genes

The exploration of aging-related gene expression across various cancer types has garnered significant attention due to its implications for understanding tumor biology and patient outcomes. Utilizing single-cell sequencing databases such as the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), researchers have conducted comprehensive analyses to identify the expression profiles of aging-related genes in a pan-cancer context. These studies have revealed both common and unique patterns of aging gene expression across different malignancies. Pan-cancer analyses using TCGA/GEO data have revealed conserved aging-related gene signatures. For instance, p16INK4a (CDKN2A) upregulation is observed in 12 or more distinct cancer

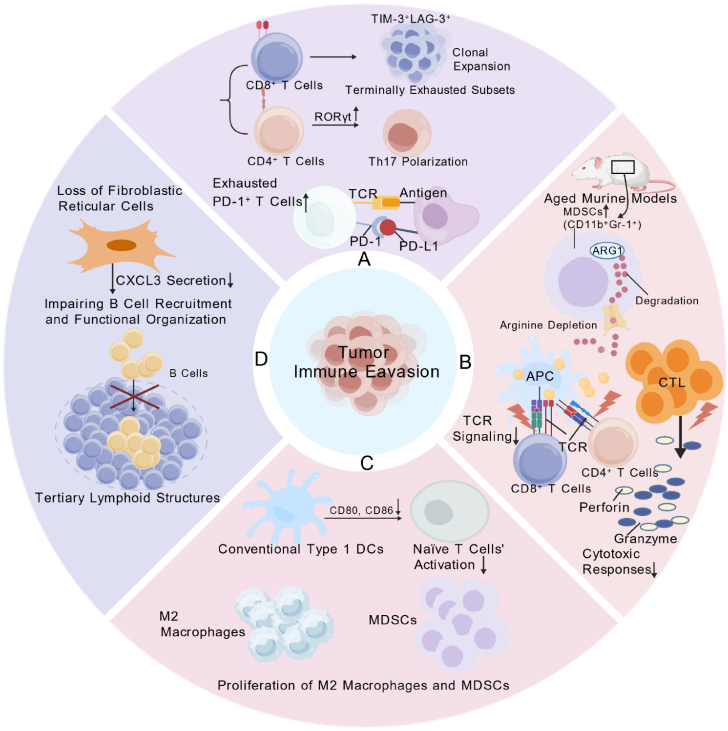

Figure 3. Immunosenescence to an immune evasion cascade.

types, suggesting a shared mechanism linked to aging processes (*44*). Conversely, certain aging-related genes may display cancer-type-specific expression profiles, indicating that the TME and genetic background can influence the expression of these genes (*45*). The integration of scRNA-seq data allows for a nuanced understanding of how aging-related genes contribute to the heterogeneity of tumors, providing insights into their roles in cancer progression and potential therapeutic targets.

5.2. Clinical significance of aging-related genes

The clinical implications of aging-related genes as prognostic markers in cancer are increasingly being recognized. Several studies have highlighted the potential of these genes to serve as biomarkers for patient outcomes, and particularly in terms of survival and treatment response. Elevated expression of specific aging-related gene signatures (*e.g.*, senescence core signatures) has been robustly correlated with 30-50% poorer survival outcomes across multiple cancer types, highlighting their potential as prognostic biomarkers reflecting tumor biological age and aggressiveness (*46*). Moreover, targeting aging-related pathways has emerged as a promising therapeutic strategy. Therapeutically, senolytic strategies designed to deplete senescent cells have demonstrated significant promise in preclinical trials, showing the potential to improve drug response by up to 3.5-fold (*47*). The ability to use aging-related genes for prognostic stratification could enhance personalized treatment approaches, allowing clinicians to tailor therapies based on the biological aging status of tumors, ultimately improving patient outcomes.

5.3. Clinical trials of personalized therapy in geriatric oncology

The growing recognition of aging as a critical determinant in cancer biology has spurred dedicated clinical investigations into personalized therapeutic strategies for older adults. Recent trials (Supplementary Table S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=274*) highlight efforts to tailor interventions by accounting for age-related physiological decline, comorbidities, and functional vulnerabilities (*48-80*). A landmark study (NCT02054741) demonstrated that integrating Comprehensive Geriatric Assessment (CGA) into oncology practice significantly reduced patient-reported symptomatic toxicity by 30% in older adults with advanced cancers (*49*). This intervention identified frailty markers (*e.g.*, cognitive impairment, and malnutrition) that predicted susceptibility to chemotherapy toxicity, enabling preemptive dose modifications or supportive care.

scRNA-seq insights are increasingly informing trial design. One study (NCT03885908) evaluated automated geriatric co-management guided by molecular senescence signatures (*e.g.*, CDKN2A↑/LMNB1↓). This approach correlated SASP factors (IL-6, TGF-β) with diminished treatment tolerance, allowing dynamic therapy adjustments. Similarly, NCT04262232 adapted the Ca-HELP intervention for rural elderly populations by stratifying patients using circulating senescence-associated microRNAs, reducing hospitalizations by 25%.

6. Current research limitations and future directions

6.1. Technical challenges

The use of sscRNA-seq technology has revolutionized the understanding of cellular heterogeneity and the complexities of aging and cancer. However, this approach is not without its challenges. One significant limitation is the presence of noise and batch effects within the scRNA-seq data. Noise can arise from various sources, including technical artifacts during library preparation and sequencing, which can obscure biological signals and complicate data interpretation. Studies have revealed that the performance of noise reduction methods can vary significantly depending on the biological and technical factors present in the data, such as the magnitude of batch effects and the complexity of cell populations (*81*). Moreover, the low abundance of senescent cells (< 5%) in most tissues poses a significant challenge for their reliable detection and characterization *via* sequencing (*82*). Given that senescent cells can represent a minor fraction of the total cell population, their detection and characterization require highly sensitive methods to ensure reliable data acquisition. Approaches like deep learning models have been proposed to address these challenges, yet the need for robust and standardized protocols remains critical (*82*). Thus, while scRNA-seq presents an unprecedented opportunity to explore the intersection of aging and cancer, overcoming these technical hurdles is essential to the advancement of the field.

6.2. Future research directions

Looking ahead, future research should focus on integrating multi-omics data to further understand the mechanisms linking aging and cancer. By combining transcriptomics with proteomics, metabolomics, and epigenomics, researchers can gain a more comprehensive view of the biological processes at play. Such integrative approaches have the potential to unveil novel biomarkers and therapeutic targets that could enhance precision medicine strategies for age-related diseases and cancer (*83*). In addition, developing targeted therapeutic strategies aimed at aging-related pathways could pave the way for innovative treatments. Drawing on insights from

multi-omics analyses may facilitate the identification of key aging-related targets that can be modulated to improve patient outcomes (*84*). Moreover, as the field of artificial intelligence continues to evolve, the use of artificial intelligence to analyze complex multi-omics datasets could lead to breakthroughs in understanding the intricate relationship between aging and cancer. Through collaboration across disciplines and use of advanced computational tools, researchers can enhance the precision of their investigations and ultimately contribute to the development of more effective, personalized therapeutic interventions (*85*).

7. Conclusion

The intersection of aging and pan-cancer research has seen remarkable advances, particularly propelled by the advent of single-cell sequencing technologies. These innovations have enabled researchers to elucidate the complexities of the aging microenvironment and its profound influence on tumorigenesis. By analyzing data at a single-cell resolution, scientists have begun to unravel the intricate relationships between cellular aging processes and cancer development, revealing potential biomarkers and therapeutic targets that were previously obscured by bulk analytical methods.

From an expert perspective, a crucial step is to recognize the transformative impact of scRNA-seq on our understanding of cancer biology. This technology allows for a nuanced exploration of cellular heterogeneity within tumors, enabling researchers to identify distinct aging signatures that may contribute to cancer progression. The ability to profile individual cells has opened new avenues for discovering aging-related biomarkers that can serve as indicators for early cancer detection. Moreover, these biomarkers have the potential to inform personalized treatment strategies, aligning therapeutic interventions with the specific aging profiles of patients.

Although progress in this field is commendable, the technical and methodological challenges that remain need to be acknowledged. The complexity of single-cell data analysis requires sophisticated computational tools and robust statistical methodologies to accurately interpret the vast amounts of information generated. Current research efforts must focus on developing more precise analytical frameworks that can integrate multi-omics data, combining genomic, transcriptomic, and proteomic insights to create a comprehensive understanding of the aging-cancer nexus.

Balancing different research perspectives is vital in this evolving landscape. As we strive for a holistic view of aging and cancer, collaboration among researchers from diverse disciplines, including molecular biology, bioinformatics, and clinical oncology, needs to be fostered. Such interdisciplinary approaches can enhance the robustness of findings and facilitate the translation of research into clinical practice. As an example, integrating insights from immunology could elucidate how age-related changes in the immune system affect the TME, thereby informing therapeutic strategies that involve immunomodulation.

Moreover, as we move toward a more personalized approach to cancer treatment, we need to consider the ethical implications of aging-related research. The identification of aging biomarkers raises questions about how this information will be used in clinical settings. Ensuring that findings are considered fairly and do not lead to age-based discrimination in treatment access is a critical consideration for researchers and clinicians alike.

Cellular senescence acts as a double-edged sword: while initially suppressing tumorigenesis *via* p53 activation, persistent senescent cells create a permissive niche through SASP-mediated inflammation. This paradox is exemplified by p16INK4a^{+} keratinocytes in aged skin, which exhibit a 5-fold higher mutational burden due to reduced DNA repair capacity.

In conclusion, the ongoing exploration of the relationship between aging and cancer, facilitated by scRNA-seq technologies, holds significant promise for advancing our understanding of tumor biology and enhancing patient outcomes. By addressing the existing challenges and fostering interdisciplinary collaboration, we can unlock new insights that pave the way for innovative diagnostic and therapeutic strategies. The future of cancer research lies in our ability to integrate diverse perspectives and methodologies, ultimately leading to more effective and personalized approaches to cancer prevention and treatment in the aging population. As we continue to navigate this complex field, the commitment to rigorous research and ethical considerations will be paramount in shaping the next generation of cancer care.

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Review

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Therapeutic potentials of mesenchymal stem cell-derived exosomes for major solid malignancies: A narrative systematic review

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SUMMARY: Treatments for solid tumors, the most common malignant neoplasms, are often confounded by tumor microenvironments that impede the achievement of uniform anti-tumor effects throughout the entire malignant mass, which contributes to recurrence and progression, negatively impacting clinical outcomes. Improved treatment methods for solid malignancies are therefore needed. Mesenchymal stromal cells (MSCs) have been investigated for treatments for various types of solid tumor cancers due to their ability to target tumor cells with similar cell surface protein profiles. MSC-derived exosomes (MSC-Exos) elicit many of the tumor cell responses produced by MSC with no potential for differentiation and reduced risks of adverse effects. We surveyed the literature and clinical trials registries to identify studies investigating MSC-Exo-based anti-cancer therapies for gastric cancer, colorectal cancer, breast cancer, lung cancer, brain cancer, pancreatic cancer, and urological malignancies, and summarize the results of relevant studies herein to provide a comprehensive description of the therapeutic effects and potential clinical applications of MSC-Exos for the treatment of solid tumor malignancies. We include a summary of relevant clinical trials performed to date in an attempt to assess the data available regarding MSC-Exo safety, and propose future efforts regarding the requirements for transitioning forward from phase-1, 2 trials.

Keywords: exosome, solid malignant tumor, mesenchymal stem cell, extracellular vesicle, epithelial-to-mesenchymal transition

1. Introduction

Solid malignant tumors, the most common malignant neoplasms, account for about 80% of the most prevalent human cancers (*1*). The treatment of solid tumors often involves multiple therapeutic strategies, including surgery, chemotherapy, radiation, targeted therapy, and immune therapy. The effectiveness of such treatments depends on a number of factors associated with the tumor microenvironment (TM) that impede the achievement of uniform anti-tumor effects throughout the entire malignant mass (*2*). The resulting therapeutic inadequacies can contribute to the persistence of residual tumor cells, leading to recurrence and progression, which negatively impact clinical outcomes (*3,4*).

The physical structure of solid tumors contributes to the reduced effectiveness of chemotherapy, internal radiotherapy, targeted therapy, and immunotherapies (*5*). The dense tissue and abnormal vascularization of solid tumors can affect the penetration and distribution of therapeutic molecules within the malignant cell mass (*2,6*). Consequently, the high dosages required to overcome these structural obstacles often result in toxicity due to the limited specificity and moderate rates of off-target effects of chemotherapy drugs (*7,8*). Primary and secondary resistance to immunotherapies and tumor-targeted therapies also occurs due to both genetic and non-genetic mechanisms that often contribute to tumor cell plasticity (*9,10*). Heterogeneity in the TM can also result in the formation of hypoxic regions that locally inhibit free radical formation, which plays critical roles in the mechanisms of action of radiotherapy, immunotherapy, and many chemotherapy drugs (*5,11-13*). Improving the effectiveness of solid tumor treatments will likely require overcoming these challenges to varying extents.

Mesenchymal stromal cells (MSCs) are multipotent stem cells capable of self-renewal and cell differentiation (*14*). MSC-based cancer therapies seek to utilize the tumor-homing properties of MSCs and their selective inhibition of tumor growth and progression (*15-17*). However, MSC can contribute to tumor progression under certain circumstances (*18-20*). MSC-derived exosomes (MSC-Exos) have been shown to elicit many of the tumor cell responses produced by MSC (*21-23*) with no potential for differentiation and reduced risks of adverse effects (*21,24*), suggesting that the use of MSC-Exo-based biologicals might represent an important new adjuvant to current treatment strategies (*21,25-27*). This review summarizes the current body of research toward the application of MSC-Exos for the anti-cancer treatments, and discusses the efficacy and safety of MSC-Exo-based treatments for solid tumor cancers.

2. Literature search

The primary goal of our review was to summarize studies investigating the use of MSC-Exos for anti-tumor treatments of the following solid tumor malignancies: gastric cancer, colorectal cancer, breast cancer, lung cancer, brain cancer, pancreatic cancer, or urological cancer. We searched the PubMed database for literature published in English between January 1, 2005 and March 1, 2025 (the approximate date of the final revisions of this manuscript) to identify studies investigating the use of MSC-Exos for anti-cancer therapies using the following search criteria: (mesenchymal stem cells [Title/Abstract]) AND ((extracellular vesicles [Title/Abstract]) OR (exosomes [Title/Abstract])) AND ((solid cancer [Title/Abstract]) OR (gastric cancer [Title/Abstract]) OR (colorectal cancer [Title/Abstract]) OR (breast cancer [Title/Abstract]) OR (lung cancer [Title/Abstract]) OR (brain cancer [Title/Abstract]) OR (pancreatic cancer [Title/Abstract]) OR (urological cancer [Title/Abstract])). The search retrieved 148 results. Endnote software (Clarivate; Philadelphia, USA) was used to search for duplicate publications, and no duplicates were identified. The title, abstract, and full-text articles were subjected to manual examination. Articles meeting any of the following criteria were excluded: (1) investigations of extracellular vesicles other than exosomes, (2) investigations involving exosomes not derived from mammalian MSC, (3) studies examining exosomes derived from tumor cells, and (4) studies using exosomes to develop diagnostic or prognostic biomarkers. We excluded 62 articles. We searched the references of the included review articles to identify additional relevant publications, which resulted in the selection of an additional 22 articles for screening, of which 20 were excluded, leaving a total of 88 articles included.

We performed a search of the US National Library of Medicine clinical trials database (*clinicaltrials.gov*) using "cancer AND exosome AND mesenchymal" to identify relevant clinical trials, and retrieved 14 results. The results were screened manually to determine whether any met the exclusion criteria above, and 5 trials were excluded. We searched the Chinese clinical trials database (*chictr.org.cn*) using "cancer" as the keyword in the public title and "exosome" as the keyword in the scientific title, and 34 results were retrieved. The results of each search were screened manually, and all of these trials were excluded. Therefore, a total of 9 clinical trials were included. All authors participated in the screenings of published articles and clinical trial descriptions, with disagreements resolved by discussion and consensus.

3. Exosome biogenesis and intercellular transfer

Having become a major focus of research over the past decade, studies continue to reveal more information regarding the complex roles exosomes play in intercellular communication, tissue regeneration, and cancer (*28-31*). Almost all human cell types are capable of producing exosomes, and MSCs produce exosomes *via* the same pathways used for exosome production in differentiated cell types (*32*). Figure 1 represents exosome production, exosome release, and exosome internalization. The production of exosomes and the transfer of their molecular cargo allow cells to influence the growth and physiological state of other cells through biochemical signaling. Much of the details of exosome biogenesis and the regulation of its complex pathways lie outside the scope of our review. Herein, we provide a summary of those features relevant to the application of MSC-Exos to the treatment of solid tumors.

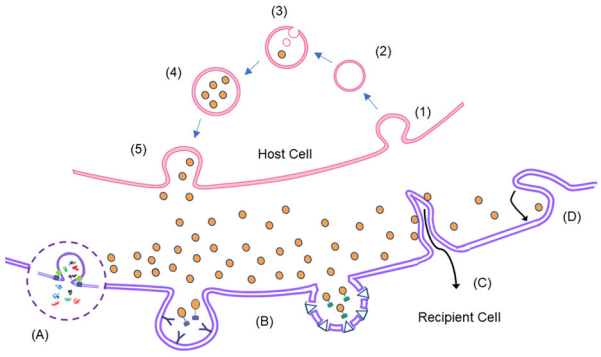

Figure 1. Features of exosome biogenesis and intercellular transfer. Exosome biogenesis is initiated by (1) invagination of the plasma membrane that ultimately forms (2) the early endosome. During endosome maturation, various biomolecules (yellow) are sorted into intraluminal vesicles (ILVs), which are visible in (3) the late endosome. The accumulation of ILVs marks the formation of (4) the multivesicular body (MVB). The MVB fuses with the plasma membrane of the host cell, and (5) scission of the limiting membrane releases the ILVs into the extracellular space as exosomes. The internalization of exosomes into recipient cells can occur by (A) direct-contact membrane fusion (magnified inset), (B) receptor-mediated endocytosis facilitated by clathrin or caveolin, (C) filopodia-mediated endocytosis, or (D) macropinocytosis.

3.1. Exosome production and secretion

Exosomes are produced and released from host cells under homeostatic and pathological conditions *via* constitutive and inducible pathways. Inducible exosome secretion is activated by various stimuli, including heat shock, hypoxia, DNA damage, increased intracellular calcium, and lipopolysaccharide exposure (*33*). Exosome biogenesis is initiated by invagination of the plasma membrane (PM) progressing to form an intracellular vesicle with a phospholipid bilayer membrane known as the early endosome. Pathways known to produce endosomes include caveolin-dependent endocytosis (*34*), clathrin-mediated endocytosis (*35*), and a less-understood third pathway occurring independent of both caveolin and clathrin (*36*). In the late endosome, asymmetric distribution of cell membrane lipids, especially ceramide and cholesterol, contribute to membrane curvature in another invagination process (*37-40*) in which the endosomal sorting complex required for transport (ESCRT) proteins drive further inward bulging and membrane fission to form the intraluminal vesicle (ILV) (*41*), which is simultaneously loaded with biochemical cargo. Upon formation of multiple ILVs, the late endosome is known as the multivesicular body (MVB). Exosome secretion occurs as the MVB fuses with the host cell membrane during exocytosis, and the ILVs become exosomes upon release into the extracellular space, as reviewed by Arya *et al*. (*42*).

Despite the lack of an international consensus, a number of proteins have been identified as exosome markers, with a list of key proteins including CD9, CD63, CD81, tumor susceptibility gene 101, apoptosis-linked gene 2-interacting protein X (Alix), ADP ribosylation factor 6 (Arf6), Flotillin, and the heat shock proteins Hsp70 and Hsp90 (*43,44*). MSC-Exos also carry the MSC-specific markers CD29, CD73, CD90, CD44, and CD105 (*45*). The tetraspanins CD9, CD63, and CD81 function in molecular cargo sorting and cell adhesion. Alix and tumor susceptibility gene 101 function in ILV formation (*46*). Arf6 functions in the formation of ILVs, cargo sorting, and exocytosis. Flotillins function in membrane trafficking, and HsP80 and HsP90 function as molecular chaperones, protecting the secondary and tertiary structures of trafficked proteins (*47*).

RabGTPases function as molecular switches in intracellular transport that allow shifting between different downstream pathways (*48*). Rab7 mediates the transport of endosomes along cytoskeleton fibers in early and late endosome development (*49*), and contributes to multiple steps in endosome development and trafficking (*50*). Three different pathways mediated by RabGTPases are known to contribute to exosome secretion. Rab11 mediates secretion *via* a ubiquitin-dependent ESCRT pathway (*51,52*). Rab27 mediates a ubiquitin-independent ESCRT pathway (*52,53*), in which Rab27 mediates fusion of the MVB with the PM (*54,55*). Rab31 mediates a third pathway in which ceramides and cholesterol associate with Flotillins to recruit Rab31 to late endosomes, resulting in exosome secretion *via* a Rab27-mediated ESCRT-independent pathway (*56,57*). Attachment of the MVB to the PM is mediated by Rab27 and Rab35 (*55,58*). Rab5 and Rab13 mediate the secretion of ILVs as exosomes (*59,60*) with ARF6 mediating membrane remodeling at the site of MVB attachment/fusion through ATP-dependent contraction of actin-myosin fibers in membrane fission (*54,61*).

Though the regulatory mechanisms of these pathways have not been fully elucidated, key elements have been identified, and alterations in one pathway are known to influence the activity of others. Rab5 is required for early endosome formation (*62*), and the transition from early to late endosome is regulated by Rab5 and Rab7, with Rab7 remaining localized with the endosome/MVB through subsequent steps in exosome development (*49*). The recruitment of the TBC1D2B protein by Rab31 inhibits the activity of Rab7 (*56*). While Rab7 is required for Rab27 mediated exosome secretion *via* the ubiquitin-independent ESCRT pathway (*63*), the inhibition of Rab7 also blocks lysosome-MVB fusion (*50*), which shifts the fate of the pathway away from autophagy and toward Rab27 mediated exosome secretion occurring independent of ESCRT pathways (*56*), demonstrating a link between the secretion and recycling pathways.

Though much has been learned regarding exosome biology as presented above, significant variation in exosome biogenesis has been shown to occur based on host cell type and physiological state, with differences in the activities RabGTPases contributing substantially to the variation observed (*57,64,65*). Examples of such variations are exemplified in the results of one study which found that Rab27a and Rab27b functioned in different steps of exosome secretion in squamous carcinoma cells (*55*), whereas studies of human umbilical vascular endothelial cells and bone marrow (BM)-derived mast cells found that Rab27b mediated exosome secretion while Rab27a did not (*64,65*). Such variation is likely influenced by host-specific differences in the predominating secretory pathway, and highlights the overlapping roles of RabGTPases in MVB formation, vesicular secretion, and lysosomal degradation.

3.2. Exosome cargo sorting

The transfer of ILV molecular cargo from host cell to recipient cells is the means by which exosomes function in intercellular communication. The molecular cargo profile of exosomes varies based on host cell type (*66*) and physiological status (*67*). Unique cell-of-origin protein and nucleic acid profiles often remain identifiable in exosomes produced by malignant cells, a property that has led to a great deal of research investigating the use of exosomes as diagnostic markers for cancers originating from a wide range of cell types (*68,69*). Though a

clear understanding of the pathways and regulatory mechanisms involved in cargo sorting is lacking (*42*), a vast array of cargo molecules and key components of the cargo sorting machinery have been identified. Most exosomal cargo sorting appears to occur during ILV production, with lipid- and protein-mediated mechanisms contributing to both processes.

Exosomal cargo can consist of a wide variety of hormones, lipids, cytokines, chemokines, growth factors, extra-nuclear and mitochondrial DNA, messenger RNA, and various non-coding RNAs, including small nuclear RNA, microRNA (miRNA), circular RNA (circRNA), PIWI-interacting RNA, and long non-coding RNA (lncRNA) (*33,70*). Consequently, though the basic physical structure of exosomes is homologous across different cell types, the lipid, protein, and nucleic acid profiles of exosomes from a single cell can vary greatly. The lipid bilayer structure of the exosomal membrane protects the cargo from degradation by sequestering it away from the proteases, lipases, and nucleases commonly found in the extracellular environment, and the self-proteins embedded in the membrane aid in preventing its destruction by host immune cells.

3.2.1. Lipid cargo sorting

Lipids play a key role in the formation of the physical structure of endosomes and ILVs, as well as contributing to exosome function (*37,38*). The ILV membranes contain a variety of lipids, with cholesterol, sphingomyelin, phosphatidylcholine, and phosphatidylserine, being most abundant (*38,71*). Most research regarding endosomal lipids has focused on enrichment of cholesterol. The oxysterol binding protein related protein 1L and steroidogenic acute regulatory protein related lipid transfer domain protein are known to mediate the transfer of cholesterol from the endoplasmic reticulum (ER) to the endosome (*72,73*). In a recent study, CD63 was shown to mediate the sorting of cholesterol into ILVs, resulting in significant cholesterol enrichment (*74*). Flotillins associate with cholesterol in lipid rafts in the cell membrane, which participate in endocytosis (*75*). Flotillins are also associated with cholesterol in ILVs, but the sorting mechanism through which this occurs remains unclear (*76*). Lipid enrichment of the ILV membrane increases lipophilicity, thereby enhancing the fusion of exosomes with recipient cell membranes (*77,78*).

3.2.2. Protein cargo sorting

Cargo proteins are trafficked from the *trans*-Golgi network (TGN) to the endosome, with the proteins involved in cargo sorting arising from the TGN as well (*79*). Important mediators of the endosomal cargo sorting of proteins include ESCRT-0, -I, -II, and -III. The subunits of these ESCRT proteins bind in sequential fashion to the limiting membrane of newly forming endosomes (*41*). ESCRT-0, -I, and -II subunits contain ubiquitin-binding domains that are used to sort ubiquitinated proteins in ILVs (*80*). Though some of these proteins are de-ubiquitinated in the endosome, approximately 15% of exosomal proteins can remain ubiquitinated (*81*). Other post-translational modifications that have been implicated in ESCRT-dependent sorting include myristoylation, ISGylation, glycosylation, and SUMOylation (*82,83*). Following translation on the ER, these modifications occur in the TGN, with ESCRT recognition resulting in sorting into developing ILVs (*84*). Other proteins sorted in ESCRT-dependent secretion include Programmed cell death protein ligand 1(PD-L1) (*85*), Major Histocompatibility Complex II (MHC II) (*86*) , CD63, Syntenin, Syndecan 1 (*63*), β-Integrin (*87*), Fibronectin (*88*), and Protease-activated receptor-1 (PAR1) (*89*).

Less is known regarding protein cargo sorting in ESCRT-independent exosome secretion. The lysosome-associated membrane protein 2 isoform A (LAMP2A) protein binds the KFERQ consensus peptide motif in other proteins, which account for approximately 20% of membrane proteins in the human proteome. A recent study showed that LAMP2A mediates the sorting of KFERQ-containing proteins into early endosomes, ultimately localizing to ILVs and being secreted *via* the Rab31-mediated pathway (*90,91*). This cargo sorting mechanism requires the cytosolic KFERQ binding protein HSC70, as well as CD63, Alix, Syntenin-1, and ceramides, and results in Flotillin enrichment (*91*). Flotillins associate with cholesterol and ceramide rich membranes (*92*), and recruit Rab31 in early endosome production (*56*). LAMP2A-mediated sorting also enriches exosomes with the hypoxia-inducible transcription factor 1 α (HIF1A) protein (*91*), which contains the KFERQ motif (*93*). HIF1A is upregulated in cells under hypoxic conditions that are known to contribute to resistance to anti-cancer treatments in solid tumors (*94*). A separate study found that EGFR, which also contains the KFERQ motif (*95*), was enriched in exosomes produced *via* the Rab31-mediated pathway (*56*).

Exosomes are enriched with the tetraspanins CD9, CD37, CD63, CD81, and CD82 (*96*), with CD63 being the most abundant regardless of the biogenesis pathway used (*97,98*). Despite the abundance of tetraspanins in exosomes, their contributions to protein sorting in exosome biogenesis are largely unclear. CD63 is trafficked to early endosomes in ESCRT-dependent exosome production, yet is required for ESCRT-independent LAMP2A-Rab31-mediated secretion (*91*). In a recent study, investigators showed that CD9 inhibited the localization of CD63 to early endosomes in ESCRT-independent exosome production (*99*). Further experiments showed that the CD9-mediated reduction of CD63 localization was reversed by blocking endocytosis using three separate inhibitors with different mechanisms

of action (*99*). Proteins that are sorted into ILVs by tetraspanins *via* ESCRT-independent pathways include CD10, β-Catenin, Ezrin, RAC (*96*), PD-L1 (*85*) PMEL17 (*100*), and LMP1 (*101*). Exosomes are also enriched with HSP70, HSP90, and HSP20, but the mechanism by which these are sorted is not clear.

3.2.3. Nucleic acid cargo sorting

Double-stranded genomic DNA (*102*), single-stranded DNA (*103*), mitochondrial DNA (*103*), and viral DNA (*104*) have been identified in exosomes. DNA molecules have been identified both encapsulated and exposed on the surface of exosomes (*104*). The sorting mechanisms and functions of exosomal DNA remain unclear, and it is the least studied aspect of endosomal trafficking in cells under homeostatic conditions. Exosomes can also contain a wide variety of cellular RNA molecules including mRNAs, lncRNAs, sRNAs, miRNAs, circRNAs, PIWI-interacting RNA, vault RNAs (vtRNAs), Y RNAs, transfer RNA fragments, small nucleolar RNAs (snoRNAs), ribosomal RNA fragments, and mitochondrial RNAs (*105,106*). Despite the wide variety of RNA species that have been identified in exosomes, relatively little is known regarding the mechanisms by which these molecules are sorted into exosomes. Passive RNA loading is dependent on the intracellular concentration of RNAs. Though selective sorting of RNAs are active processes, the quantity of molecules sorted into exosomes is also influenced by the intracellular concentration of the RNA species involved .

The sorting of many RNAs has been shown to be mediated, at least in part, by sequence motifs contained within them. Exosomes are enriched with miRNA containing the CGGGAG sequence motif, whereas miRNAs with AUUA, AGAAC, and CAGU motifs are most often limited to cells (*107*). In addition, miRNA isoforms with 3' uridylation are over-represented in exosomes (107). The sorting of mRNAs into exosomes is likely mediated by the primary and secondary structures of the transcript. A 25 nucleotide sequence was found to be enriched in exosomal mRNAs. This sequence forms a stem-loop structure which contains a core CUGCC sequence and an miR-1289 binding site (*108*). However, many of the exosomal mRNA containing these motifs have been found to exist as non-functional fragments of transcripts, and their function in recipient cells is unclear (*109*). The enrichment of exosomes with circRNAs has been proposed to be mediated by the GMWGVWGRAG degenerate sequence motif in hepatoma cells (*110*). At least 21 RNA motifs have been identified that mediate the sorting of RNAs by association with lipid rafts in the limiting membrane of developing ILVs, constituting a passive RNA-specific sorting mechanism (*111*).

A number of heterogeneous nuclear ribonucleoproteins (hnRNPs) have been identified that mediate the sorting of miRNAs with specific motifs, including the following hnRNPs and motif sequences: hnRNPA2B1 (GGAG, AGG, UAG or A/G-rich sequences); hnRNPK (UC_3–$4[U/A]_2$); hnRNPC1 (AU-rich sequences); hnRNPG (CC[A/C]-rich); hnRNPH1 (GGGA); and hnRNPQ (GGCU, AYAAYY, or UAUYRR) (*107,109,112*). The sorting of lncRNAs has also been shown to be mediated by hnRNPA2B1 in a number of cancer cell lines (*110,113,114*). This sorting mechanism is dependent on the GGAG motif in the lncRNAs in at least some of these cells (*115*). At least 14 other proteins that function in other cellular processes have also been shown to contribute to the sorting of exosomal RNAs, as reviewed in Fabbiano *et al*. (*112*). Nine of these do so through the recognition of RNA sequence motifs. Among these is the Argonaut 2 (AGO2) protein, which functions in the miRNA induced silencing complex (miRISC)-related pathway, and mediates sorting through the recognition of GCACUU and G-rich sequences in various RNAs, such as miR-320a, miR-100, and miR-let-7a (*116*). One of the RNA sorting proteins for which an RNA motif has not been detected is Alix, an exosome biomarker that also functions as an ESCRT accessory protein in ILVs (*117*). Alix has been shown to contribute to the sorting of miR-24, miR-31, miR-125b, miR-99b, miR-221, miR-16, and miR-451 (*118*).

3.3. Exosome uptake

Fusion of the MVB with the PM of the host cell and scission of the limiting membrane of the MVB results in the release of ILVs into the extracellular space as exosomes. Upon contact with other cells, integrins in the exosome membrane interact with recipient cell adhesion molecules to facilitate attachment (*119*). Exosomal membrane proteins can stimulate phagocytosis by immune cells and influence immuno-surveillance *via* the detection of antigens presented in MHC proteins in the exosome membrane (*120*). Exosomal membrane surface proteins may act as ligands interacting with cell-surface receptors of the recipient cell, which can contribute to the target-cell specificity demonstrated by exosomes *in vivo* (*121*). Such interactions may also result in juxtacrine transduction of signaling pathways (*122*) or localize exosomes to sites of endocytosis machinery in the PM of the recipient cell (*123*).

As shown in Figure 1, the internalization of exosomes into recipient cells can occur by (A) direct-contact membrane fusion (*124*), (B) receptor-mediated endocytosis facilitated by clathrin or caveolin (*125,126*), (C) filopodia-mediated endocytosis (*119,127*), or (D) macropinocytosis (*126*). Though membrane fusion mediated uptake results in the release of exosomal molecular cargo (Figure 1, magnified inset), endocytosed exosomes remain intact and are encapsulated in endosomes. The fate of internalized endosomes is primarily dictated by the cell type and physiological state of the recipient cell, but the various mechanisms

involved are poorly understood. These endosomes may fuse with lysosomes for degradation or antigen processing, or may become permeabilized by recipient cell lipases (*128*). Encapsulated exosomes can fuse with the limiting membrane of the endosome (*127,129*), but it is not entirely clear whether this releases exosomal cargo (*128*). The endosome may localize to the ER, making the exosomal cargo available for trafficking elsewhere within the recipient cell, but evidence of this is lacking. Alternatively, the encapsulated exosomes and functional cargo may be secreted by the recipient cell (*130*).

4. Effects of MSC-Exos on cancer cells and potential therapeutic effects

MSCs primarily exert their regulatory functions through paracrine pathways, and MSC-Exos have been implicated in various tumor-related processes, including tumor cell proliferation, apoptosis, metastasis, and treatment resistance (*131,132*). Studies using MSC-Exos for miRNA delivery have reported both the enhancement and inhibition of tumor cell proliferation, with one such study reporting that miR-130b-3p, promoted cell proliferation, migration, and invasion in human lung cancer cell lines (*133*). Similar tumorigenic effects have been observed in other types of malignant tumors, including renal, breast, and nasopharyngeal cancers, where specific miRNAs within MSC-Exos contributed to carcinogenesis (*23*). *In vitro* studies have demonstrated that BM-derived MSCs (BM-MSCs) enhance vascular endothelial growth factor (VEGF) expression in tumor cells, activating the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway to exert pro-cancer effects (*134*). Factors present in exosomes, such as cytokines and adhesion molecules, may also promote tumor development, as reviewed in Moeinzadeh *et al*. (*135*).

By contrast, MSC-Exos have also demonstrated anti-cancer properties. The IRF2/INPP4B pathway, which promotes apoptosis in acute myeloid leukemia cells, was inhibited by BM-MSC-Exos carrying miR-222-3p (*136*). Exosomes produced by human adipocyte (AT)-derived MSCs (hAT-MSC-Exos) and umbilical cord-derived MSCs (hUC-MSC-Exos) also demonstrate anti-cancer effects. The delivery of miR-145 by AT-MSC-Exos was shown to inhibit prostate cancer cell proliferation by reducing Bcl-xL activity and promoting apoptosis through the caspase-3/7 pathway (*137*). Furthermore, MSC-Exos are linked to the epithelial-to-mesenchymal transition (EMT), enhancing tumor cell migration and invasion (*138*), and hUC-MSC-Exos have been shown to induce EMT *via* the ERK pathway in breast cancer cells, contributing to tumor progression and metastasis (*139*).

MSC-Exos have also been shown to promote angiogenesis in mouse model experiments by inducing vascular endothelial growth factor (VEGF) expression and stimulating MSC-Exos-mediated cell-cell interactions that contribute to tumor progression (*140*). A previous study demonstrated that exosomes isolated from the conditioned medium of BM-MSCs can transfer various pro-angiogenic miRNAs to human umbilical vein endothelial cells *in vivo*, thereby enhancing angiogenesis and facilitating communication between stem cells and endothelial cells (*141*). Wnt proteins are potent angiogenic factors, and their signaling pathways significantly influence angiogenesis and vascular remodeling. MSC-Exos have been shown to stimulate fibroblast proliferation and promote angiogenesis *in vitro* (*142*). The anti-vascular remodeling properties of MSC-Exos have been corroborated by several studies. One such study revealed that exosomal miR-16, which is enriched in MSC-Exos, inhibits angiogenesis in breast cancer cells by downregulating VEGF and CD31 expression and modulating the *mTOR/HIF-1α/VEGF* signaling axis (*143,144*).

MSC-Exos possess immunomodulatory capabilities similar to those of MSCs. Functioning not only as natural antigen carriers, but also act as antigen presenters, MSC-Exos regulate both direct and indirect antigen presentation to stimulate both adaptive and innate immune responses. Furthermore, exosomes facilitate the transfer of antigenic peptides or bioactive molecules, thereby influencing other immune cell subpopulations (*145*). Notably, BM-MSC-Exos have been shown to downregulate interferon (IFN)-γ expression in dendritic cells (DCs) and T cells (*146*). Other research has also indicated that BM-MSC-Exos can activate immature DCs, leading to increased secretion of IL-10, increased numbers of $Foxp3^+$ regulatory T cells (Treg), and the inhibition of inflammatory T helper cell responses (*147*). MSC-Exos derived from other sources also play roles in immune regulation. AT-MSC-Exos have been reported to inhibit the proliferation and activation of stimulated T cells (*148*), while hUC-MSC-Exos induce the expression of immunosuppressive cytokines through interactions with peripheral blood monocytes, which promotes the development of M2 macrophages (*149*).

Chemotherapy remains a cornerstone treatment for many solid tumors, yet the emergence of multidrug resistance in tumor cells presents a significant challenge to its efficacy. To develop effective strategies that can overcome this resistance, understanding its underlying mechanisms is essential. Recent evidence suggests that MSC-Exos play a pivotal role in promoting treatment resistance (*150*). Ji *et al*. (*151*) demonstrated that MSC-Exos can enhance tumor cell resistance to 5-fluorouracil *in vitro* and *in vivo* by activating the CaM-Ks/Raf/MEK/ERK signaling cascade and modulating MDR-related proteins, thereby preventing apoptosis. Furthermore, tumor dormancy, the process wherein cancer cells in metastatic sites remain in the G0 phase after the primary tumor's removal, is strongly linked to cancer recurrence, metastasis, and chemotherapy resistance. Phan and Croucher (*152*) have suggested that MSC-Exos from cancer cells can induce dormancy, which contributes

to cisplatin resistance. By contrast, MSC-Exos have also been shown to increase chemotherapy sensitivity in various types of cancer (*23*). Though AT-MSC-Exos have been shown to reverse cisplatin resistance in breast cancer cells (*153*), hUC-MSC-Exos have been shown to restore sensitivity to docetaxel and paclitaxel by regulating LAMC2 expression and the PI3K/Akt signaling pathway (*154*).

The EMT has also been implicated in chemotherapy resistance, and inhibiting the EMT represents a promising strategy for resistance reversal. Treatment with hUC-MSC-Exos containing miR-451a has been shown to suppress EMT *in vitro* by inhibiting disintegrin-metalloproteinase-10 (ADAM10) protein expression, thereby increasing the sensitivity of tumor cells to paclitaxel (*155*). Targeted therapies have been developed in which antibodies are used to target specific molecular pathways involved in chemoresistance. These include cetuximab and panitumumab, which target epidermal growth factor receptor (EGFR), as well as trastuzumab, which targets HER-2 (*156,157*). However, despite the significant improvements these therapies offer, resistance remains a major obstacle to long-term survival. MSC-Exos have been implicated in mediating resistance to targeted therapies. For example, changes in the contents of BM-MSC-Exos produced by acute myeloid leukemia malignancy have correlated with resistance to tyrosine kinase inhibitors (*158*). However, in chronic myeloid leukemia, treatment with hUC-MSC-Exos enhanced sensitivity to the tyrosine kinase inhibitor imatinib by promoting apoptosis through the activation of caspase-9 and caspase-3 (*159*).

The exploration of immune factors in the TM has opened new possibilities for immunotherapy, which directs the immune system to target cancer cells to promote cell cycle progression regulation, cell death, and the inhibition of metastasis and angiogenesis (*25,160*). However, a significant proportion of patients fail to respond to immunotherapy due to primary, adaptive, or acquired resistance (*161*). Recent research suggests that exosomes play an immunomodulatory role in the TM by influencing the activity of natural killer cells, T cells, and B lymphocytes (*162*). MSC-Exos have been shown to modulate immune cell functions, and alter the secretion of inflammatory factors, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In one study, MSC-Exos promoted the differentiation of monocytic myeloid-derived suppressor cells into immunosuppressive M2 macrophages (*163*). Future studies are needed to clarify whether MSC-Exos have an overall immunosuppressive or immuno-stimulatory role in the TM, and to establish how they might influence resistance to immunotherapy. In pancreatic ductal adenocarcinoma (PDAC), BM-MSC-Exos carrying galectin-9 small interfering RNA (siRNA) and oxaliplatin prodrugs have been found to induce immunogenic cell death, and reverse immunosuppression in the TM by reducing M2 macrophage polarization and recruiting cytotoxic T lymphocytes, suggesting that enhancement of immunotherapy (*164,165*).

MSC-Exos have also gained attention as promising carriers for biomolecules and chemical agents in solid-tumor malignancies due to their role intracellular communication, low immunogenicity, minimal toxicity, high bioavailability, evasion of immune cell phagocytosis, and potential to cross biological barriers, as review by Lin *et al*. (*23*). Engineered MSC-Exos can encapsulate therapeutic miRNAs, proteins, and drugs, offering novel therapeutic avenues. Mechanistically, MSC-Exos can deliver specific miRNAs that promote caspase activity and induce apoptosis by inhibiting the expression of drug efflux proteins, such as the multidrug resistance-1 (MDR1) protein (*166*). In addition, MSC-Exos provide a platform for delivering siRNAs, enhancing drug sensitivity and therapeutic outcomes in various cancer treatments (*167*). Their potential in chemotherapy drug delivery is also significant, as MSC-Exos can improve the effects of drugs inhibiting tumor growth and improve tumor site targeting precision compared to that of traditional chemotherapy (*168*). Furthermore, MSC-Exos can cross critical physical barriers, such as the blood-brain barrier, likely by endocytosis, thus facilitating the delivery of therapeutic drugs and thereby increasing chemotherapy drug concentrations in challenging tumor site locations, as reviewed by Sen *et al*. (*169*). We have included a summary of our discussion of the effects of MSC-Exos and miRNAs on solid tumor malignancies in Figure 2.

4.1. Breast cancer

Recent studies have highlighted the multifaceted role of MSC-Exos in breast cancer, particularly in influencing angiogenesis, cell proliferation, migration, immune evasion, and chemotherapy resistance. MSC-Exos regulate angiogenesis primarily through the delivery of specific miRNAs. Treatment with BM-MSC-Exos containing miR-16 reduces VEGF expression, which inhibited angiogenesis and tumor progression in 4T1 breast cancer cells (*143*). Further research also demonstrated that treatment with BM-MSC-Exos carrying miR-100 downregulated VEGF expression by modulating the *mTOR/HIF-1α* axis, which also suppressed angiogenesis *in vitro* (*170*).

In addition to their role in angiogenesis, MSC-Exos also exhibit anti-tumor properties by regulating gene expression. Treatment with MSC-Exos containing miR-148b-3p and miR-145 inhibited breast cancer cell proliferation by downregulating the expression of the oncogenes *erb-b2 receptor tyrosine kinase 2* (*ERBB2*), *tripartite motif containing 59* (*TRIM59*), *matrix metallopeptidase 9* (*MMP9*), *Rho associated coiled-coil containing protein kinase 1* (*ROCK1*), and *tumor protein p53* (*TP53*) oncogenes (*171*). The treatment of the breast cancer cell line MCF-7 with MSC-Exos containing

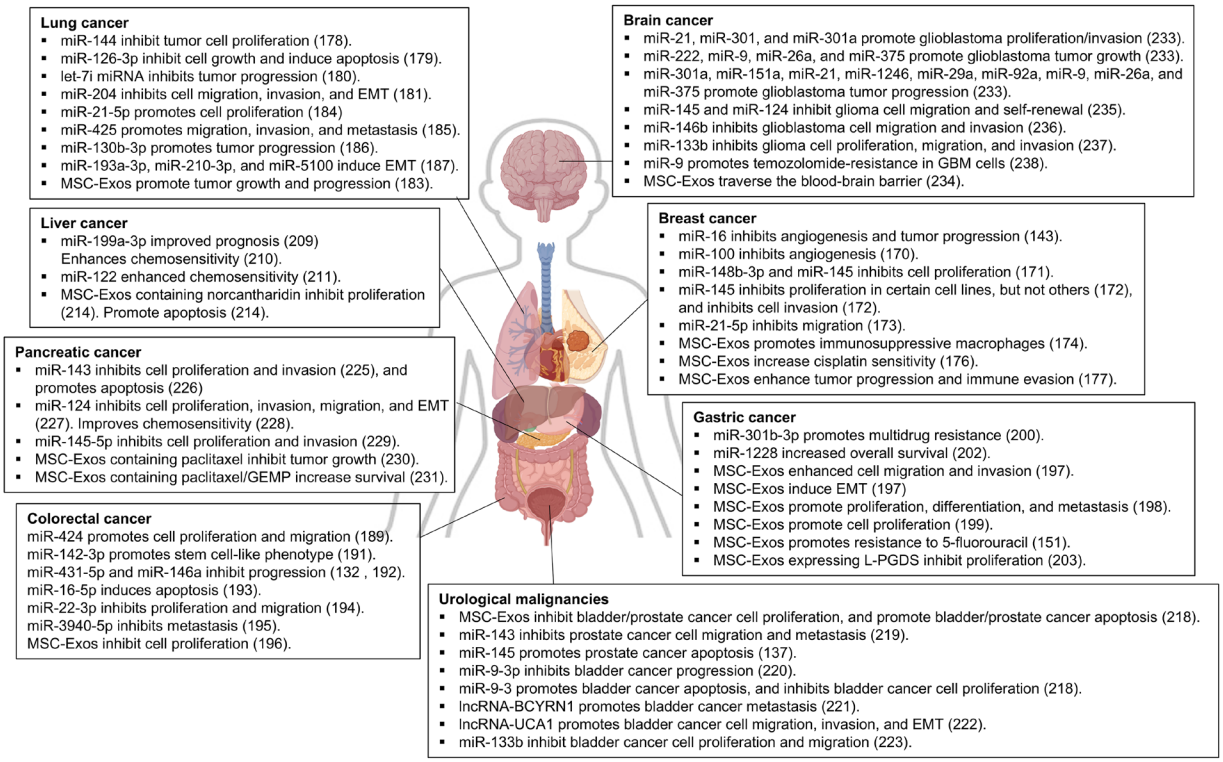

Figure 2. Visual summary of the effects of mesenchymal stromal cell-derived exosomes (MSC-Exos) and non-coding RNAs on solid tumor malignancies. The effects of various miRNAs, lncRNAs, and MSC-Exos on brain cancer, lung cancer, breast cancer, liver cancer, gastric cancer, colorectal cancer, pancreatic cancer, and urological malignancies are presented to summarize both the pro-tumor and anti-tumor effects of each, as discussed in section *4. Effects of MSC-Exos on cancer cells and potential therapeutic effects*.

miR-145 inhibited *membrane mucin-1* (*MUC1*) gene expression, which is closely associated with metastasis, and suppressed cell growth. By contrast, miR-145 did not suppress cell growth in the synonymous breast cancer cell lines MDA-MB-231 and LM2-4142, but it did inhibit cell invasion in both (*172*). Similarly, MSC-Exos inhibited *zinc finger protein 367* (*ZNF367*) gene expression through a miR-21-5p-mediated mechanism, which suppressed the migration of breast cancer cells (*173*). These findings suggest that MSC-Exos containing selected miRNAs can regulate multiple pathways to contribute to the inhibition of tumor progression and metastasis.

MSC-Exos can contain immune-regulatory factors, including transforming growth factor (TGF)-β, that influence various signaling proteins that drive *Programmed cell death 1 ligand 1* (*PD-L1*) gene overexpression in BM monocyte precursors. This leads to the differentiation of immunosuppressive macrophages, creating a tumor-favorable immune environment that ultimately accelerates cancer progression (*174*) by contributing to the immune evasion properties of breast cancer cells (*175*). These results demonstrate the importance of PD-L1 inhibition as a target for enhancing anti-tumor immunity in breast cancer. In addition, MSC-Exos have been shown to increase cisplatin sensitivity by downregulating *solute carrier family 9* (*SLC9A1*) gene expression and inactivating the Wnt/β-catenin pathway in breast cancer cells, representing a possible means of overcoming chemotherapy resistance (*176*). However, the effects of MSC-Exos on breast cancer progression is context-dependent, and their effects can vary based on the cell type of origin. For instance, though hUC-MSC-Exos inhibit breast cancer development, AT-MSC-Exos enhance tumor progression and immune evasion (*177*). These results underscores the need to further characterize the properties of exosomes produced by MSC derived from different source cell-types in order to bolster the development of MSC-Exo based therapeutics for breast cancer.

4.2. Lung cancer

Understanding the role of MSC-Exos in lung cancer is crucial, as these exosomes significantly influence the development and progression of this aggressive malignancy. Advances in this field could pave the way for new therapeutic strategies. BM-MSC-Exos carrying miR-144 have been shown to target the cell cycle proteins cyclin E1 (CCNE1) and cyclin E1 (CCNE2), which inhibited colony formation and the proliferation of lung cancer cells (*178*). Exosomes rich in miR-126-3p have been shown to suppress lung cancer cell growth and induce apoptosis by downregulating non-receptor

protein tyrosine phosphatase 9 (PTPN9) (*179*). The let-7i miRNA inhibits lung cancer progression through the *KDM3A/DCLK1/FXYD3* axis, which functions in the regulation of ion transport (*180*), and MSC-Exos containing miR-204 inhibit cell migration, invasion, and the EMT in lung cancer cells *via* the *KLF7/AKT/HIF-1α* axis (*181*). MSC-Exos containing specific circRNA or miRNAs, such as circRNA-100395, miR-631, miR-598, and miR-320A, also exhibit potent anti-tumor effects, as review by Feng *et al*. (*182*).

MSC-Exos have also been shown to contribute to the lung cancer microenvironment through processes that support tumor growth and progression (*183*). MSC-Exos enriched with miR-21-5p suppress *SMAD7* gene expression thereby promoting lung cancer cell proliferation (*184*). Treatment with BM-MSC-Exos containing miR-425 suppresses *cytoplasmic polyadenylation element binding protein 1* (*CPEB1*) gene expression, which contributes to cell migration, invasion, and lung metastasis in lung cancer cells (*185*). Treatment with hUC-MSC-Exos enriched with miR-130b-3p also promote lung cancer progression through the *FOXO3/NRF2/TXNRD1* axis in the human lung cancer cell lines H292 and H1299 (*186*), and hypoxic BM-MSC-Exos containing miR-193a-3p, miR-210-3p, and miR-5100 induced the EMT *in vivo* in a mouse syngeneic lung cancer tumor model by activating the signal transducer and activator of transcription 3 (STAT3) signaling pathway, which promotes invasion and metastasis in lung cancer (*187*). These results highlight potential targets for MSC-Exo based therapeutics in future studies.

4.3. Colorectal cancer

Studies have reported that MSC-Exos can promote the tumor proliferation, migration, and invasion properties of colorectal cancer cells through the delivery of specific miRNAs (*188*). MSC-Exos carrying miR-424 enhance colorectal cancer cell proliferation and migration, while inhibition of miR-424 induces apoptosis in colorectal cancer cell lines (*189*). The expression of miR-424 and TGFBR3 are linked to tumor differentiation, infiltration depth, TNM staging, vascular invasion, lymph node metastasis, and distant metastasis, making miR-424 a potential therapeutic target for the treatment of colorectal cancer (*190*). MSC-Exos delivering miR-142-3p promote a stem cell-like phenotype in colorectal cancer cells, thereby exacerbating disease progression (*191*). The results of these studies highlight the value of these miRNA as potential targets for MSC-Exo based therapies through the enrichment of MSC-Exos with siRNAs designed to silence the expression of miR-424 and miR-142-3p.

MSC-Exos enriched with certain other miRNAs can exhibit inhibitory effects on colorectal cancer. MSCs containing miR-431-5p have been shown to suppress colorectal cancer progression by downregulating *peroxiredoxin 1* (*PRDX1*) gene expression (*192*), and MSC-Exos containing miR-16-5p were shown to selectively suppress the expression of integrin subunit alpha 2 (ITGA2) mRNA, inducing apoptosis in colorectal cancer cells (*193*). In addition, miR-22-3p in MSC-Exos has been shown to downregulate expression of the *RAS* family oncogene *RAP2B*, which inhibited the PI3K/AKT signaling pathway, reducing both the proliferation and migration of colorectal cancer cells (*194*). MSC-Exos containing miR-3940-5p have been shown to inhibit colorectal cancer metastasis by inhibiting *integrin α6* (*ITGA6*) gene expression (*195*), whereas the miR-146a/*SUMO1* axis contributes to both the alleviation of colitis and inhibition of colorectal cancer progression (*132*). MSC-Exos also suppress the expression of aquaporin-5 and EGFR proteins in colorectal cancer cell lines (*196*). These results highlight the value of specific miRNAs as promising therapeutic targets for MSC-Exo based treatments for colorectal cancer.

4.4. Gastric cancer

Gu *et al*. (*197*) found that treatment with hUC-MSC-Exos enhanced cell migration and invasion in the HGC-27 gastric cancer cell line by inducing the EMT through activation of Akt pathway signaling. In another study, hBM-MSC-Exos promoted proliferation, differentiation, and metastasis in MG63 and SGC7901 gastric cancer cell lines by activation of the Hedgehog signaling pathway (*198*). Later, Chen *et al*. (*199*) found that conditioned media containing hBM-MSC-Exos promoted gastric cancer cell proliferation by increasing the expression of the retroviral oncogene *c-Myc* in the MGC-803 and BGC-823 gastric cancer cell lines. These findings suggest that MSC-Exos could serve as promising therapeutic targets for the treatment of gastric cancer.

Research has highlighted the role of MSC-Exos as a key mediator of chemotherapeutic drug resistance in gastric cancer. Ji *et al*. (*151*) showed that hUC-MSC-Exos induced resistance to 5-fluorouracil in HGC-27, MGC-803, and SGC-7901 gastric cancer cell lines and a subcutaneous xenograft tumor model in mice by increasing the expression of the multidrug resistance genes *ATP-binding cassette, sub-family B member 1A* (*P-gp*/*MDR*), *ATP-binding cassette, sub-family C member 1* (*MRP*), and *major vault protein* (*LRP*). *In vitro* experiments showed that treatment with hUC-MSC-Exos activated calcium/calmodulin-dependent protein kinases (CaM-Ks) and the Raf/MEK/ERK kinase cascade in the gastric cancer cell lines. Treatment with MSC-Exos containing miR-301b-3p was also shown to promote drug resistance by inhibiting the expression of *thioredoxin-interacting protein* (*TXNIP*), which downregulated the expression of *P-gp*/*MDR* and *MRP* in gastric cancer cells (*200*). Targeting the interaction between MSC-Exos and cancer cells may offer novel strategies to improve the effectiveness of chemotherapy for the treatment of

gastric cancer.

MSC-Exos have shown great promise as an efficient nano-carrier for targeted drug delivery in gastric cancer therapy. Preclinical studies have demonstrated that MSC-Exos can deliver miR-1228 into gastric cancer cells, where it inhibits NF-κB activity (*201*). Additional research has revealed that MSC-Exos enriched with miR-1228 reduced gastric cancer cell growth by downregulating *matrix metalloproteinase-14* (*MMP-14*) gene expression, and analysis of serum exosomes showed miR-1228 was associated with increased overall survival in gastric cancer patients (*202*). MSC-Exos engineered to overexpress the *lipocalin-type prostaglandin D2 synthase* (*L-PGDS*) gene have been shown to inhibit the proliferation of SGC-7901 gastric cancer cells by inducing PPARγ expression and suppressing STAT3 phosphorylation (*203*). These results show that MSC-Exos have great potential for the development of an efficient drug delivery system for the treatment of gastric cancer.

Adenocarcinomas are common at both esophageal and gastric sites. Therefore, some MSC-Exos mediated effects on gastric cancer might also apply to esophageal cancer due to similar etiological characteristics (*204*), such as the effects of L-PGDS enriched MSC-Exos on the SGC-7901 gastric cancer cell line, an adenocarcinoma derivative. Though squamous cell carcinoma (SCC) is the most common type of esophageal cancer, the etiology of esophageal SCC is more poorly understood than that of adenocarcinoma (*204*), which might make it less attractive for MSC-Exo-related studies, given that fewer studies of MSC-Exos in esophageal cancer have been performed thus far (*205*). However, studies have shown that the EMT is also induced in esophageal SCC (*206*), and that treatment with hUC-MSC-Exos containing miR-375 promoted apoptosis and inhibited cell proliferation, invasion, migration, and tumorsphere formation in the KYSE70, ECA109, and EC9706 esophageal SCC cell lines (*207*).

4.5. Liver cancer

The acquisition of drug resistance poses a significant challenge for liver cancer treatment, leading to unsatisfactory clinical outcomes and reduced survival, which highlights the urgent need for novel therapies to improve the sensitivity of liver cancer to chemotherapy and targeted treatments. Evidence suggests that exosomal miRNAs have critical functions in liver cancer progression and MDR in hepatocellular carcinoma (HCC) (*208*). Notably, miR-199a-3p has been identified as a regulator of hepatocyte apoptosis and hepatocarcinogenesis, and lower levels of this miRNA has been correlated with poor prognosis in HCC (*209*). Another study showed that AT-MSC-Exos enriched with miR-199a could effectively enhanced the sensitivity of HCC cells to chemotherapy drugs by targeting the mTOR signaling pathway (*210*). A similar study showed that treatment with miR-122 enriched AT-MSC-Exos enhanced the efficacy of 5-fluorouracil and sorafenib treatments in the HepG2 liver cancer cell line (*211*). Therefore, targeting specific miRNA through MSC-Exos has represents a possible strategy for enhancing chemosensitivity in treatments for HCC.

Researchers have also explored the potential of MSC-Exos as functional drug carriers in combination therapies for HCC. Norcantharidin, a demethylated derivative of zebularine, has been shown to inhibit the proliferation and migration of HCC cells when used in combination with 2-deoxyglucose (*212*). Previous studies have demonstrated that conjugating norcantharidin with biomaterials significantly enhanced its therapeutic efficacy against various types of HCC (*213*). Treatment with norcantharidin-enriched BM-MSC-Exos resulted in greater cellular uptake of norcantharidin, which induced cell cycle arrest and reduced tumor proliferation and increased apoptosis of HepG2 cells (*214*). Liang *et al*. (*214*) also found that treatment with norcantharidin-enriched BM-MSC-Exos promoted the repair of damaged non-cancerous L02 hepatocytes. These therapeutic properties of BM-MSC-Exos enriched with key miRNA offers a promising avenue for future cancer therapies.

4.6. Urological malignancies

A previous review highlighted the critical need for more effective therapies for advanced urological cancers (*215*). Studies have proposed advanced strategies to mitigate cancer progression by depleting circulating exosomes in the systemic circulation as a treatment for urological tumors (*216*). Increasing evidence suggests that MSC-Exos can play a therapeutic role, offering distinct advantages over traditional cell therapies (*217*). A recent study evaluated the impact of hAT-MSC-Exos on the 5637 bladder cancer cell line, the LNCaP hormone-sensitive prostate cancer cell line, and the PC3 hormone-refractory prostate cancer cell line, and the results strongly suggested that exosomes exhibited significant changes in the expression of cancer-related genes across all of the cell lines tested (*218*). Though hAT-MSC-Exos increased *tumor protein p53* (*P53*) expression and reduced *BCL2 apoptosis regulator* (*BCL2*) expression in all of the cell lines, the differential expression of other genes was observed across cell lines.

In the 5637 cells, the expression level of *vascular endothelial growth factor A* (*VEGFa*) and *BCL2 associated X apoptosis regulator* (*BAX*) were also significantly reduced by hAT-MSC-Exos treatment. In the LNCaP cells, the expression levels of *VEGFc* and *BAX* were significantly reduced. In PC3 cells, expression levels of the OPNb and OPNc isoforms of the *secreted phosphoprotein 1* (*OPN*) gene were significantly increased, and *BAX* was significantly reduced. Cell

culture experiments showed the hAT-MSC-Exos treatment reduced cell proliferation in all three cell lines, suggesting the hAT-MSC-Exos-induced changes in gene expression exerted apoptotic effects on the bladder and prostate cancer cell lines (*218*). These preclinical findings further suggest that hAT-MSC-Exos might inhibit the occurrence and progression of urological tumors by enhancing cell apoptosis. Future research should provide important findings to support development of MSC-Exo based therapies for urological cancers.

Other studies have shown that exosomal miRNAs play crucial roles in post-transcriptional gene regulation in urological tumors. For instance, studies suggest that treatment with miR-143-enriched BM-MSC-Exos can prevent cell migration and metastasis in prostate cancer (*219*). Preclinical research has also shown that AT-MSC-Exos reduced cell proliferation and induced apoptosis in a tumor xenograft mouse model of metastatic prostate cancer, and cell culture studies showed the apoptotic effect of AT-MSC-Exos resulted from a reduction in BCL2 like 1 (BclxL) protein expression through a mechanism mediated by miR-145 (*137*). These results suggest that miR-145 enriched AT-MSC-Exos represents a novel therapeutic strategy to treat prostate cancer. In another study, treatment with BM-MSC-Exos containing miR-9-3p can inhibit bladder cancer progression by downregulating endothelial cell-specific molecule 1 (ESM1), presenting an additional therapeutic target (*220*). Furthermore, MSC-Exos has been shown to enhance apoptosis and necrosis while inhibiting cell proliferation in the BIU-87, EJ-1, T24, 5637, and UMUC-3 bladder cancer cell lines and a tumor xenograft mouse model of bladder cancer (*218*). Cell culture experiments showed that miR-9-3 mediated the effects of BM-MSC-Exo treatment by reducing *endothelial cell-specific molecule 1* (*ESM1*) gene expression.

Investigations of non-MSC exosomes isolated from bladder cancer patients have shown that exosomal lncRNA-BCYRN1 contributes to bladder cancer metastasis in a mouse popliteal lymph node metastasis model through VEGF-C/VEGFR3 signaling (*221*), and that lncRNA-UCA1 contributes to cell migration, invasion, and the EMT in 5637, T24, and UMUC2 bladder cancer cell lines by inhibiting miR-145 expression and enhancing the expression of *zinc finger E-box binding homeobox 1 and 2* (*ZEB1* and *ZEB2*), which induces the EMT (*222*). In another study, exosomal miR-133b was shown to inhibit cell proliferation and migration in the 5637 and T24 bladder cancer cell lines and inhibited tumor growth in a mouse tumor xenograft model through an increase in *dual-specificity protein phosphatase 1* (*DUSP1*) gene expression (*223*). These results identify additional possible targets for future research in the development of novel treatments for urological malignancies in which MSC-Exos can be enriched with lncRNA-BCYRN1, lncRNA-UCA1, and/or miR-133b.

4.7. Pancreatic cancer

A series of preclinical studies have demonstrated that treatment with MSC-Exos enriched with siRNA specific for the $KRAS^{G12D}$ GTPase, a mutant of the *KRAS* proto-oncogene, resulted in suppressed tumor progression and increased overall survival in a mouse model of pancreatic cancer (*224*). Notably, the expression of miR-143 is significantly decreased in pancreatic cancer (*225*), while treatment with exosomal miR-143 can inhibit cell proliferation and promote apoptosis in a mouse model of pancreatic cancer (*226*) Additionally, miR-143 induces apoptosis in pancreatic cancer cells and inhibits their growth, invasion, and migration by downregulating COX-2 and KRAS (*225*).

In another study, treatment with miR-124 enriched BM-MSC-Exos were shown to inhibit the proliferation, invasion, migration, and EMT of AsPC-1, PANC1, BxPC-3 and SW1990 pancreatic adenocarcinoma (PAC) cell lines and a mouse tumor xenograft model of PA. Cell culture experiments showed that miR-124 reduced *Zeste 2 polycomb repressive complex 2 subunit* (*EZH2*) gene expression, which induced apoptosis in PAC cells (*227*). Treatment with BM-MSC-Exos carrying miR-124 also enhanced the sensitivity of PAC cells to chemotherapy. Additionally, treatment with MSC-Exos containing miR-1231 was shown to inhibit cell proliferation BxPC-3 and MIA PaCa-2 pancreatic ductal adenocarcinoma (PDAC) cell lines and a mouse tumor xenograft model (*228*). These results suggest that MSC-Exos present a promising therapeutic approach for delivering PA-inhibitory miRNAs to pancreatic tumors.

Exosomes can also serve as an attractive nanoscale drug delivery platform, which has been actively explored for the treatment of pancreatic cancer. Researchers have utilized hUC-MSC-Exos to deliver exogenous miR-145-5p *in vitro* and in a mouse model, where it inhibited the proliferation and invasion of PDAC cells, promoted apoptosis and induced cell cycle arrest by reducing *SMAD family member 3* (*Smad3*) gene expression (*229*). In another study, investigators successfully loaded MSC-Exos with the anti-cancer drug paclitaxel, and demonstrated that treatment with paclitaxel-enriched MSC-Exos significantly inhibited tumor growth *in vitro*, which provided the first evidence of active drug encapsulation and delivery through MSC-Exos (*230*).

Subsequently, various other drug delivery platforms were developed based on the MSC-Exos design. One study treated PDAC in a mouse tumor xenograft model with BM-MSC-Exos enriched with paclitaxel and gemcitabine monophosphate (GEMP), which resulted in increased overall survival (*231*). This novel exosome drug delivery system demonstrated high selectivity for PDAC cells and effective penetration of tumors (*231*). Research is also underway to explore dual-delivery biological systems using MSC-Exos, lectin 9 siRNA loaded *via* electroporation, and surface modification with

oxaliplatin. These approaches enhance the accumulation of chemotherapeutic agents within pancreatic tumors while reducing their systemic distribution (*232*). Exosomes produced by these novel MSC-Exos systems are both modified with targeted ligands and genetically engineered to retain their original characteristics, and hold great promise for efficient chemotherapy drug delivery to tumor cells with significantly enhanced tumor targeting.

4.8. Brain cancer

In a comprehensive review, Ordóñez-Rubiano *et al*. (*233*) noted that exosomal miRNAs play a significant role in the progression of glioblastomas, as they are released during disease progression, and contribute to tumor growth and invasion. Exosomal miRNAs, such as miR-21, miR-301, and miR-301a, are transferred to the surrounding cells, wherein they disrupt homeostasis in normal cells and enhance the proliferation and invasion of malignant cells. Compared to exosomes from normal brain tissue, tumor cell-derived exosomes exhibited significantly elevated levels of miRNAs, including miR-222, miR-9, and miR-26a, which activate various signal transduction pathways that stimulate tumor growth. Notable exosomal miRNAs involved in glioblastoma progression include miR-301a, miR-151a, miR-21, miR-1246, miR-29a, miR-92a, miR-9, miR-26a, and miR-375. These miRNAs represent potential biomarkers and therapeutic targets in glioblastoma.

MSC-Exos have been shown to effectively regulate immune responses and promote the repair and regeneration of damaged neurons. Through the ability to traverse the blood-brain barrier, MSC and their exosomes can be used as carriers for effector molecules in therapeutic applications for brain cancer treatments (*234*). Using flow cytometry and *in situ* hybridization, researchers have demonstrated that MSC-secreted exosomes localized to co-cultured glioma cells, delivering exosomal miR-145 and miR-124, which decreased glioma cell migration and self-renewal by reducing *synaptonemal complex protein 1* (*SCP-1*) and *SRY-box transcription factor 2* (*Sox2*) gene expression (*235*). Additional experiments in a mouse glioma xenograft model showed that intracranially administered MSC secreted exosomes transferred a fluorescent-labelled miR-124 mimic to tumor cells *in vivo* (*235*). The transfection of MSCs with extracellular vesicles containing miR-146b and subsequent injection of them into tumors has been shown to reduce the motility and invasiveness of glioblastoma cells in a rat model (*236*). In a similar study, MSC-Exos carrying miR-133b were shown to reduce EZH2 expression and downregulate the Wnt/β-catenin pathway in co-cultured U87, U251, LN229, and A172 human glioma cells, which inhibited cell proliferation, migration, and invasion (*237*).

The treatment of glioblastoma tumors with radiation and chemotherapy often results in the development of resistance to these therapies. The limitations of current treatments are in large part due to the inability of clinicians to precisely deliver therapeutic drugs to the glioblastoma multiforme (GBM) tumor site. To overcome this difficulty, researchers are investigating the use of MSC-Exos for efficient effector molecular delivery. Munoz *et al*. (*238*) reported that expression of miR-9 in temozolomide-resistant GBM cells was greater than that in healthy cells, and that non-resistant cells were transformed to resistant cells by the intercellular transfer of miR-9 *via* GBM-derived exosomes. They used BM-MSCs engineered to secrete exosomes carrying an anti-miR-9 molecule to silence the expression of miR-9 in co-cultured temozolomide-resistant GBM cells, which led to temozolomide sensitization and increased cell death accompanied by increased caspase activity (*238*).

5. MSC exosomes in clinical applications

The ability to sterilize exosomes by filtration and their lower immunogenicity (*104*) results in lower risks of adverse effects compared with MSC transplantation (*21*). The lack of replication and differentiation result in more predictable biological responses and shorter half-life compared with transplanted MSCs (*239*), and the small size and lipophilic membrane of exosomes can contribute to greater penetrance of physiological barriers (*240*). The over-expression of CD47 in exosomes produced by human BM-MSCs and AT-MSCs allows evasion of immune cell phagocytosis, which ensures the bioavailability of MSC-derived exosomes at the tumor site (*241*). The ability to load MSC-Exos with therapeutic molecules allows delivery of a wide range of biologically active cargo (*242*).

Exosomes are preferentially internalized by recipient cells of the same cell-type as the secreting host cell due to the conservation of cell membrane protein signatures (*243*). Cell signature based targeting also occurs with exosomes produced by cancer cells (*244*), with exosomes targeting both malignant and non-malignant cells of similar origin (*245*). Thus, the parent cell can be selected to ensure the sorting of target-cell ligands into exosomes to mediate delivery to specific target cell types (*243*). MSC-Exos also demonstrate cell signature targeting specificity, and have shown substantial potential for targeting tumor cells for anti-cancer drug delivery, enhancing efficacy under low toxicity conditions (*246*).

5.1. Potential tumor-related effects of MSC-Exos

Studies have shown that MSC-based treatments can modulate both tumorigenic and tumor-suppressive processes (*247*), and that MSC-Exo transplantation can suppress the growth and progression of histologically different types of cancer (*132,248*). However, tumor-derived exosomes have been shown to contribute to

tumor progression (*249*) and resistance to chemotherapy (*250*), and similar studies found that MSC-Exos also contributed to resistance (*151*), angiogenesis (*251*), cell proliferation (*252*), and migration (*253*). By contrast, other studies concluded that MSC-Exos inhibited the development of resistance (*254*), increased chemosensitivity (*255*), inhibited angiogenesis (*143*) and tumor cell proliferation (*256*), and promoted tumor cell apoptosis (*257*). Due to these conflicting findings, the tumorigenic potential of transplanted MSC-Exos remains a safety concern for the development of anti-cancer therapies (*258*).

5.2. Safety of MSC-Exo anti-cancer therapeutics in clinical trials

Most clinical trials investigating exosomes in cancer patients have focused on the analysis of plasma exosomes as diagnostic or companion diagnostic biomarkers (*259*). Investigations of MSC-Exos for anti-cancer therapies are few in number. Phase 1 clinical trials have been performed to investigate the safety of various MSC-Exos transplantation protocols. Using our search and exclusion criteria, we identified nine registered trials investigating applications of MSC-Exos for cancer treatments (Table 1). Only one of these trials, NCT03608631 (n = 9), uses MSC-Exos in anti-cancer therapy for a solid tumor malignancy. In NCT03608631, patients with metastatic pancreatic cancer are treated with exosomes derived from hBM-MSCs containing siRNA that silence the expression of $Kras^{G12D}$, which is a primary driver of tumor progression in PDAC (*260,261*).

The results of NCT03608631 have not yet been published, but the preprint report of the phase 1b clinical data was recently made available (*262*). The NCT03608631 investigators reported no adverse reactions or toxicity despite using a dosing regimen designed to determine the highest tolerable dosage. Analysis of post-treatment tumor tissue samples showed that Ras signaling was downregulated by suppression of ERK phosphorylation, the number of $PanCK^+$ cancer cells were reduced, and the numbers of $aSMA^+$ stromal cells were increased or remained stable compared to the results of the pretreatment analysis. Further post-treatment analysis of tissue samples showed increases in intratumoral $CD8^+$ T cells, $CD4^+$ $Foxp3^+$ Tregs, and $CD4^+$ $Foxp3^-$ cells compared to the pre-treatment analysis, suggesting that a favorable anti-tumor immune response was induced within the TM. Six patients experienced disease progression after three treatment cycles. One patient experienced disease progression after five treatment cycles. One patient experienced disease progression after six treatment cycles, and one patient experienced disease progression at 3 months following six treatment cycles (*262*). Though the efficacy findings of NCT03608631 are mixed, the safety data are an important contribution to the overall body of research in this field.

With regard to evaluating the safety of MSC-Exos for anti-cancer treatments, caution must be exercised in comparisons of safety data from studies investigating the application of MSC-Exos for pathologies other than cancer, due to the need to evaluate the potential tumorigenic properties of MSC-Exo therapeutics. One of other trials identified, NCT06245746, is investigating the use of MSC-Exos in an intervention for acute myeloid leukemia (Table 1). However, this study is still in the recruiting stage. Six of the remaining trials are also in the recruiting or pre-recruiting stages. The last remaining phase 1 trial, NCT04134676, has been completed (Table 1). However, this study administered MSC-Exos in a topical treatment for wound healing, thereby limiting the suitability of its safety data for comparison with those of solid tumor studies, which most often administer anti-tumor therapeutics intravenously (IV) or intratumorally (IT). In the NCT03608631 and NCT06245746 trials, the MSC-Exos were administered by IV infusion.

With so few studies of MSC-Exos for anti-cancer treatments available for analysis, we considered comparisons with studies using non-MSC derived exosomes for the evaluation of adverse effects related to administration route and toxicity. A recent systematic review identified 10 clinical trials investigating the use of exosomes for anti-cancer treatments (*259*). Among these were NCT01159288, NCT05375604, NCT04592484, and NCT05559177 (Table 1), which investigated non-MSC derived exosome treatments for solid tumor cancers. The NCT01159288 trial, which was completed in 2015, used exosomes derived from IFN-γ-maturated DCs to treat non-small-cell lung cancer (NSCLC) (*263*). The NCT05375604 trial used human embryonic kidney cell (HEK293) derived exosomes (HEK-Exos) containing anti-sense oligonucleotides that inhibit expression of the *signal transducer and activator of transcription 6* (*STAT6*) gene (*264*) for the treatment of patients with advanced hepatocellular carcinoma and those with gastric cancer or colorectal cancer with liver metastases.

The NCT04592484 trial also used HEK-Exos that were loaded with exogenous cyclic dinucleotide (CDN) agonists of the stimulator of interferon genes (STING) pathway (*265*) for the treatment of solid tumors, including cutaneous squamous cell carcinoma, head and neck squamous cell cancer, anaplastic thyroid carcinoma, and triple negative breast cancer. The NCT05559177 trial used chimeric exosomes in patients with recurrent or metastatic bladder cancer. These exosomes were derived from cells produced by the fusion of nuclei of bladder cancer cells with peripheral blood antigen presenting cells, with both types of cells obtained from each patient to produce a personalized cancer vaccine.

Of these four additional clinical trials using non-MSC derived exosomes, safety data are available for NCT01159288 only. Of the seven patients included in

Table 1. Summary of clinical trials investigating various exosome-based therapies

Patient criteria (sample size)	Study phase (status)	Intervention summary (route)	Clinical safety results	Trial registration (*Ref.*)
Metastatic pancreatic cancer (n = 9)	Phase 1b (ongoing, not recruiting)	hBM-MSCs carrying siRNA to silence $Kras^{G12D}$ expression (IV)	No adverse reactions or toxicity	NCT03608631 (*262*)
Acute myeloid leukemia (n = 9)	Phase 1 (recruiting)	hUC-MSC-Exos (IV).	Not available	NCT06245746
Rectal cancer (n = 20)	Phase 1 (not yet recruiting)	Human placenta-derived MSC-Exos to prevent early anastomosis leakage	Not available	NCT06536712
Multiple organ failure (n = 120)	Phase 1 (not yet recruiting)	MSC-Exos (IV)	Not available	NCT04356300
Pilonidal sinus/pilonidal disease (n = 120)	Phase 1 (recruiting)	Drug+/-MSC+/-MSC-Exos (site injection)	Not available	NCT06391307
Dystrophic epidermolysis bullosa (n = 10)	Phase 1 (recruiting)	Allogeneic MSC-Exos (topical)	Not available	NCT04173650
Amyotrophic lateral sclerosis (n = 38)	Phase 1 (recruiting)	hUC-MSC-Exos (nasal drop)	Not available	NCT06598202
Patients undergoing hematopoietic stem cell transplantation (n = 120)	Phase 1 (recruiting)	MSC-Exos (topical oral and bladder irrigation)	Not available	NCT06599346
Chronic ulcer (n = 38)	Phase 1 (completed)	Wharton jelly-MSC-Exos (topical)	No safety data	NCT04134676 (*272*)
Advanced NSCLC (n = 41)	Phase 2 (completed)	IFN-γ-maturated DC-Exos (intradermal) after oral cyclophosphamide	Grade-3 hepatotoxicity (n = 1)	NCT01159288 (*263*)
Advanced HCC, gastric cancer, colorectal cancer (n = 9)	Phase 2 (terminated)	HEK-Exos containing siRNA to silence STAT6	Not available	NCT05375604 (no publication)
Advanced solid tumors (n = 27)	Phase 2 (completed)	HEK-Exos loaded with CDN agonists of STING	Not available	NCT04592484 (no publication)
Recurrent or metastatic bladder cancer (n = 9)	Phase 1 (unknown)	Personalized chimeric exosome cancer vaccine (not reported)	Not available	NCT05559177 (no publication)
Advanced NSCLC (n = 9)	Phase 1 (completed)	DC-Exos (intradermal)	10 events, grade-1,2	Not registered (*266*)
Metastatic melanoma (n = 15)	Phase 1 (completed)	DC-Exos (intradermal)	None ≥ grade 2	Not registered (*273*)
Advanced colorectal cancer (n = 40)	Phase I (completed)	Autologous exosomes from patient ascites (intradermal)	42 events, grade-1,2	Not registered (*268*)

NCT01159288, one experienced grade-3 hepatotoxicity with no other adverse events reported (*263*). Through our screening of review articles retrieved in our literature search, we identified three additional trials that were not registered with *clinicaltrials.gov* (Table 1). These studies used intravenous infusions of DC-derived exosomes to treat advanced NSCLC (*266*), metastatic melanoma (*267*), and advanced colorectal cancer (*268*), with all reporting that the various treatments were well tolerated (*269*).

The sum of these results for non-MSC derived exosomses and MSC-Exos based treatments for solid tumors suggest that the risk of adverse effects associated with intravenous infusions of exosome therapeutics is low. However, more safety data are needed. The addition of healthy control arms in future studies might provide important information regarding possible adverse effects related to administration routes and dosing regimens. Moreover, the sample sizes of most of these clinical trials are quite small. Future multicenter studies and international collaborations could increase the number of patients participating in the development of these urgently needed improvements in treatments for solid tumor malignancies. Improved reporting of safety data and updating of online registry entries would benefit investigators planning new studies. The NCT03608631 investigators proposed continuing the trial with a combination therapy using the $Kras^{G12D}$-suppressing exosomes and an unspecified inhibitor of immune checkpoint CTLA-4 (*270*), a known driver of immunosuppression in pancreatic cancer (*271*). This approach is supported in part by the results of *in vitro* and animal experiments (*262*). We look forward to the publication of their future clinical findings.

6. Conclusions

Through our search of the available literature describing the therapeutic potential of MSC-Exos for the treatment of solid malignancies, we found that preclinical and phase-1 clinical studies provide exciting evidence regarding the safety and potential efficacy of MSC-Exos in the treatment of various solid tumors, including breast, lung, gastrointestinal, pancreatic, colorectal, brain, and urological cancers. These findings suggest that MSC-Exos based therapies have the potential to revolutionize cancer treatment. However, we also recognized that MSC-Exos possess certain dual characteristics by which their properties have been observed to promote tumor progression, while also being observed to suppress it under other conditions. This complexity may be related to the origin of MSC exosomes, the types of molecules they carry, and specific conditions within the TM. Such conflicting properties need not diminish their potential as therapeutic tools. The selective loading of MSC-Exos with tumor-suppressive miRNAs or anti-cancer drugs achieved thus far represents great potential for the development of an effective delivery system for precisely targeting cancer cells. Ultimately, genetic engineering of the contents of exosomes will likely be required to favor the expression of anti-tumor molecules and suppress those linked to tumorigenic processes. Future research should focus on elucidating the mechanisms of MSC-Exos and their functions in different TMs, as well as exploring how to optimize the composition of MSC-Exos to enhance their therapeutic efficacy to further lay the groundwork for novel clinical applications.

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Review

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Therapeutic strategies in traditional Chinese medicine for premature ovarian failure: Modulation of oxidative stress and autophagy–apoptosis *via* the AMPK/mTOR pathway

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SUMMARY: Premature ovarian failure (POF), also referred to as premature ovarian insufficiency (POI), is a multifactorial reproductive endocrine disorder characterized by amenorrhea, infertility, hypoestrogenism, and elevated gonadotropin levels before the age of 40. Emerging evidence links its pathogenesis to oxidative stress and dysregulation of the autophagy–apoptosis balance in ovarian cells. Excessive accumulation of reactive oxygen species (ROS) impairs mitochondrial function in oocytes, while aberrant autophagy and granulosa cell apoptosis accelerate the depletion of primordial follicles. The AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) pathway serves as a critical nexus between energy metabolism, oxidative stress, and cell survival. Traditional Chinese medicine (TCM), with its multi-component and multi-target characteristics, has demonstrated unique advantages in modulating the AMPK/mTOR pathway to restore ovarian function. This review synthesizes recent findings on single herbs, classical formulas, and non-pharmacological therapies (acupuncture and moxibustion). Mechanistic studies have revealed that these interventions can activate AMPK, inhibit mTOR overactivation, enhance Nrf2-mediated antioxidant defenses, reduce ROS production, and rebalance autophagy and apoptosis *via* pathways such as PI3K/Akt and SIRT1/p53. By aligning stage-specific regulation of AMPK/mTOR signaling with the TCM principle of syndrome differentiation, this integrative approach provides theoretical guidance for precise, personalized treatment to optimize multi-target strategies for POF management.

Keywords: premature ovarian failure, AMPK/mTOR, oxidative stress, autophagy, apoptosis, traditional Chinese medicine, acupuncture, moxibustion, Nrf2, PI3K/Akt

1. Introduction

Premature ovarian failure (POF), also known as premature ovarian insufficiency (POI), is a multifactorial and heterogeneous disease. It is characterized by amenorrhea, infertility, lower estrogen levels, and elevated gonadotropin levels in women as a result of ovarian failure before the age of 40. These conditions significantly impair female reproductive function and quality of life (*1,2*). Clinical diagnostic criteria are amenorrhea (lasting ≥ 4 months), reduced estradiol (E_2) levels, abnormally high follicle-stimulating hormone (FSH) levels (more than 4 weeks between consecutive tests, FSH > 40 IU/L), and a significant reduction in fertility (*3,4*). Worldwide, the rate of POF is between 0.9% and 1.2% (*5*). Only 5–10% of these women can conceive naturally and give birth (*6*). The causes of POF are complex, and the exact mechanisms are unclear. The generally accepted causes currently include genetic factors, autoimmune factors, medical factors such as surgery, radiotherapy, and chemotherapy, and environmental factors (*7,8*). As the pace of life has accelerated, psychological pressure on women has increased, and many adverse factors such as tumor radiotherapy have increased over the past few years, the incidence of POF has increased yearly and it tends to affect younger women (*9*). POF not only affects a woman's fertility but can also lead to osteoporosis, cardiovascular disease, and other long-term complications (*10*), seriously affecting the patient's

quality of life. Currently, hormone replacement therapy (HRT) is the main treatment for POF in clinical practice (*11*). Still, there are problems of unsatisfactory efficacy, many limitations in the form of contraindications, and an increased risk of other gynecological diseases with long-term use (*12,13*). Therefore, the pathogenesis of POF needs to be investigated and new therapeutic strategies need to be developed. Stem cells have self-repairing and regenerative capabilities, can stimulate follicles and increase hormone levels, and are effective in treating infertility and ovarian failure (*14,15*). In a study, Fu *et al.* found that adipose-derived stem cells promoted the repair of chemotherapy-induced POF by inhibiting granulosa cell apoptosis and aging (*16*). Nevertheless, the clinical use of stem cells still faces significant challenges, including high culturing costs, a limited supply, and immune rejection and ethical concerns in patients (*1*).

In recent years, the role of oxidative stress and dysregulation of cellular autophagy and apoptosis in the pathogenesis of POF has been increasingly emphasized with more in-depth molecular and cellular biology studies. Under physiological conditions, the generation and scavenging of reactive oxygen species (ROS) is dynamically balanced in the ovaries, and ROS act as signaling molecules to drive cellular regulatory pathways (*17,18*). However, stimulation by various internal and external factors leads to oxidative stress, which damages oocytes and granulosa cells and accelerates follicular atresia. Dysregulation of autophagy, an important cellular protective mechanism, can affect follicle development and survival. Apoptosis is the major form of follicular atresia. Excessive apoptosis leads to premature loss of primordial follicles, resulting in ovarian failure. Abnormal apoptosis and autophagy of granulosa cells are considered to be the key pathological mechanisms of POF (*19*).

Traditional Chinese medicine (TCM) has long been used in China for the treatment of a wide variety of illnesses (*20*). In TCM, there is no clear and systematic discussion of POF, but from the point of view of its disease characteristics, it is similar to the ancient description of a "pre-menstrual closure" and "premature menstrual cessation" type of disease, so it can be categorized as "amenorrhea," "infertility," or the like (*21*). Due to its multi-component and multi-target properties, TCM has shown unique advantages in improving ovarian function (*22*). Studies have shown that the effectiveness of TCM combined with HRT in the treatment of POF is more effective than HRT alone (*23*). Research has found that Chinese herbs can improve ovarian function by modulating the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) signaling pathway, reducing oxidative stress damage and restoring the balance between autophagy and apoptosis. AMPK, a key regulator of cellular energy metabolism, can be activated by a variety of stress conditions and then promote cellular autophagy by inhibiting mTOR signaling. This pathway plays an important role in linking oxidative stress to cellular autophagy and apoptosis (*8*).

In this study, we summarized the mechanisms of oxidative stress and cellular autophagy and apoptosis in POF and explored the therapeutic strategies based on the AMPK/mTOR pathway to provide new ideas for the clinical treatment of POF in TCM.

2. Oxidative stress and POF

Oxidative stress, characterized by excessive production of ROS or impaired antioxidant regulation, occurs in biological systems under normal and pathological conditions (*24*). ROS are the general term for oxygen derivatives with high oxidative capacities, including superoxide anion, hydrogen peroxide, and hydroxyl radicals (*25*). ROS accumulation promotes the generation of advanced oxidation protein products (AOPPs), which are key biomarkers indicative of oxidative stress. Abnormally elevated ROS attack biological macromolecules and organelles, resulting in oxidative damage to DNA, proteins, and lipids. Oxidative stress has a chronic impact on many diseases, such as diabetes, cardiovascular disease, and polycystic ovary syndrome (*26,27*). The mechanisms by which oxidative stress impairs ovarian function are complex and diverse. Excessive ROS attack oocytes and granulosa cells, leading to granulosa cell damage (*28*), weakening of nutritional support for oocytes from granulosa cells, and accelerated follicular atresia (*29*). In addition, oxidative stress inhibits steroid hormone synthases (*e.g.,* CYP19A1, which is responsible for estrogen synthesis), leading to reduced estrogen levels (*30*). Moreover, ROS promote the release of inflammatory factors (TNF-α, IL-6, *etc.*) (*31*), leading to deterioration of the ovarian microenvironment, exacerbation of ovarian fibrosis, and reduction of the available follicular reserve, as shown in Figure 1. Oxidative stress activates a variety of signaling pathways, such as the NF-κB and MAPK pathways, and promotes the release of inflammatory factors that exacerbate the deterioration of the ovarian microenvironment. This ultimately leads to a decline in ovarian function (*32-34*).

A variety of factors can induce oxidative stress in the ovaries. Environmental factors such as the plasticizer diisononyl phthalate can induce oxidative stress in ovarian granulosa cells, which in turn triggers autophagy and apoptosis. Bisphenol A (BPA) is a widespread endocrine-disrupting chemical with estrogen-like effects that has been found to promote autophagy in ovarian granulosa cells by inducing the AMPK/mTOR/ULK1 signaling pathway (*35*). Medical factors, such as metabolites of the chemotherapy drug cyclophosphamide, can induce the production of large amounts of ROS in the ovaries, leading to a dramatic reduction in follicular reserve (*36,37*). In addition, the

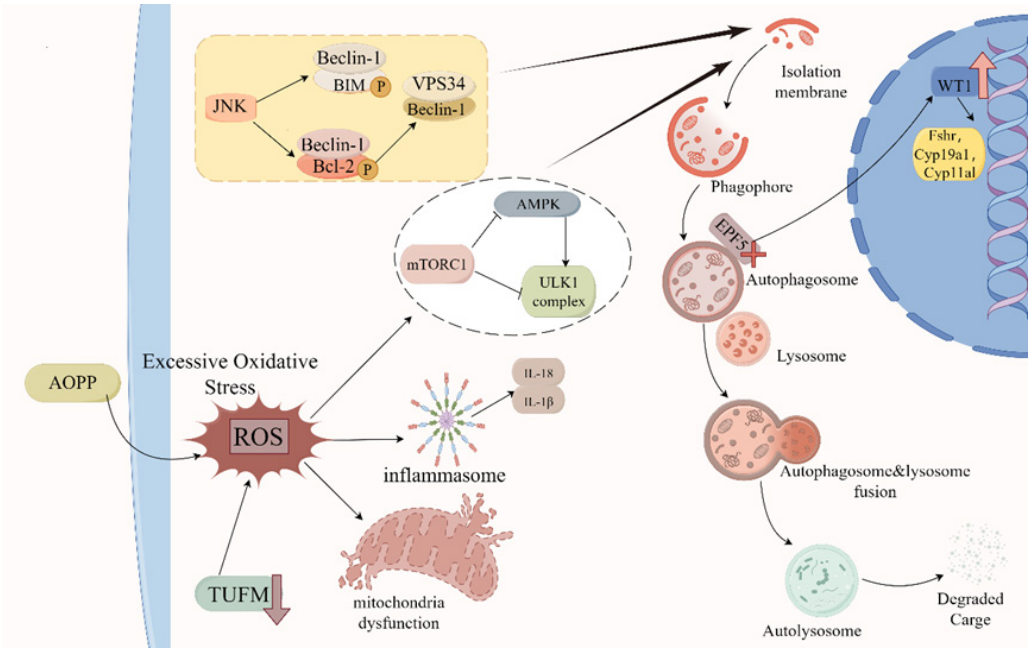

Figure 1. Signaling pathways involved in the pathogenesis of premature ovarian failure.

antioxidant capacity of the ovaries naturally declines with age, which is one of the major causes of ovarian hypoplasia in older women (*38*).

TCM may improve POF by scavenging ROS or by enhancing the antioxidant defense system. A number of active ingredients in TCM and Chinese herbal compounds have been shown to have significant antioxidant effects. For example, Angelica sinensis polysaccharide (AP) is the main chemical component of Angelica sinensis, with a content of up to 15%. AP tonifies the blood, regulates immunity, combats viruses, and is antioxidative. Li *et al.* found that (*39*) AP significantly increased the ovarian index, increased the activity of superoxide dismutase (SOD), and decreased the activity of malondialdehyde (MDA), thus inhibiting the level of oxidative stress in immune POF mice. It also reduced the levels of IL-1β and IL-6 and suppressed the level of inflammation. The Astragal polysaccharide in Astragalus can upregulate the expression of SOD, thereby scavenging ROS and slowing the development of POF. Lyceum barbarum polysaccharide (LBP) can inhibit the production of MDA and protect mitochondrial function (*40*). Salidroside can increase the proliferation of the granulosa-like tumor cell line (KGN) induced by dihydrotestosterone (DHT), activate the phosphorylation of AMPK, induce the translocation of nuclear factor E2 (Nrf2)-related factors, and target antioxidant proteins to inhibit oxidative stress damage (*41*). Danggui Shaoyao San can inhibit oxidative stress and reduce apoptosis by regulating the SIRT1/p53 signaling pathway, thereby protecting against cyclophosphamide-induced POF (*42*), and Zishen Tongmai Decoction inhibits granulosa cell apoptosis through the PI3K/Akt/mTOR pathway (*43*). These studies provide a scientific basis for TCM to combat POF by reducing oxidative stress.

3. Cellular autophagy-apoptosis and POF

Cellular autophagy and apoptosis are two important cellular processes that regulate ovarian function, and their balance is essential for maintaining normal follicular development and ovarian reserve (*19,44*). Autophagy is a complex process involving the degradation of abnormal proteins and organelles by autophagosomes (*45*). It maintains metabolism and homeostasis by selectively and non-selectively sequestering macromolecules and organelles, recognizing autophagy-selective substrates through specific receptors (*46*). Not only does autophagy play a key role in normal cellular metabolism, but it is also closely related to the onset and progression of many diseases (*47*). In the ovaries, autophagy is involved in the regulation of several physiological processes, including follicular development, atresia, and luteal degeneration, where it acts as a balance to maintain cellular homeostasis (*48*). Studies have shown that abnormal levels of autophagy are closely associated with the development of POF, and its specific role is as a "double-edged sword." Basic levels of autophagy can help remove damaged mitochondria and proteins and maintain granulocyte activity, but over-activated autophagy can lead to programmed cell death. Apoptosis, the main form of programmed cell death, is closely associated with POI and is thought to be the primary mechanism of cell death in oocytes lost during maturation from primordial follicle to antral follicle or secondary to chemotherapy (*49*). Normally, apoptosis is involved in regulating follicular atresia and maintaining the homeostasis of the primordial follicle pool. However, excessive activation of apoptosis

is an important feature of POF and can lead to premature depletion of the primordial follicle pool (*50,51*).

There is a complex relationship between autophagy and apoptosis. During mild stress, autophagy is activated as a protective mechanism to promote cell survival. However, when an injury is severe, autophagy can switch to a pro-death mechanism that acts in concert with apoptosis. This transition is regulated by multiple signaling pathways, as shown in Figure 1. Under nutrient-rich conditions, the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which is activated by rapamycin, inhibits the Unc-51-like autophagy-activating kinase 1 (ULK1) complex. When nutrient levels are low, AMP-activated protein kinase (AMPK) becomes activated, which leads to the inhibition of mTORC1 and the subsequent activation of the ULK1 complex (*52,53*). Under endoplasmic reticulum stress, JNK activation phosphorylates Bcl-2, causing the release of Beclin-1. Further disruption of Bcl-2/Beclin-1 interaction is caused by JNK-phosphorylated BIM, resulting in the activation of the PI3K complex, consisting of VPS34 and Beclin-1, by ULK1 (*54*). This generates PI3P and initiates autophagic nucleation (*55*). Dysregulation of the autophagy-apoptosis balance is a key component in the pathogenesis of POF. Studies have shown that the autophagy-related proteins LC3-II and Beclin-1 are abnormally expressed in the ovarian tissue of POF patients, along with increased activity of the apoptosis marker caspase-3 (*42,56*). This dysregulation of autophagic apoptosis accelerates the depletion of the follicle pool.

Experimental studies have confirmed that a variety of herbal components and compounds can regulate the autophagy/apoptosis balance through different pathways. Baicalein inhibits excessive apoptosis through the PI3K/Akt pathway; curcumin can simultaneously activate protective autophagy through the AMPK/mTOR pathway while inhibiting apoptosis-associated protein expression (*57*). Resveratrol-βcd was able to restore the proportion and function of macrophages in the ovarian environment, delay cell autophagy and apoptosis, inhibit the progression of POF, and maintain normal ovarian function (*58*). Bushen Ningxin Soup can significantly reverse the VCD-induced reduction of primary follicles in ovarian tissue, increase FSH and luteinizing hormone (LH) concentrations, decrease serum E_2 and anti-Mullerian hormone (AMH) concentrations, decrease oocyte number and oocyte mitochondrial dysfunction, and alleviate POF (*59*). These findings provide a cell biological basis for TCM intervention in POF.

4. Therapeutic strategies based on the AMPK/mTOR pathway in TCM

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine kinase consisting of the catalytic subunit α (α1/α2), the regulatory subunit β (β1/β2) and γ (γ1/γ2/γ3) (*8*). AMPK acts as an intracellular energy receptor present in all eukaryotic cells and maintains cellular energy homeostasis mainly by regulating metabolic and immune functions. When intracellular energy levels decrease, AMPK is activated, which in turn inhibits energy-consuming anabolic processes (*e.g.*, mTOR-dependent anabolic pathways) and activates energy-producing catabolic processes (*e.g.*, autophagy) (*60*).

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase belonging to the phosphatidylinositol kinase-associated kinase (PIK) family and is a key regulator of cellular growth and metabolism that functions primarily by binding to cofactors to form two complexes, mTORC1 and mTORC2 (*61,62*). mTORC1 has been widely studied; it is sensitive to rapamycin and is mainly involved in ribosome and protein synthesis (*63*), cell autophagy (*64*), and lipid and glucose metabolism. The activity of mTORC1 is regulated by a variety of factors, including the availability of nutrients, growth factors, and energy status. When nutrients are sufficient, mTORC1 is activated to promote cell growth and anabolism, whereas when energy is insufficient, mTORC1 activity is inhibited and the cell enters a catabolic state. There are currently limited studies on mTORC2 in regard to cytoskeletal regulation, cell migration, and apoptosis.

Studies have shown that AMPK and mTORC1 are both key regulators of autophagy in response to various stressful conditions (*8*). mTORC1 activity is inhibited by AMPK through phosphorylation of tuberous sclerosis complex 2 (TSC2) (*8,65*), which inhibits cell growth and anabolism and promotes autophagy. Conversely, mTORC1 can inhibit the activity of AMPK by phosphorylating the α-subunit of AMPK, thereby maintaining the anabolic state of cells. This reciprocal regulatory mechanism allows cells to flexibly adjust their metabolic activity according to energy and nutrient status to maintain cellular homeostasis.

4.1. The AMPK/mTOR pathway and oxidative stress

The AMPK/mTOR signaling pathway is an important bridge between cellular energy metabolism and oxidative stress. As a cellular energy receptor, AMPK is activated during oxidative stress and mitigates oxidative damage through multiple pathways. On the one hand, AMPK can directly phosphorylate and activate the antioxidant transcription factor Nrf2 to promote the expression of antioxidant enzymes such as HO-1 (Heme Oxygenase-1) and NQO1 (NAD(P)H Quinone Dehydrogenase 1) (*66*). On the other hand, AMPK can reduce the source of ROS production by inhibiting mTOR, as overactivation of mTOR increases mitochondrial electron transport chain activity and ROS generation (*67*).

Research has found that in animals with POF, reduced AMPK activity associated with mTOR overactivation

led to increased oxidative damage in ovarian tissue. The use of AMPK agonists such as AICAR can significantly improve this situation. TCM renal tonics such as Icariin have been shown to enhance ovarian antioxidant capacity through activation of the AMPK/Nrf2 pathway (*68*).

Notably, activation of AMPK has dose- and time-dependent effects. Moderate activation of AMPK enhances cellular antioxidant defenses, but excessive or prolonged activation can lead to energy depletion (*69*). This explains why certain TCMs have antioxidative effects at low doses, while high doses may have the opposite effect. Therefore, the concept of 'balancing yin and yang' emphasized in the treatment of POF by TCM is highly compatible with the bidirectional regulation of the AMPK/mTOR pathway.

4.2. The AMPK/mTOR pathway and cellular autophagy-apoptosis

The AMPK/mTOR pathway is a central signaling pathway that regulates autophagy in cells, with AMPK initiating autophagosome formation by directly phosphorylating ULK1 (*70*) and simultaneously deregulating autophagy by inhibiting mTOR. Under conditions of energy stress, this regulation ensures that cells are able to maintain survival by recycling damaged components through autophagy. In apoptosis, AMPK reduces the expression of pro-apoptotic proteins by inhibiting mTOR while activating anti-apoptotic pathways such as CREB (*71*).

In POF, dysregulation of the AMPK/mTOR pathway leads to an imbalance between autophagy and apoptosis. Ovarian tissue showed blocked autophagic flux and excessive activation of apoptosis. TCMs such as Zuo Gui Wan can dual-regulate autophagy and apoptosis through the mTOR pathway: promoting protective autophagy to remove damaged mitochondria and inhibiting the caspase cascade reaction to reduce cell death (*72*). Tanghinin IIA, in contrast, restored autophagic flux and attenuated granulocyte apoptosis in an AMPK-dependent manner (*73*).

Of particular interest are possible differences in the regulatory role of the AMPK/mTOR pathway in different follicle types. Moderate autophagy in primordial follicles is important for maintaining dormancy, whereas growing follicles require more energetic support. The holistic regulatory features of TCM may be better suited to this complex situation, *e.g.*, kidney-tonifying drugs may regulate AMPK activity globally, while blood-activating drugs may be more targeted to the local microenvironment of specific follicle groups.

4.3. AMPK/mTOR pathway-based TCM for treatment of POF

Based on the multi-target regulatory properties of the AMPK/mTOR pathway, TCM has demonstrated unique therapeutic advantages in the treatment of POF. Its mechanism of action may regulate the AMPK/mTOR pathway through multiple links and pathways (Figure 2).

Kidney-tonifying herbs such as Radix rehumanize, Ginsenoside Rg1, Corni Fructus (*74*), and Epimedium glycoside are rich in polysaccharides and flavonoids, which can activate AMPK to boost antioxidant defenses. Experiments have shown that polysaccharides from Radix rehumanize can dose-dependently increase p-AMPK expression and improve ovarian reserve. Blood-activating drugs such as Salvia miltiorrhiza, Rhizome Ligusticum Chuanxiong, and Saffron contain phenolic acid components that inhibit mTOR overactivation and regulate autophagic flux. Liver-sparing drugs such as Chaihu and Xiangfu may indirectly affect AMPK activity by improving ovarian microcirculation.

In terms of compound studies, Yougui Pills may

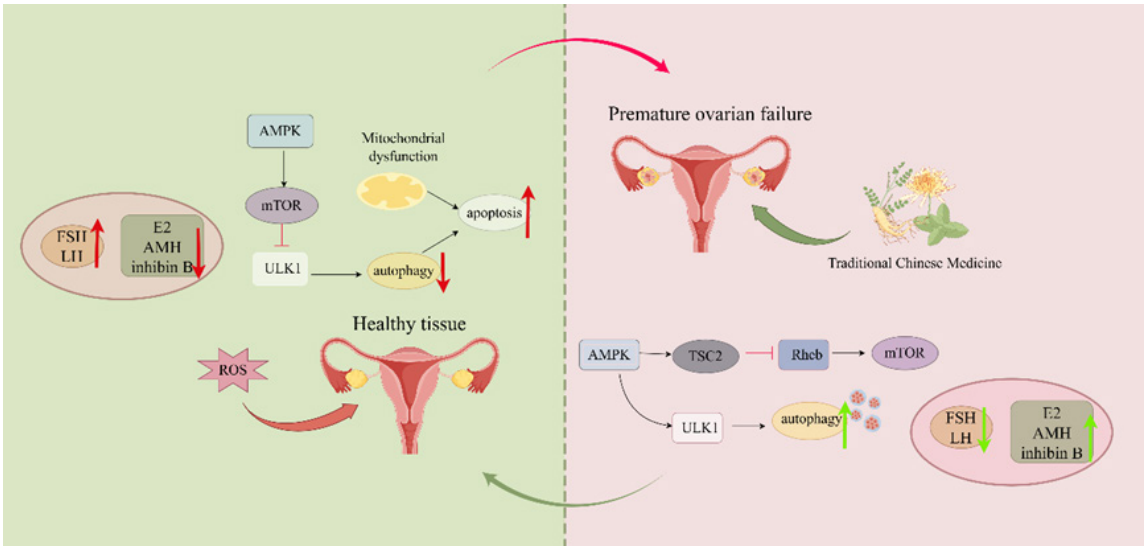

Figure 2. Schematic diagram of traditional Chinese medicine based on the AMPK/mTOR signaling pathway for the treatment of premature ovarian failure.

protect cyclophosphamide-injured ovaries by promoting autophagy and inhibiting apoptosis *via* the AMPK/mTOR pathway. The two immortal gums, turtle and deer, displayed the ability to regulate the AMPK/Nrf2/HO-1 pathway and reduce oxidative stress injury. These combinations reflect the 'multi-component, multi-target' nature of TCM, which is better suited to regulating complex network pathways such as AMPK/mTOR.

In addition, TCM can also treat POF through acupuncture and moxibustion (*20*). Acupuncture and moxibustion treatment for POF is based on the principle of "tonifying the kidney and filling the essence, regulating Chong Ren," and the main acupoints such as Guan Yuan, the Uterus, and Sanyinjiao, with evidence-based acupuncture points that are used to regulate the function of the hypothalamus-pituitary-ovary axis (HPOA) through acupoint stimulation, while directly affecting the local microenvironment of the ovaries. Acupuncture stimulation activates the phosphorylation of the AMPKα subunit, promotes the expression of Nrf2-mediated antioxidant enzymes (*e.g.*, SOD, CAT), and reduces the level of MDA in ovarian tissue. At the same time, it promotes autophagosome formation and scavenges protein aggregates that have accumulated due to oxidative damage by inhibiting mTORC1 signaling and upregulating Beclin-1 and LC3-II protein expression. Randomized controlled clinical trials have shown that acupuncture combined with HRT can significantly increase serum AMH levels in patients with POF and that its efficacy is superior to that of HRT alone, without hormone-related adverse effects.

Moxibustion is a traditional Chinese therapy using a burned moxa stick made from dried mugwort. The combustion of the mugwort permits transmission of heat to the body that causes various pathologic changes (*75*). Available research suggests that moxibustion therapy has significant therapeutic value in the areas of arthritis (including osteoarthritis of the knee) and pain management (*76,77*). This pleiotropic effect includes improvement of immune function and inhibition of oxidative stress and apoptosis (*78*). Moxibustion has also displayed unique benefits in the treatment of POF. Research has shown that moxibustion can help to regulate the menstrual cycle and increase blood flow to the ovaries (*79*). It can also balance hormone levels (such as E_2, aromatase, and testosterone) and enhance ovarian function (*80*). Its therapeutic principle is based on the TCM theory of 'warming the kidney and filling the essence, regulating the Chong ren,' which improves ovarian function by stimulating specific acupoints with warmth and heat. Commonly used acupuncture points include Guanyuan, Qihai, and Sanyinjiao. Together with back acupuncture points such as Ren Yu and Vital Gate, it constitutes a therapeutic program of anterior and posterior matching points. It increases ATP levels in ovarian tissue, activates the AMPK-sensing energy state, and then inhibits the over-activation of mTOR. Studies (*78,79,81*) have shown that moxibustion can effectively increase levels of E_2 and AMH while decreasing levels of the hormones FSH and LH. Moxibustion can also activate the Nrf2/HO-1 and PI3K/Akt signaling pathways in the ovaries, thereby reducing inflammatory injury and improving ovarian reserve function. Activation of the AMPK/mTOR pathway by moxa displays "biphasic regulation." In the early phase (1-2 weeks of treatment), it mainly inhibits mTOR and promotes autophagy to remove the damaged components; in the late phase (3-4 weeks), it maintains cellular homeostasis through the balance of AMPK/mTOR, which is highly compatible with the therapeutic principle of TCM, which is "first pass and then replenish."

5. Conclusion and future perspectives

POF is a complex gynecological endocrine disease with a pathogenesis that is closely related to the imbalance of oxidative stress and autophagy and apoptosis (*37,82*), and the AMPK/mTOR signaling pathway, as a core regulatory network linking energy metabolism, oxidative stress, and autophagy and apoptosis (*83*), plays a key role in the development of POF. This review systematically described the mechanisms of oxidative stress and autophagy-apoptosis in POF. This work has synthesized current evidence on TCM interventions targeting the AMPK/mTOR pathway and, for the first time, highlighted how the stage-specific regulatory roles of AMPK/mTOR signaling align with the TCM principle of syndrome differentiation and treatment.

Numerous studies have shown that TCM can effectively regulate the AMPK/mTOR pathway, reduce oxidative stress damage, and restore the balance of cellular autophagy and apoptosis through its multi-component and multi-target properties, thus improving ovarian function. These findings provide an important theoretical basis and practical guidance for the combined treatment of POF with Chinese and Western medicine (Table 1).

However, the current study had several limitations. First, most of the cited studies are still at the animal experiment or cellular level, with insufficient evidence for clinical translation. Second, studies on the mechanisms by which TCM regulates the AMPK/mTOR pathway lack depth, and there is a lack of studies on spatio-temporal specific regulation in particular. In addition, network pharmacological studies on the interactions between the complex components of TCM complexes and the AMPK/mTOR pathway need to go into further depth.

That said, combining Chinese and Western medicine in the treatment of POF is worth exploring. Examples include combining TCM with assisted reproductive technology to improve oocyte quality and endometrial tolerance by regulating the AMPK/mTOR pathway or combining TCM with HRT to reduce adverse effects and

Table 1. Summary of traditional Chinese medicine interventions for POF *via* oxidative stress and autophagy-apoptosis pathways

Name	Formula	Mechanism of action	Signaling pathway	Type of research	Experimental model	Key findings	*Ref.*
Red Ginseng, RG	Saponins, sugars, amino acid derivatives	Antioxidant, promotes granular cell proliferation	Nrf2/HO-1,PI3K-Akt	Animal experiments, cell experiments	Cyclophosphamide/ D-galactose (D-Gal)-induced POF model	Reduces the expression of aging-related proteins and has estrogen-like effects	(*84, 85*)
Dodder	Flavonoid, quercetin	Inhibits cell apoptosis	/	Animal experiments	Tripterygium glycoside /cyclophosphamide-induced POF	Promotes the restoration of hypothalamic-pituitary gonadotropic function, enhances the responsiveness of the pituitary gland and ovaries to hormones, and promotes follicular development	(*86, 87*)
Curcuma longa Linn	Curcumin	Reduce oxidative stress and inhibits apoptosis	AMPK/mTOR,Nrf2/ HO-1,PI3K-Akt	Animal experiments, cell experiments	Human ovarian granulosa cell line + mice with POF model	Alleviate ovarian dysfunction and apoptosis caused by oxidative stress	(*57, 88*)
Astragalus	Astragaloside IV	Inhibits oxidative stress and improves ovarian reserve function	Nrf2/ARE	Animal experiments	Cyclophosphamide induced POF in rats	Reduces apoptosis of granulosa cells in rats with POF and increases the number of primordial follicles, primary follicles, and antral follicles	(*89*)
Danggui Shaoyao Powder	*Paeoniae Alba Radix* (Bai Shao), *Angelicae Sinensis Radix* (Dang Gui), *Atractylodes Macrocephala Koidz* (Bai Zhu), *Poria* (Fu Ling), *Chuanxiong Rhizoma* (Chuan Xiong), *Alismatis Rhizoma* (Ze Xie)with a 1:3:4:4:8:8 ratio	Inhibits follicular atresia	AMPK/mTOR, PI3K/Akt/FOXO3a	Animal experiments	Cyclophosphamide/ D-galactose (D-Gal)-induced POF model	Raises estrogen levels, decreases the Bax/Bcl2 ratio	(*42, 90, 91*)
Dingkun Pill	*Ginseng Rubra Radix*(Hong Shen), *Cervi Pantotrichum Cornu* (Lu Rong), *Croci Stigma*(Xi Hong Hua), *Spatholobi Caulis*(Ji Xue Teng Gao), *Paeoniae Alba Radix* (Bai Shao), *Rehmanniae Praeparata Radix* (Shu Di Huang), *Angelicae Sinensis Radix* (Dang Gui), *Scutellariae Radix* (Huang Qin), *etc.*	Reduce oxidative stress and inhibit apoptosis	PI3K/Akt/mTOR	Animal experiments, Randomized controlled trial	Tripterygium glycoside induced POF	Upregulate the expression levels of Bax, Cyt C, and Caspase-3, downregulate the expression level of Bcl-2, and downregulate the ratio of Bcl-2 to Bax	(*92, 93*)

Table 1. Summary of traditional Chinese medicine interventions for POF *via* oxidative stress and autophagy-apoptosis pathways (continued)

Name	Formula	Mechanism of action	Signaling pathway	Type of research	Experimental model	Key findings	*Ref.*
Bu-Shen-Ning-Xin decoction	*Rehmanniae Praeparata Radix* (Shu Di Huang), *Paeoniae Alba Radix* (Bai Shao), *Corii Asini Colla*(E Jiao), *Coptidis Rhizoma* (Huang Lian), *Scutellariae Radix* (Huang Qin), *Poria* (Fu Ling), *Nelumbinis Plumula* (Lian Zi Xin), *Schisandrae Chinensis Fructus* (Wu Wei Zi), *Cornus*(Shan Yu Rou), *Uncariae cum Uncis Ramulus* (Gou Teng) and *Concha Cypraeae Violacae* (Zi Bei Chi)	Inhibition of oxidative stress	PI3K/AKT/mTOR, rno_circRNA_012284/ rno_miR-760–3p/ HBEGF(Heparin-binding epidermal growth factor-like growth factor)	Animal experiments, cell experiments	VCD induced the *in vivo* and *in vitro* POF model.	Reduces mitochondrial oxidative stress and apoptosis in ovarian germ cells (OGCs), improves ovarian microenvironment	(*59, 94*)
Yu Linzhu	*Panax ginseng*(Ren Shen)6g, *Poria* (Fu Lin)6g, *Atractylodes Macrocephala Koidz* (Bai Zhu)6g, Radix *Glycyrrhizae* (Gan Cao)3g, *Angelica Sinensis* (Dang Gui)12g, *Rehmanniae Preparata Radix* (Shu Di Huang)12g, *Ligusticum sinense* (Chuan Xiong)3g, *Paeoniae Alba Radix* (Bai Shao)6g, *Cuscutae Semen* (Tu Si Zi)12g, *Eucommia Ulmoides Oliv* (Du Zhong)6g, *Cervus nippon Temminck* (Lu Jiao Shuang)6g, *Zanthoxyli Pericarpium* (Hua Jiao)6g	improve oocyte mitochondrial function, ovarian oxidative stress, and ovarian microenvironment	hif1α/cx43,mTOR	Animal experiments, Randomized controlled trial	Mouse zona pellucida 3 (Zp3)/VCD induced POF model	Improves ovarian function by alleviating hormone levels, ovarian morphology, follicular development, the proliferation and energy metabolism of OGCs	(*95-98*)
Acupuncture and moxibustion	Guanyuan point, Sanyinjiaopoints	Inhibition of excessive autophagic damage in ovarian granulosa cells and reduction of follicular atresia	PTEN/AKT,PI3K/ Akt	Animal experiments, Randomized controlled trial	Cyclophosphamide-induced POF model	Regulate the expression of autophagy signaling pathways and key proteins, adjust the autophagy level of ovarian granulosa cells	(*99-102*)
Moxibustion	Guanyuan point, Zhongwan point, Shenque point	Improvement of reproductive hormone levels, reduction of inflammatory responses, modulation of immunity	PI 3 K/Akt/mTOR	Randomized controlled trial	Tripterygium glycoside induced POF	Increases estrogen levels and reduces inflammatory cell infiltration, thereby reducing inflammatory damage	(*78, 99*)

improve efficacy. As understanding of the AMPK/mTOR pathway grows and research into modernizing TCM continues, new avenues will open up for the prevention and treatment of POF.

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Review

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The medical metaverse in China: Current applications and future prospects

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SUMMARY: The medical metaverse, with its potential for efficient care delivery, improved patient outcomes, and reduced healthcare costs, is profoundly impacting global healthcare systems. Scholars researching this topic primarily focus on exploring specific scenarios for its use. This article aims to analyze the development trajectory, potential applications, and directions for management optimization within the medical metaverse. Through a case study of China, we review the current status of use of the medical metaverse and systematically examine its future prospects and challenges. We contend that the medical metaverse offers significant value in enabling equitable distribution of healthcare resources, enhancing medical care efficiency, promoting the integration of medical education, research, and clinical practice, and assisting in public health management. To ensure sustainable development, however, the imperative task is to proactively devise technical standards and legal regulatory frameworks and to dynamically monitor the effectiveness of medical metaverse technologies, with the ultimate aim of maximizing the value of the medical metaverse.

Keywords: medical metaverse, current uses, prospects, challenges, China

1. Introduction

The digital healthcare revolution is poised to create new opportunities for global health (*1*), addressing the escalating demand for care, the inequitable distribution of resources, and the urgent need for more efficient and resilient healthcare models (*2*). Within this transformative landscape, the metaverse, an emergent paradigm integrating technologies such as extended reality, artificial intelligence, blockchain, cloud computing, and digital twins, is progressively demonstrating its profound potential to reshape healthcare delivery and management (*3,4*). The metaverse's intrinsic characteristics are immersion, persistence, and decentralization. These offer innovative solutions to many persistent challenges in conventional healthcare. Such issues include geographical limitations, high training costs, and suboptimal patient experiences (*5*).

A global consensus on the medical metaverse concept has not yet been established (*6*). A multidisciplinary expert group, consisting of physicians and IT specialists from Asia, the United States, and Europe, published the *Expert Consensus on the Metaverse in Medicine* (*7*). Metaverse in medicine is considered to be the medical Internet of things implemented through augmented reality technology in this consensus (*8*). In this space, doctors, patients, medical devices, and data interact *via* avatars. This facilitates a wide range of medical activities, including remote diagnosis and treatment, medical training, simulated surgery, and health management. Currently, various countries are actively exploring pathways for use of the metaverse in healthcare. The United States (*9*) and Europe (*10*) lead in areas like product approval and setting data standards. Meanwhile, Asian nations, exemplified by South Korea (*11*) , Japan (*12*), and China (*13*), are proactively advancing pilot projects. This is often under strategic government guidance. However, global challenges persist. These include technological immaturity, data privacy concerns, and ethical issues. This is driven by pressing challenges: an aging population, a heavy burden of chronic diseases, and unequal distribution of medical resources.

While its practical applications are not yet fully mature, its development model offers unique research value. Existing research primarily focuses on the medical metaverse's potential (*14-16*) or uses related to specific diseases (*17*). However, most studies are limited to describing scenarios for use of a single technology. They thus lack systematic integration (*18*). Moreover, policy research is still highly limited. The unique Chinese

policy environment, and particularly its role in driving or constraining metaverse development in healthcare, has not been adequately discussed. This study therefore aims to address these critical gaps. We will utilize a systematic analytical framework covering policy, technology, and applications. This framework will comprehensively assess China's medical metaverse. It will evaluate its policy-driven mechanisms, current uses of technology, core scenarios, and key future development trends. The ultimate goal is to maximize the value of the medical metaverse in China.

2. Current uses of the medical metaverse in China

2.1. Policy support for the medical metaverse

2.1.1. National strategic guidance

The Chinese Government highly prioritizes both the digital economy and national health. It has successively issued key documents, including the Healthy China 2030 Planning Outline (*19*) and the 14th Five-Year Plan for Digital Economy Development (*20*). Their aim is to profoundly advance the integration of digital technology and healthcare. The 14th Five-Year Plan for Digital Economy Development explicitly promotes the integrated application of virtual reality (VR) and similar technologies. It also supports the innovative use of metaverse-related technologies within the medical sector. Moreover, the State Council outlined a strategic plan to accelerate metaverse development in its Three-Year Action Plan for Metaverse Industry Innovation and Development (2023–2025) (*21*). This plan focuses on key areas that include technological innovation, industrial applications, governance frameworks, and infrastructure development.

Key areas of support for the medical metaverse include: *i*) Digital Twins and Precision Medicine: Promoting the use of digital twin technology in clinical research, disease simulation, and personalized treatment. *ii*) Immersive Medical Training: Encouraging medical facilities to utilize VR or augmented reality (AR) technology. This involves creating virtual operating rooms and medical education environments. The aim is to enhance the training quality of healthcare professionals. *iii*) Smart Telemedicine: Supporting the development of remote diagnosis, virtual doctors, and AI medical assistants. These advances are based on metaverse technologies that aim to improve healthcare accessibility in remote areas. *iv*) Data Security and Privacy Protection: Formulating robust data security and privacy protection frameworks. This ensures that patient information is secure within the medical metaverse environment.

2.1.2. Regional support policies

Under national strategic guidance, local governments have actively responded (*22*) . They have successively issued industrial support policies. These policies prioritize "metaverse + healthcare" as a key direction of development (*23*). Local support policies are categorized into different phases based on differences in the timing of policy issuance and the frequency with which they are cited. Regional support policies are characterized by initial deployment in economically developed Eastern cities. This forms medical metaverse industrial clusters.

Different regions possess varying industrial structures. Consequently, their specific support approaches differ (*24*) . Some regions have incorporated the medical metaverse into their development plans. Examples include Shanghai's Action Plan for Cultivating New Metaverse Tracks (2022–2025) and Fujian Province's Three-Year Action Plan for Metaverse Industry Innovation and Development (2023–2025). Other regions are building medical metaverse industrial bases and ecological parks. For instance, Zhejiang Province's Digital Economy Development Leading Group Office issued the Guiding Opinions on the Construction of Future Industry Pilot Zones in Zhejiang Province. Some regions also implement measures to support the medical metaverse ecosystem. An example is Beijing's Eight Measures on Accelerating Innovation-led Development of the Metaverse in the Beijing Urban Sub-center. These policies explicitly support specific scenarios for use, such as remote diagnosis, virtual surgery, and intelligent monitoring. They also encourage collaborative innovation among medical facilities, technology companies, and universities. This fosters technological research, development, and practical application (*25*).

2.1.3. Industry standards

The National Health Commission actively promotes "Smart Hospital" and "Internet + Healthcare" projects. Similarly, the National Administration of Traditional Chinese Medicine and the National Administration of Disease Control and Prevention have developed relevant guidelines, such as the Reference Guide on Scenarios for Use of Artificial Intelligence in the Healthcare Sector. Though not explicitly mentioning metaverse technology, these policies' core requirements align closely with the medical metaverse. They encourage technologies like 5G, the Internet of things (IoT), and VR/AR to optimize diagnostic, treatment, and care models. Examples include remote surgical guidance and 3D digital imaging archives. These policies collectively remove obstacles for deployment of metaverse technology in hospital settings. They also provide clear entry points for its use.

2.2. Technological progress in the medical metaverse

2.2.1. Core driving technologies

The development of China's medical metaverse relies on a multi-dimensional technological ecosystem.

This ecosystem integrates core technologies, digital infrastructure, interactive terminals, and a foundation. Its progress stems not from a single technological breakthrough, but from the innovation and integration of multiple technology clusters (*26*). Figure 1 illustrates the overall technical architecture of the medical metaverse. It emphasizes how foundational technologies support upper-layer applications. The definitions of these technologies, along with their digitalization processes, have been thoroughly explored in similar studies (*27,28*).

Unlike conventional communication technologies, the medical metaverse relies on a core cluster of technologies. These include extended reality, digital twins, and blockchain (*29*). Extended reality (XR), VR, AR, and mixed reality (MR) bridge the gap between virtual and physical worlds. VR creates fully immersive virtual environments through computer-simulated visuals and audio *via* head-mounted devices (*30*). Its key features are immersion, interactivity, and imagination. VR plays a vital role in medical education, psychotherapy, and rehabilitation. AR overlays virtual information onto real-world scenes using localization algorithms (*31*). It is characterized by presence, augmentation, and relevance. AR is widely used in remote surgical guidance, intraoperative navigation, and patient education. MR combines VR's immersion with AR's overlay capabilities for extensive interaction of the virtual world and real world (*32*) . Its features include presence, blending, and realism. MR is utilized in drug discovery and expert virtual consultations. Digital twin technology uses sensors to gather real-world data, digitizing it to create corresponding virtual models. Researchers develop human digital twin models from physical data for medical diagnosis and assessment. Blockchain technology provides data security and privacy protection for the medical metaverse. It uses distributed ledgers and cryptographic algorithms to ensure secure storage of and authorized access to medical data. Blockchain also creates unique digital identities. This prevents identity fraud and data tampering while enhancing system transparency and efficiency.

Support from infrastructure like the IoT and 5G/6G networks is crucial (*33*). They ensure real-time transmission of massive data between terminals and cloud servers. This guarantees smooth immersive experiences and synchronized remote operations. Interactive terminals, such as wearables and digital tools like 3D modeling, provide direct user experiences within the medical metaverse. Notably, existing digital healthcare systems also form a foundational basis for metaverse development (*34*). Mature telemedicine platforms, for instance, have resolved issues with cross-regional medical collaboration. Their established communication standards and workflows lay the groundwork for more immersive metaverse consultations. Digital hospitals and smart wards enable administrators to simulate and optimize resource allocation, personnel flow, and infection control within the metaverse (*35*). These systems serve as early forms of the metaverse. They also provide gathering of crucial data, process validation, and user education for its full-scale development.

2.2.2. Technology landscape

Building on foundational technological breakthroughs, China's medical metaverse technology ecosystem is steadily expanding, as detailed in Table 1. Leading Chinese companies, including Tencent, Huawei, and

Real-World Information								Policies, Regulations, and Standards
	Application Layer	R&D	Prevention	Diagnosis	Treatment & Rehabilitation	Education & Innovation	Industrial Applications	
	Tool Layer	Modeling Tools	Rendering Tools	Development Engines	Interaction Tools	Peripheral Tools	Virtual Asset Tools	
	Data Layer	Data Acquisition	Data Transmission	Data Storage	Data Analysis	Data Processing	Data Validation	
	Perception Layer	Visual: Extended Reality Devices	Auditory: Speech Recognition Systems	Haptic: Somatosensory & Wearable Devices	Olfactory: IoT Sensors	Neural Devices: Brain-Computer Interface	Virtual Humans, Bionic Robots	
	Hardware Layer	Hardware Components	Computing	Communication Networks	Operating Systems	Technologies		
	Foundation Layer	Chips	Cloud Computing	5G	Desktop OS	AI Technology	Blockchain Technology	
		Sensors	Edge Computing	6G	Mobile OS	IoT Technology	XR Technology	
		Optical Components	Spatial Computing	WiFi	Server OS	Web3.0	Digital Twin	

Figure 1. Technical architecture of the medical metaverse. The figure details its layered technical architecture, from the foundation to applications. It includes hardware, data, tools, and specific medical use cases. This illustrates the metaverse's comprehensive strategy for technological integration and healthcare innovation.

Table 1. Uses of core technologies in the medical metaverse in China*

Technology layer	Core technology	Key players	Key devices	Areas of use
Foundation layer	Chips	Huawei, Cambricon, Sugon	Domestic GPU/ASIC chips	Medical AI computing, Image processing
	Cloud/Edge computing	Alibaba Cloud, Tencent Cloud, Huawei Cloud	Medical cloud servers, Edge nodes	Imaging cloud platforms, Remote surgery
Hardware layer	XR devices	Huawei, Pico, DPVR	AR/VR headsets, Holographic devices	Surgical navigation, Medical education
	BCI	Zhejiang Univ., Tsinghua Univ.	EEG signal devices	Neuro-rehabilitation, Consciousness disorder treatment
	Medical wearables	Huami Tech, Yuwell Medical	Smart bands, Vital sign monitors	Chronic disease management, Post-operative monitoring
Data layer	Medical big data	National Health Medical Big Data Center, Ping An Health	Federated learning, Blockchain systems	EHR sharing, Epidemic prediction
	Privacy computing	Ant Group, WeBank	MPC frameworks	Cross-institutional data collaboration, Privacy protection
Tool layer	Digital twin modeling	Siemens Healthineers, United-Imaging	Unity/Unreal, Medical imaging software	Organ 3D reconstruction, Surgical simulation
	Virtual surgery engine	Dmicoda, Weishu Zhiyuan	VR surgical planners, Mechanics simulators	Surgical training, Surgical plan rehearsal
	AI-assisted diagnostic tools	Infervision, Deepwise	Imaging AI platforms	Lung cancer screening, Fundus lesion identification
Application layer	Prevention	Ping An Good Doctor, DXY.cn	VR fitness devices, Health apps	Virtual health communities, Disease prevention education
	Diagnosis	Tencent Health, Ali Health	AI+VR imaging systems	Early tumor diagnosis, Remote imaging consultation
	Treatment & rehabilitation	Beijing Xuanwu Hospital, Shanghai Ruijin Hospital	VR therapy pods, BCI rehab devices	Anxiety treatment, Parkinson's rehabilitation
	Medical education	Shanghai Jiao Tong Univ., West China Hospital	VR anatomy labs, Surgical simulators	Medical student skill training, Clinical operation assessment
	Medical innovation	Baidu, WuXi AppTec	Virtual drug lab platforms	Molecular structure simulation, AI-assisted drug design

**Data Sources*: *Metaverse Development Research Report 3.0* (2022). Metaverse Culture Lab, Tsinghua University; *Global and China Metaverse Industry Analysis Report* (2023). Huajing Research Institute.

Abbreviation: XR: extended reality; AI: artificial intelligence; VR: virtual reality; GPU: graphics processing unit; ASIC: application-specific integrated circuit; EHR: Electronic Health Record; EEG: Electroencephalography; MPC: multi-party computation; BCI: brain-computer interface; Tech: technology; Univ: university.

Note: The entities listed are illustrative examples of active participants in the development of medical metaverse technologies in China, identified from public sources. Their inclusion does not indicate endorsement or commercial affiliation. The authors declare no conflicts of interest.

Alibaba, are at the forefront of technological research and development, and implementation of applications. Uses of different technologies involve tiered characteristics. For instance, technologies such as remote diagnosis and virtual medical education have been used on a large scale. They demonstrate significant social value and are now core scenarios within the medical metaverse. Scenarios for use of AI+XR diagnostics and intelligent monitoring are continuously expanding. Technologies such as medical non-fungible tokens and brain-computer interfaces remain in the early exploratory stages. They hold immense potential but face numerous challenges. Other technologies, including hardware devices and blockchain data sharing, are mature in their application. However, they require further upgrading and optimization.

2.3. Typical scenarios for use of the medical metaverse

The healthcare ecosystem involves diverse participants, including patients, clinicians, practitioners, government, academia, and industry. Thus, uses of the medical metaverse span the entire gamut of healthcare. They range from R&D, prevention, diagnosis, treatment, and rehabilitation to education and innovation. This promotes collaborative healthcare development (*15,36,37*), as shown in Figure 2.

A. Drug development and clinical translation: Metaverse technology accelerates drug screening and clinical trials. For instance, simulating drug-target

Figure 2. Typical scenarios for use of the medical metaverse. The figure illustrates the metaverse's innovative uses in drug R&D, surgery, diagnosis, and treatment, rehabilitation, medical education, and patient monitoring, highlighting the extensive integration of virtual technology into healthcare.

interactions significantly reduces early drug discovery cycles (*38*). A cutting-edge direction combines brain-computer interfaces with VR for clinical translation. In 2020, Zhejiang University completed a clinical translation study of China's first implanted BCI (*39*). A high-level quadriplegic patient successfully controlled a virtual arm in a VR environment *via* thought and then manipulated an external robotic arm for complex 3D movements.

B. Disease diagnosis and surgical treatment: Medical diagnosis and treatment represent the most impactful area for use of the medical metaverse. Its core value lies in making invisible lesions and anatomical structures visible and interactive (*40,41*) . In 2019, Jin *et al.* (*42*) proposed the Holographic Digital Human. This concept involves using 100 ZB of medical data to build full diagnostic and treatment process models, aiming to upgrade care systems. A Zhongshan Hospital team at Fudan University conducted lung cancer screening *via* the IoT (*43*). Their auxiliary application, PNapp5A, uses cloud computing for deep analysis, intelligent assessment, and management of lung nodules. This significantly reduces the healthcare system burden (*44*). A Beijing Chang Gung Hospital team, affiliated with Tsinghua University, used AR for intraoperative simulations and guidance in complex neurosurgery. This enabled surgeons to clearly visualize deep structures during lesion removal, effectively avoiding damage to critical functional areas (*45*). Research has shown that VR-assisted planning for pancreatic cancer surgery achieved 100% treatment accuracy and sensitivity compared to conventional 2D imaging planning. Average intraoperative blood loss was also effectively controlled (*46*).

C. Rehabilitation medicine and mental health: The metaverse can aid patients in receiving contextual and behavioral information. Its immersive quality offers unparalleled advantages for rehabilitation and psychotherapy compared to conventional methods. A meta-analysis on post-stroke upper limb dysfunction showed that, at the 6-month long-term follow-up, the motor function scores of the VR therapy group improved significantly more compared to the conventional rehabilitation group (mean improvement of 3.9 *vs.* 1.5 points, p=0.045) (*47*). The Shanghai Mental Health Center, a leading Chinese facility, has incorporated VR exposure therapy into routine clinical practice. It independently developed a series of virtual scenarios for specific phobias (*48,49*). Examples include simulated public speaking, virtual elevators, and high-rise edges.

D. Medical education and training: The metaverse marks a significant milestone in medical education. It is addressing the challenges of the high risk and high cost of practical medical instruction. In 2022, Yang *et al.* (*50*) developed a virtual training project for rigorous closed chest drainage in clinical nursing. This project integrates theoretical learning, human-computer interaction, and intelligent assessment. It effectively enhances nurses' technical skills, critical thinking, and emergency response capabilities. Shanghai Jiao Tong University's School of Medicine created an oral and maxillofacial surgery simulation training system. By creating personal digital twin training profiles for each trainee, it enabled personalized and precise training. Data have shown that trainees who used this system had a more standardized technique and better understanding of complex anatomical structures, effectively reducing the time to transition from a novice to a qualified surgeon (*51*).

E. Disease prevention and health management: The medical metaverse brings significant benefits to individual health management and broader public health governance. He's team applied the XGBoost algorithm to EHR and wearable device data (*52*). They created an electronic frailty index to predict frailty risk and adverse event rates in elderly patients during and after hospitalization. This allows a shift from passive treatment to proactive prevention. A comparative study on uses of VR health education indicated significant improvements (*53*). Users of VR had 22% higher comprehension scores and a nearly 30% higher retention rate a week later compared to a 2D video control group. They also reported significantly higher intent to be vaccinated, demonstrating vast potential for promoting healthy behaviors.

F. Collaborative healthcare development: Both international and domestic regional healthcare development currently face resource imbalances. The medical metaverse, through its remote presence, data integration, and immersive collaboration features, is overcoming geographical barriers. It promotes a more equitable and efficient collaborative healthcare ecosystem. In 2021, Professor Ye's team at Huazhong University of Science and Technology's Tongji Medical College successfully conducted three remote consultation surgeries for frontline soldiers and border residents, respectively located 3,600 km and 4,500 km away, using MR cloud platform technology (*54*). The average network latency during the entire remote collaborative process was below 30 ms, fully meeting real-time surgical requirements, with no complications observed.

3. Prospects for medical metaverse development in China

3.1. Prospects for use

Global statistics indicate the metaverse's global healthcare market was valued at $5.06 billion in 2021. It is projected to reach $ 71.97 billion by 2030, with a compound annual growth rate (CAGR) of 34.8% during the forecast period (2022–2030) (*55*). China's initial exploration in medical metaverse policy, technology, and scenarios for use suggests the trajectory for its future development is already emerging.

3.1.1. Balancing healthcare resource allocation

China urgently needs medical metaverse support. This is due to highly concentrated and regionally imbalanced quality healthcare resources. Remote collaborative surgical networks are essential to achieving equitable access to premium medical resources (*56*). In the future, a mature medical metaverse could foster a collaborative healthcare system. Oncology, imaging, and pathology experts from different countries could conduct synchronized consultations. They could jointly manipulate a patient's 3D digital twin model. This would enable collaborative development of optimal treatment plans (*57*). Expert digital humans, trained on vast high-quality diagnostic and treatment data, could continuously provide standardized, expert-level auxiliary diagnostic advice to doctors on the ground. This would greatly enhance the homogenization of local medical care. This represents not just a technological extension, but a reshaping of healthcare equity.

3.1.2. Enhancing healthcare efficiency

Metaverse technology will drive healthcare transformation from passive treatment to proactive health management. The core lies in a comprehensive improvement of efficiency and personalization. Individual digital models will integrate all patient data, from genomics, lifestyle, and environmental exposure to medical history. These models will dynamically simulate health trajectories. They could precisely predict major disease risks, such as cardiovascular disease and diabetes, years in advance. They will also simulate the long-term impact of various interventions on health. This will truly provide predictive and preventive medicine (*58*). To some extent, it can alleviate China's dual challenges of the chronic disease burden and an aging population.

3.1.3. Promoting integrated medical education, research, and practice

Current challenges in China's healthcare system include difficulty promoting quality core technologies. There are also long training cycles for large-scale elite personnel and limited translation of clinical research. The metaverse holds the promise of bridging clinical practice, education, and research (*59*). An iterative metaverse medical education, research, and practice platform is envisioned. Experts could perform complex surgeries, with all operative processes — from minute instrument movements to critical decision points — anonymously recorded. These data could be instantly fed to teaching and research platforms, significantly reducing the clinic-to-lab-to-clinic translation cycle.

3.1.4. Assisting public health decision-making

China faces severe challenges due to its massive population base, high-density urban clusters, and increasing openness. Conventional public health emergency response systems are becoming insufficient. The medical metaverse can provide city-level, and even national-level, public health digital twin systems (*60*). Specifically, three functions can be performed: *i*) Real-time monitoring: Viral transmission hotspots, population mobility trends, and real-time utilization of medical resources can be depicted in 3D. *ii*) Precise prediction: Simulations can assess the potential impact of various interventions. This allows selection of strategies with the lowest socio-economic cost and optimal effectiveness. *iii*) Efficient command: Wearable devices can deliver visualized, precise instructions to frontline personnel. Those devices can also acquire real-time feedback. This will enable a new model of prediction, precise decision-making, and efficient collaborative governance.

3.2. Development challenges

The metaverse integrates numerous advanced technologies. It holds immense potential across medical diagnosis, treatment, rehabilitation, and education. It promises an unprecedented smart healthcare ecosystem, capable of accelerating self-evolution and knowledge iteration. However, this profound paradigm shift also introduces new ethical, governance, and social challenges (*61*).

The first is the technological cost. Effectively transforming existing healthcare systems with the metaverse requires robust hardware. This includes specialized glasses, sensors, and other devices to accurately ascertain a patient's condition. However, such equipment is costly (*62*). Moreover, the metaverse demands high-level connectivity for efficient operation. This results in massive infrastructure costs for providers. It places significant financial demands on healthcare administrators.

The second challenge is technical bottlenecks. Healthcare interactions between patients and doctors, or patients and treatment resources, are frequent and complex. This leads to immense volumes of data stored and transmitted within the metaverse. It also lowers the sensitive threshold for network latency. Computing power demands will consequently surge (*63*). In-depth research on and use of edge computing, distributed systems, and blockchain technology are potential solutions to this issue.

The third challenge is the cognitive divide. As an emerging technological paradigm, the medical metaverse differs significantly from conventional healthcare models in its operational logic and methods of interaction. This leads to clear disparities in acceptance and ability to use technology among doctors and patients. The cognitive divide is essentially a challenge of cultural adaptation to the digital transformation of healthcare practices. Research indicates that level of education, years in the profession,

income, and social norms all influence acceptance (*64*).

The fourth challenge is data privacy and security. The medical metaverse collects and extensively shares sensitive patient data, including EEG, biometrics, and health preferences. This raises significant data privacy and surveillance concerns (*65*). Existing policies primarily promote industry growth, offering only principle-based statements on risks like data sovereignty and virtual medical liability. Actionable details on implementation are lacking. Consequently, addressing emergent risks, such as XR medical accidents or BCI ethical disputes, may lead to regulatory evasion and delayed emergency responses.

3.3. Directions for policy optimization

Rapidly developing due to policy and innovation, the medical metaverse optimizes healthcare but also introduces risks. While strong in resource integration, current policies need enhanced technical security, cross-border data flow, and ethical frameworks. An updated governance framework is thus essential, following historical expansions seen with EHRs (*66*) and online services (*67*).

Technologically, top-level design must embed security and privacy. Scholars suggest minimal privilege access (*68*) and biometric blockchain authentication for data protection (*69*). Ethically, dedicated committees should set clear standards for digital human doctors, affective computing, and immersive therapies. Policy adaptation involves revising existing laws. For example, specific metaverse clauses under China's Personal Information Protection Law could allow blockchain-encrypted cross-border research data, similar to EU digital health strategies for data classification and whitelisting.

Cross-departmental collaboration is crucial. Key regulators (the National Health Commission, MIIT, and CAC) must come together on implementation, data governance, and industry standards. Joint innovation hub selection, offering limited policy exemptions for data sharing and new care models, is one example. Formulating joint emergency plans for novel risks like XR accidents or data breaches clarifies responsibilities and ensures consistent R&D, clinical use, and compliance.

A dynamic evaluation system enhances governance and manages market expectations given rapid metaverse expansion. The Gartner hype cycle can assess technical maturity, guiding differentiated policies (*70,71*). In terms of its value, a quantifiable system, based on scenario maturity, clinical need, market potential, and ethical risks, should identify high-value, sustainable uses, ensuring evidence-based decisions (*72*).

4. Conclusion

The development of China's medical metaverse is characterized by policy-driven growth, technological integration, and scenario innovation. Under the national "14th Five-Year Plan for Digital Economy" and "Healthy China 2030" strategies, local governments actively issue specific support policies. These policies drive the use of core technologies like XR, AI, and 5G. This includes scenarios such as drug R&D, remote diagnosis, surgical treatment, medical education, and intelligent monitoring.

Despite ongoing challenges in computing power bottlenecks, data privacy, and cognitive divides, the medical metaverse holds great future value. This can be realized through cross-departmental collaboration. Such cooperation will jointly advance technical standardization, legal and ethical regulation, and dynamic scientific evaluation. The metaverse is expected to enhance healthcare accessibility, precision, inclusiveness, and equity. This will provide a valuable reference for global digital healthcare development.

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Original Article

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Neurotropin alleviates Alzheimer's disease pathology by inhibiting FUS-mediated Calhm2 transcription, blocking the Calhm2/EFhd2 interaction, to improve mitochondrial dysfunction-associated microglia polarization

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SUMMARY: Neurotropin, a non-protein extract widely used for the treatment of neuropathic pain, has recently been reported to protect against ischemic brain injury, enhance remyelination in demyelinating diseases, and ameliorate neuroinflammation and memory deficits. However, its role in microglial polarization and mitochondrial dysfunction in Alzheimer's disease (AD) remains poorly understood. In this study, we investigated the therapeutic potential of Neurotropin in the 5xFAD mouse model of AD. Neurotropin administration alleviated cognitive decline, reduced amyloid-β (Aβ) deposition, suppressed neuroinflammation, and preserved neuronal density. Mechanistically, Neurotropin improved mitochondrial morphology, restored ATP production, increased mitochondrial DNA copy number, and reduced oxidative stress while promoting a shift in microglial polarization from the pro-inflammatory M1 phenotype toward the anti-inflammatory M2 phenotype. Transcriptomic and molecular analyses revealed that calcium homeostasis modulator family member 2 (Calhm2) was markedly upregulated in 5xFAD mice, colocalized with microglia, and transcriptionally regulated by fused in sarcoma (FUS), while Calhm2 interacted with EF-hand domain containing protein D2 (EFhd2). Neurotropin suppressed FUS-mediated Calhm2 transcription and attenuated Calhm2–EFhd2 interaction. Importantly, overexpression of Calhm2 in both microglial cells and 5xFAD mice abolished the beneficial effects of Neurotropin, leading to exacerbated mitochondrial dysfunction, oxidative stress, and inflammatory cytokine release. Together, these findings identify Calhm2 as a critical mediator of Neurotropin's neuroprotective effects and demonstrate that Neurotropin alleviates AD pathology by suppressing FUS-dependent Calhm2 transcription and blocking the Calhm2/EFhd2 interaction. This study provides new insights into the mechanism of Neurotropin action and highlights its therapeutic potential for AD.

Keywords: Neurotropin, Alzheimer's disease, FUS, Calhm2, EFhd2, mitochondrial dysfunction, microglia polarization

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia worldwide (*1*). With the ongoing increase in the aging population, both the prevalence and incidence of AD continue to rise (*2*). By 2050, the number of individuals affected by dementia is projected to triple globally, placing an immense emotional and financial burden on patients, families, and healthcare systems (*3*). Clinically, AD is characterized by progressive memory loss, cognitive impairment, and behavioral disturbances (*4*). The main pathological hallmarks of AD include extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles composed of phosphorylated Tau protein (*5*). The intricate interaction between Aβ and Tau synergistically contributes to AD pathogenesis (*6*). Despite extensive efforts, clinical trials targeting Aβ or Tau alone have yielded limited success, underscoring the urgent need to identify and validate novel therapeutic targets for AD.

Neurotropin, an established analgesic derived from inflamed rabbit skin inoculated with the vaccinia virus, has been widely prescribed for the treatment of neuropathic pain in Japan and China for the past 50 years, with a well-documented safety profile (*7*). Beyond its analgesic effects, emerging evidence from animal studies suggests that Neurotropin also exerts notable neuroprotective properties (*8*). For example, three months of Neurotropin administration significantly improved spatial cognitive impairment in a Down syndrome mouse

model carrying triplication of 65% of human trisomy-21 genes (*8*). Despite these promising findings, research on Neurotropin in the context of AD remains scarce, and its potential therapeutic value and underlying mechanisms in AD have yet to be fully elucidated.

Microglia, the innate immune cells of the central nervous system (CNS), play a complex role in AD by mediating neuroinflammation, phagocytosis, and neurodegeneration (*9*). Upon activation, microglia can polarize into two major phenotypes: the pro-inflammatory M1 type, which releases cytokines that exacerbate neuronal damage, and the anti-inflammatory M2 type, which promotes repair and tissue homeostasis (*10*). Upon accumulation of Aβ plaques, microglia become excessively activated and secrete pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), IL-6, and necrosis factor-alpha (TNF-α), thereby aggravating AD pathology (*11*). As AD progresses, the phagocytic capacity of microglia to clear Aβ plaques declines, resulting in increased plaque accumulation and disease progression (*12*). Furthermore, Aβ interacts with receptors of microglial cells, such as triggering receptors expressed in myeloid cells 2 (TREM2), initiating downstream pathways that cause mitochondrial injury and amplify cytotoxicity and inflammation, thereby accelerating AD progression (*13*). Based on these observations, we hypothesize that Neurotropin may attenuate M1-type microglial activation and protect against mitochondrial dysfunction in AD.

Calcium homeostasis is intricately connected to microglial activation. Recent research has increasingly focused on the role of calcium homeostasis modulator family proteins (Calhm), including Calhm1, Calhm2, and Calhm3, in AD (*14*). Calhm1, the most studied member, regulates calcium homeostasis, Aβ production, and neuronal vulnerability to Aβ-induced toxicity (*15*). The P86L mutation in Calhm1 is linked to a higher incidence of AD (*16*). Additionally, studies have shown that Calhm3 interacts with Calhm1, and the absence of Calhm3 abolishes taste-induced ATP release (*17*). Calhm2, highly expressed in the mouse brain, regulates ATP release in astrocytes (*14*). Loss of Calhm2 results in a depression-like phenotype in mice that significantly reduces Aβ deposition and neuroinflammation, and alleviates AD-related cognitive impairments (*14*). Our preliminary data indicated that Calhm2 expression was elevated in 5xFAD mice, whereas treatment with Neurotropin reduced Calhm2 expression in 5xFAD mice. These findings indicate that Calhm2 may play a critical role in mediating the therapeutic effects of Neurotropin in AZ.

Results from the bioinformatic prediction tool also suggest that fused in sarcoma (FUS) may potentially target Calhm2. FUS is an RNA/DNA-binding protein known to induce mitochondrial damage and mediate neurodegenerative pathogenesis (*18*). Additionally, FUS plays a critical role in stabilizing the mRNAs of its downstream target genes and can also function as a transcription factor (*19*). However, whether FUS participates in mediating microglial polarization and mitochondrial injury in AD and the underlying mechanism remains to be investigated.

Based on these findings, we hypothesize that Neurotropin alleviates AD by improving mitochondrial dysfunction and reducing microglial polarization through the inhibition of FUS-mediated transcriptional activation of Calhm2. This study may provide a theoretical basis for using Neurotropin as a treatment for AD.

2. Materials and Methods

2.1. Ethics statement

This research was conducted in strict accordance with the ARRIVE guidelines. The study protocol was thoroughly evaluated and received approval from the Ethics Committee of The First Affiliated Hospital of Nanchang University (Approval No. CDYFY-IACUC-202401QR002). To ensure humane treatment, mice were anesthetized using 3% isoflurane with oxygen as the carrier gas and subsequently euthanized with carbon dioxide.

2.2. Animal model of AD (5×FAD mice) and treatment

Five-month-old male 5xFAD transgenic (Tg) mice were purchased from Jackson Laboratory (stock no. 034848-JAX, Bar Harbor, ME, USA) and housed under a 12-hour light/12-hour dark cycle (lights on at 5:00 am) with free access to water and food; cages were cleaned weekly.

For the first experiment, 12 wildtype (WT, non-Tg) mice and 12 Tg mice were divided into four groups: non-Tg, non-Tg + Neurotropin, Tg, and Tg + Neurotropin (6 mice per group). Neurotropin (200 NU/kg, Nippon Zoki Pharmaceutical Co., Osaka, Japan) was administered orally using a Zonde needle daily from 5 to 7 months of age, while control groups received an equivalent volume of NaCl. At 8 months, these mice were also examined using behavioral tests for a continuous 5 days and were sacrificed. Afterward, the hippocampi from the mice were collected.

In the second experiment, 12 Tg mice were divided into Tg + Neurotropin + oe-NC and Tg + Neurotropin + oe-Calhm2 groups (6 mice each). Along with daily Neurotropin administration, stereotactic intracerebral injections of lentiviral particles (oe-NC or oe-Calhm2) were performed in month 6 of age based on a previously published protocol (*20*). Mice were anesthetized with 80 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride and fixed on a stereotactic frame. Lentiviral particles were injected into the cerebral cortex (anteroposterior = −0.3 mm, mediolateral = 2 mm, dorsoventral = −1.5 mm; anteroposterior = −2 mm, mediolateral = 1.2 mm, dorsoventral = −1.2 mm) and

hippocampi (anteroposterior = −2 mm, mediolateral = 1.2 mm, dorsoventral = −2 mm) using a micropipette attached to a 10-μL Hamilton syringe. At 8 months, these mice were also examined using behavioral tests for a continuous 5 days and were sacrificed. Afterward, hippocampi from the mice were collected. Hippocampi from 3 mice of each group were used for histological staining. The left hippocampi from 3 mice were reserved for transmission electron microscopy (TEM), and the right hippocampi were kept for other analyses. The detailed study design is summarized in Chart 1 of Supplementary materials (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=272*).

2.3. Morris water maze

Hippocampal-dependent memory and cognitive abilities were evaluated using established procedures. Mice were placed in a circular swimming pool with an 81 cm diameter, filled with water maintained at 24-25°C. An escape platform, 10 cm in diameter, was positioned 0.5 cm beneath the water's surface in the center of one quadrant. During the acquisition phase, mice were individually released into the water from random starting points along the pool wall in each of the four quadrants. This process was repeated four times daily for five consecutive days. Each trial allowed a maximum of 90 seconds for the mouse to locate the hidden platform. Once the platform was found, the mouse was allowed to remain on it for 15 seconds. If the mouse failed to find the platform within 90 seconds, it was guided to the platform by an experimenter. The time taken to reach the platform (escape latency) was recorded, with a maximum limit of 90 seconds. Swimming speed and the number of times the mice crossed the previous platform location were also recorded and analyzed using ANY-maze behavioral tracking software (Stoelting Co., Wood Dale, IL, USA).

2.4. Immunohistochemistry (IHC) staining.

Hippocampi were post-fixed in 4% paraformaldehyde for 24 hours, then paraffin-embedded and sectioned at a thickness of 4 μm. Sections were rehydrated through xylene and graded ethanol, treated with 3% hydrogen peroxide, and blocked with 10% normal goat serum for 45 minutes at room temperature. Amyloid deposits were detected using an anti-β-Amyloid 1-16 antibody (# SIG-39155, 1:1000, Biolegend, San Diego, CA, USA). Before antibody incubation, hippocampal sections were pre-incubated with 70% formic acid at 4°C for 24 hours, then rinsed in PBS. The sections were then incubated with a biotinylated anti-mouse IgG1 antibody (#ab97240, 1:250, Abcam, Cambridge, UK) for 90 minutes at room temperature. Following washes in PBS, sections were treated with DAPI for 10 minutes. Finally, sections were coverslipped with permanent mounting medium (Vector Laboratories, Burlingame, CA, US). All stained sections were observed and photographed under a microscope (Eclipse Ni-U, Nikon Instruments Inc., Tokyo, Japan), and areas of Aβ1-42 staining were quantified using Image Pro Plus 6.0.

2.5. Immunofluorescence (IF) staining

Hippocampal sections were rehydrated, treated, and blocked as previously described. Slides were then incubated overnight at 4°C with one of the following primary antibodies: anti-neuronal nuclei (NeuN, ab177487, 1:100; Abcam), anti-ionized calcium-binding adapter molecule 1 (Iba-1, ab178846, 1:1000; Abcam), anti-CD16/32 (45-0161-82, 1:1000; Thermo Fisher Scientific), anti-Arginase 1 (Arg1, 711765, 1:1000; Thermo Fisher Scientific), anti-Calhm2 (19931-1-AP, 1:200; Proteintech), or anti- EF-hand domain-containing protein D2 (EFhd2, PA5-78575, 1:500; Thermo Fisher Scientific). The following day, sections were washed three times with PBS and then incubated with secondary antibodies (Abcam) for 2 hours at room temperature. Nuclear staining was performed using DAPI (Life Technologies, Waltham, CA, USA). Fluorescently labeled cells were visualized using an LSM 710 ZEISS microscope (Jena, Germany). Ten images per sample were captured and quantified using ImageJ.

2.6. Enzyme-linked immunosorbent assay (ELISA) assay

ELISA kits (ab108865, ab100713, and ab108910 from Abcam) were utilized to measure expression levels of IL-1β, IL-6, and TNF-α in serum and hippocampal tissues, according to the manufacturer's instructions. Regarding hippocampus tissue, they were homogenized in a mixture of phenylmethylsulfonyl fluoride (PMSF) and radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) at a ratio of 10 μL PMSF to 1 mL RIPA lysis buffer, kept on ice. The homogenates were centrifuged at 12,000 rpm for 5 minutes at 4°C, and supernatant protein concentrations were determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). For each sample, 5 μL of extracted protein was used for detection. Absorbance was measured at 450 nm using a spectrophotometer, and concentrations were calculated using a standard curve.

2.7. TEM

The CA1 region of the left hippocampus was carefully dissected and fixed in 3% buffered glutaraldehyde. This was followed by post-fixation in 1% osmium tetroxide. The samples were then dehydrated using a series of increasing ethanol concentrations and embedded in Epon 812. The ultrastructure of the hippocampal CA1 subregion was visualized with a TEM (Philips,

Amsterdam, Netherlands). Images were taken from five randomly selected sections. These images were analyzed for morphometric parameters using Image Pro Plus 6.0. Stereological methods used for analyzing synapses and mitochondria were as previously described. Measurements included mitochondrial length (mito length)/mitochondrial width and copy number of mitochondrial DNA (mtDNA).

2.8. Mitochondrial membrane potential (MMP) assay

A mitochondrial membrane potential assay kit with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (C2006; Beyotime Institute of Biotechnology) was used to detect MMP. JC-1 accumulates in mitochondria with higher membrane potentials, forming J-aggregates that emit red fluorescence (Cy3, excitation/emission wavelength of 525/590 nm). In mitochondria with lower membrane potentials, JC-1 remains in monomeric form, emitting green fluorescence (fluorescein isothiocyanate [FITC], excitation/emission wavelength of 490/530 nm). A decrease in the red: green fluorescence ratio indicates a decrease in MMP. For this assay, 0.1 mL of purified mitochondria (protein concentration 0.2 mg/mL) from different groups were incubated with 0.9 mL of 0.2X JC-1 staining working solution. A time scan was performed using a fluorescence microplate reader (Gemini EM Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) with an excitation/emission wavelength of 485/590 nm and observed under an Olympus BX5 fluorescence microscope imaging system (Olympus America, Melville, NY, USA).

2.9. Malondialdehyde (MDA) and superoxide dismutase (SOD) measurement

MDA is a byproduct of lipid peroxidation and serves as a reliable indicator of oxidative stress. Levels of MDA in the hippocampi of mice were measured using commercial assay kits from Beyotime Biotechnology Institute (S0131S) following the manufacturer's instructions. The measurement is based on reaction of one molecule of MDA with two molecules of thiobarbituric acid, yielding a pink-colored chromogen. Color intensity was measured at 532 nm, with a reference wavelength of 450 nm. The activity of SOD in hippocampus tissue samples was measured using commercial assay kits from Beyotime Biotechnology Institute (S0086), according to the manufacturer's instructions. The assay involves the reaction of nitroblue tetrazolium with superoxide anion, producing a blue-colored chromogen.

2.10. ATP content and activity of the respiratory chain complex I

ATP content of hippocampal tissues was measured using the Enhanced ATP Assay Kit (S0027, Beyotime Biotechnology) and the Electron Transport Chain Complex I Assay Kit (S0026, Beyotime Biotechnology), following the manufacturer's instructions. Results are presented as fold-change values relative to the control group (non-Tg mice).

2.11. Cell culture, treatment, and transfection

Mouse microglia BV2 cells were obtained from the American Type Culture Collection (ATCC) and routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco, Carlsbad, CA) in 6 cm plates. BV-2 cells were seeded in 6-well plates at a density of 1×10^5 cells per well for 24 hours. Cells were then treated with or without Neurotropin (0.1 NU/mL) for 12 hours before being exposed to lipopolysaccharide (LPS, 100 ng/mL, L2630, Sigma-Aldrich, St Louis, MO, USA) for an additional 12 hours. Cells were plated 24 hours before transfection at 70-80% confluency. Custom-designed small interfering RNA (siRNA) targeting FUS (si-FUS) and control siRNA (si-NC) were provided by Genesee Biotech and integrated into pCD513B-U6 plasmids by Genepharma (Shanghai, China) to construct siRNA vectors. For Calhm2 overexpression, the full-length Calhm2 sequence was amplified by PCR and cloned into pcDNA3.1 plasmids obtained from SBI (Mountain View, CA, USA). Empty plasmids were used as negative controls. Calhm2 overexpression transfection was carried out using Lipofectamine 3000 reagent according to the manufacturer's protocol. The small RNA interference transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.12. Mitochondrial reactive oxygen species (mtROS) production assay

To measure mitochondrial superoxide levels, cells were incubated with MitoSOX™ Red Mitochondrial Superoxide Indicator (11579096, Invitrogen, Ltd., UK) at 30°C for 10 minutes, then washed three times with PBS. SOD was added at a concentration of 40 U/mL as a negative control for superoxide after the MitoSOX™ Red treatment. Levels of mtROS were analyzed using a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.13. RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Trizol reagent (15596026, Invitrogen). One microgram of RNA was reverse transcribed into cDNA using the PrimeScript cDNA synthesis kit (6110A, Takara, Osaka, Japan) following the manufacturer's instructions. Quantitative PCR was then performed using the TaqMan® Universal PCR Master

Mix (4305719, Thermo Fisher Scientific, Waltham, MA, USA) and primers from Origen Biotech (Wuxi, Jiangsu, China), as listed in Supplementary Table S1 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=272*). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH expression.

2.14. Western blot

Proteins were extracted using radio-immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors at 4°C for 30 minutes (Beyotime Inc.). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (10741395, Thermo Fisher Scientific). For each sample, 30 μg of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore). Membranes were blocked and rinsed with PBS before being incubated with primary antibodies against Calhm2 (PA5-53219, 1:10,000, Thermo Fisher Scientific), EFhd2 (PA5-78575, 1:1,000, Thermo Fisher Scientific), FUS (PA5-52610, 1:1,000, Thermo Fisher Scientific), and β-catenin (13-8400, 1:200, Thermo Fisher Scientific). After additional PBS washing, the membranes were incubated with secondary antibodies (31402, 1:2,000, Invitrogen) and visualized using an ECL kit (WBULS0100, Merck Millipore, MA, USA).

2.15. Chromatin immunoprecipitation (ChIP) assay

BV2 cells were harvested and lysed with RIP lysis buffer (Merck Millipore). Following cell cross-linking and sonication, samples were supplemented with 20 μL of 50× protease inhibitor cocktail (PIC), 900 μL of ChIP Dilution Buffer, 20 μL of 50× PIC, and 60 μL of Protein A Agarose/Salmon Sperm DNA. After incubation, the samples were centrifuged, and the supernatant was carefully transferred to a new tube. Next, 1 μL of anti-FUS antibody (11570-1-AP, 1:200, Thermo Fisher Scientific) or IgG antibody (ab181569, 1:50, Abcam) was added to the supernatant and incubated overnight at 4°C. Following precipitation and washing steps, 1 μL of RNase A was added to each tube and incubated at 37°C for 1 hour. Subsequently, 10 μL of 0.5 M EDTA, 20 μL of 1 M Tris-HCl, and 2 μL of 10 mg/mL proteinase K were added to each tube and incubated at 45°C for 2 hours. Finally, DNA samples were collected and quantified using qPCR.

2.16. Dual-luciferase reporter assay

The experimental protocol followed the procedures outlined in a previously published study. Briefly, BV-2 cells were transfected with si-FUS or si-NC using Lipofectamine 3000 transfection reagent (Invitrogen). After 48 hours of incubation, luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit (Promega, Shanghai, China) to assess the luciferase activity of Calhm2.

2.17. Co-immunoprecipitation (Co-IP) assay

Hippocampal tissues were washed twice with PBS, followed by treatment using a buffer containing 50 mM NaCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 0.05% Triton X-100. Resulting lysates were sonicated on ice in an IP buffer and centrifuged at 12,000 rpm for 10 minutes. A 30 μL aliquot of the supernatant was collected as the input sample. Additionally, 420 μL of the supernatant underwent overnight immunoprecipitation at 4°C with an anti-HA-tag-Calhm2 antibody (customized by Thermo Fisher Scientific) or a control nonspecific IgG antibody (1:1000, ab18413, Abcam). Protein A was then added and incubated for 1 hour at 4°C. After four washes with IP buffer, the samples were centrifuged for 2 minutes, and the supernatant was discarded. Finally, 30 μL of 2X SDS sample buffer was added and incubated for 10 minutes. The samples were then subjected to immunoblotting.

2.18. Statistical analysis

Data analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA). Data are presented as the mean ± standard deviation (SD) from at least three independent experiments. An unpaired Student's *t*-test was used to assess differences between the two groups. For comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was initially performed, except for MVM test data, which was analyzed using two-way ANOVA. This was followed by the Tukey post hoc test for further analysis. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Neurotropin ameliorated Aβ pathology and cognitive decline in 5xFAD mice

To elucidate the effect of Neurotropin in AD, 5xFAD transgenic (Tg) mice and WT (non-Tg) controls were administered Neurotropin. In non-Tg mice, Neurotropin treatment did not alter escape latency, swimming speed, or the number of target quadrant crossings during the probe trial (Figure 1A-1C). In contrast, Tg mice exhibited markedly longer escape latency, faster swimming speed, and fewer target quadrant crossings compared to non-Tg mice. Importantly, Neurotropin treatment significantly attenuated these deficits, indicating a protective effect on cognitive performance. To further assess pathology, IHC staining was performed to evaluate amyloid plaque burden in the hippocampus. As expected, no plaques

were detected in the hippocampus of non-Tg mice, regardless of treatment (Figure 1D). By contrast, Tg mice displayed a substantial increase in hippocampal plaques, which was significantly reduced by Neurotropin treatment. These results collectively indicate that Neurotropin alleviated Aβ pathology and associated cognitive decline in 5xFAD mice.

Building on these findings, we next investigated whether Neurotropin modulates microglial activation and neuroinflammation in Tg mice. IF staining for NeuN (neuronal marker) and Iba-1 (microglial marker) was performed on hippocampal sections. No differences in neuronal density or microglial activation were observed in non-Tg mice, regardless of treatment (Figure 1E–1F). However, Tg mice exhibited neuronal loss and increased Iba-1 immunoreactivity, indicative of microglial activation and neuronal damage, which were ameliorated by Neurotropin treatment. Furthermore, analysis of inflammatory cytokines revealed no significant changes in IL-1β, IL-6, and TNF-α levels in either the serum or hippocampal tissues of non-Tg mice with or without treatment (Figure 1G-1H). In Tg mice, these pro-inflammatory markers were significantly elevated, and this elevation was notably suppressed by Neurotropin administration. Taken together, Neurotropin not only reduced Aβ pathology but also mitigated neuroinflammation and microglial activation in 5xFAD mice, further supporting its therapeutic potential in AD.

3.2. Neurotropin improved impaired mitochondrial dysfunction, repressed oxidative stress, and alleviated energy crises in 5xFAD mice

Given that mitochondrial dysfunction is closely linked

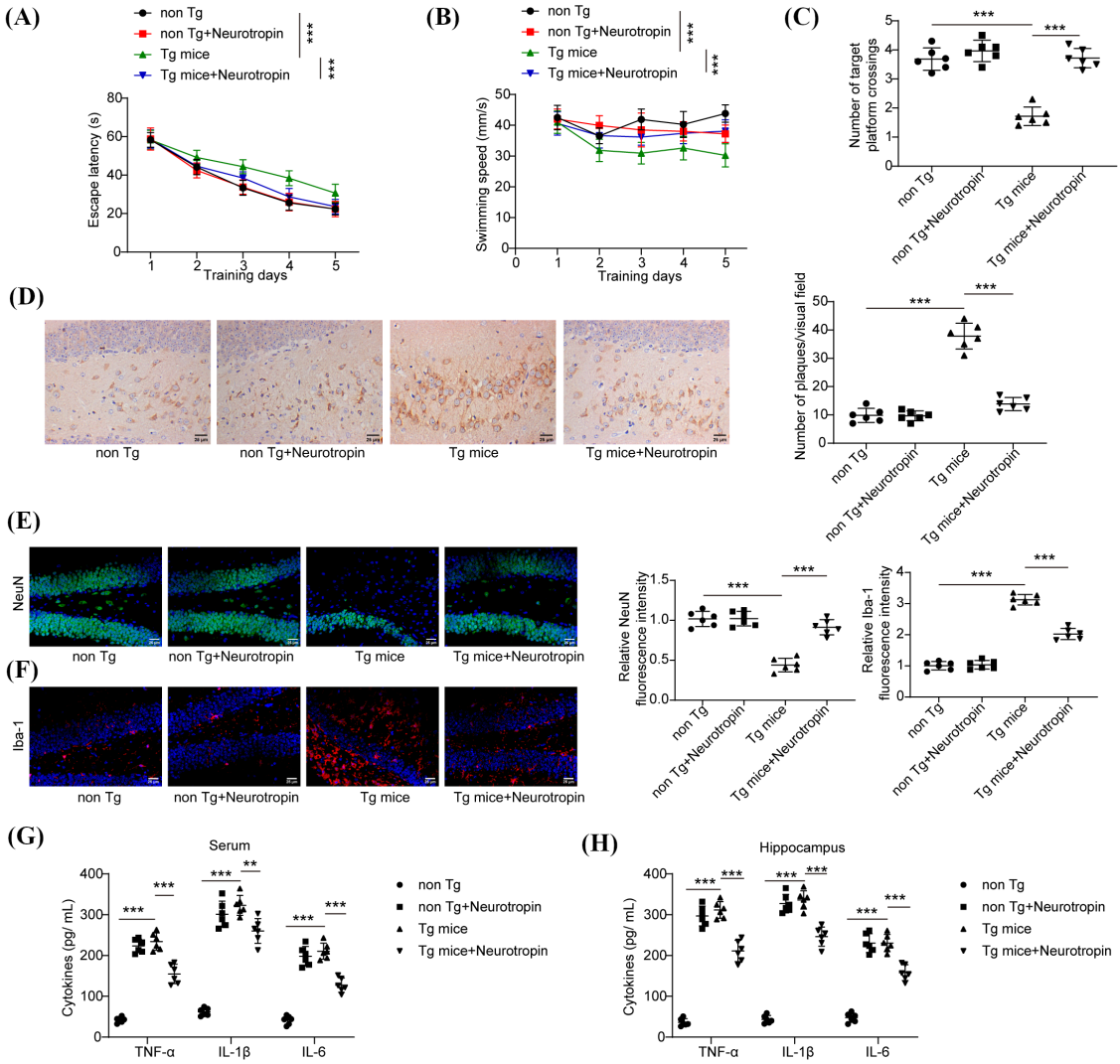

Figure 1. Neurotropin ameliorates Aβ pathology and cognitive decline in 5xFAD mice. WT mice (non-Tg) or 5xFAD mice (Tg mice) were treated without or with Neurotropin. **(A)** Escape latency and **(B)** swimming speed during 5 days of hidden platform tests were measured. **(C)** Crossings of the target quadrant during the probe trial were measured. **(D)** Immunohistochemical staining of 6E10 in the hippocampus of mice, and the number of plaques was quantified. **(E)** Neuronal cell damage in hippocampal sections was detected by IF staining. **(F)** Iba-1-positive cells in hippocampal sections were detected by IF staining. IL-1β, IL-6, and TNF-α levels in **(G)** serum and **(H)** hippocampal tissues were measured using ELISA assays. $n = 6$. $^{**}p < 0.01$, $^{***}p < 0.001$.

to AD pathology (*21*), we next examined whether Neurotropin influences mitochondrial homeostasis. As expected, the mitochondrial length/width ratio and the copy number of mtDNA in the hippocampus were significantly decreased in Tg mice compared to non-Tg mice (Figure 2A-2B). Similarly, hippocampal ATP levels and respiratory chain complex I activity were markedly decreased in Tg mice but significantly restored by Neurotropin (Figure 2C-2D). In addition, Tg mice showed elevated oxidative stress, as indicated by increased MDA production and reduced SOD activity (Figure 2E-2F), both of which were ameliorated by Neurotropin. Altogether, Neurotropin protected against mitochondrial dysfunction, oxidative stress, and bioenergetic deficits in 5xFAD mice.

3.3. Neurotropin suppressed LPS-induced M1 microglia polarization but promoted microglia polarization to the M2 phenotype of microglia

We next investigated whether Neurotropin regulates microglial polarization. In the hippocampus of non-Tg mice, LPS treatment increased the proportion of CD16/CD32 (M1 marker)/Iba1 cells without affecting the proportion of Arg (M2 marker)/Iba1 cells, irrespective of Neurotropin administration (Figure 3A-3D). In contrast, Tg mice exhibited a significant reduction in CD16/CD32/Iba1 cells and an increase in Arg/Iba1 cells following Neurotropin treatment. Consistent with these results, Neurotropin reduced hippocampal gene expression of iNOS and CD86, as well as iNOS protein levels, while enhancing Arg and TGFβ1 gene expression and Arg1 protein levels (Figure 3E-3F). These findings suggest that Neurotropin suppressed M1 microglial polarization and promoted a shift toward the M2 phenotype in Tg mice.

3.4. Calhm2 overexpression reversed neurotropin's protective effects against LPS-induced microglial inflammation and mitochondrial stress

The underlying mechanism of Neurotropin-mediated effects in AD was also explored. No significant differences in Calhm1 expression were detected among

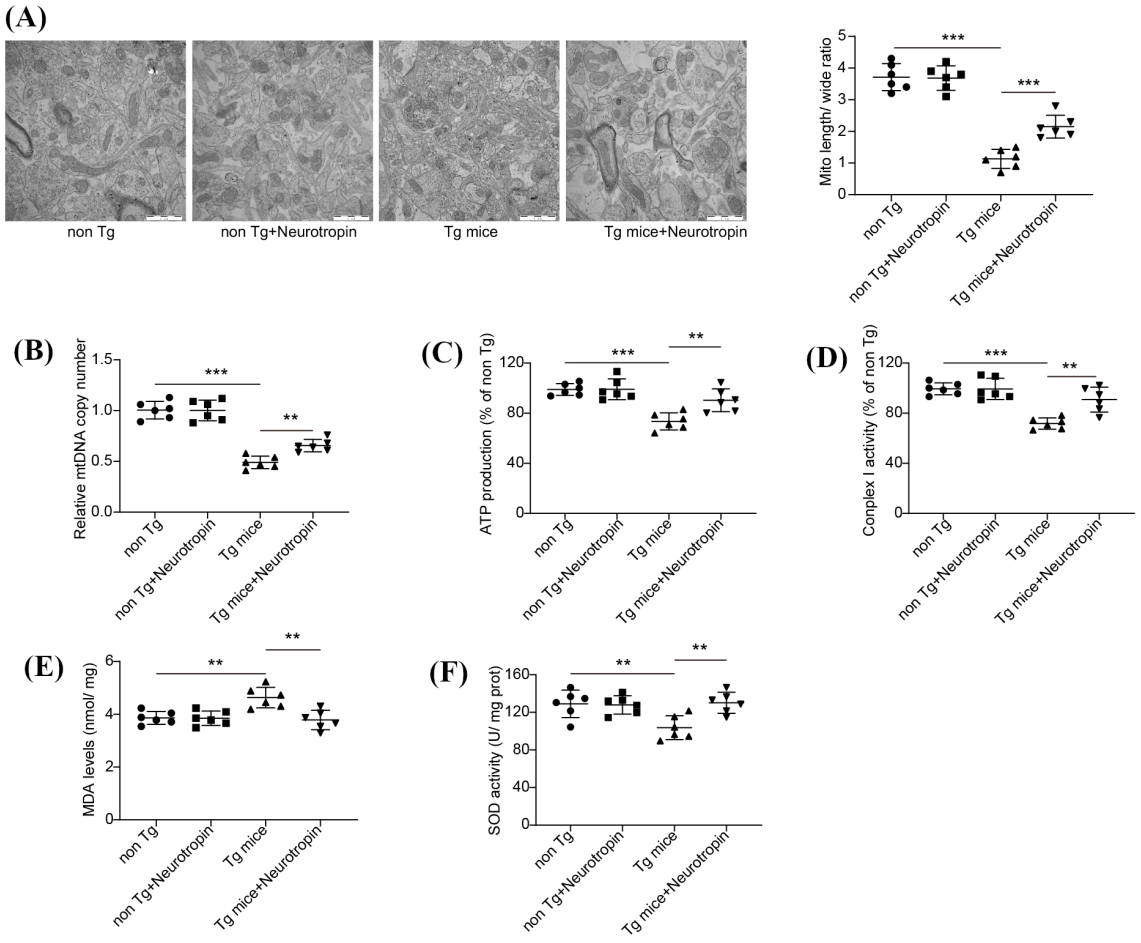

Figure 2. Neurotropin improved impaired mitochondrial dysfunction, repressed oxidative stress, and alleviated energy crisis in 5xFAD mice. WT mice (non-Tg) or 5xFAD mice (Tg mice) were treated without or with Neurotropin. **(A)** The morphology of mitochondria in the hippocampus was observed by TEM. **(B)** The copy number of mitochondrial DNA (mtDNA) in the hippocampus was measured. Energy metabolism was estimated based on **(C)** ATP levels and **(D)** respiratory chain complex I activity in the hippocampus. **(E)** MDA production and **(F)** SOD activity were assessed. $n = 6$. $^{**}p < 0.01$, $^{***}p < 0.001$.

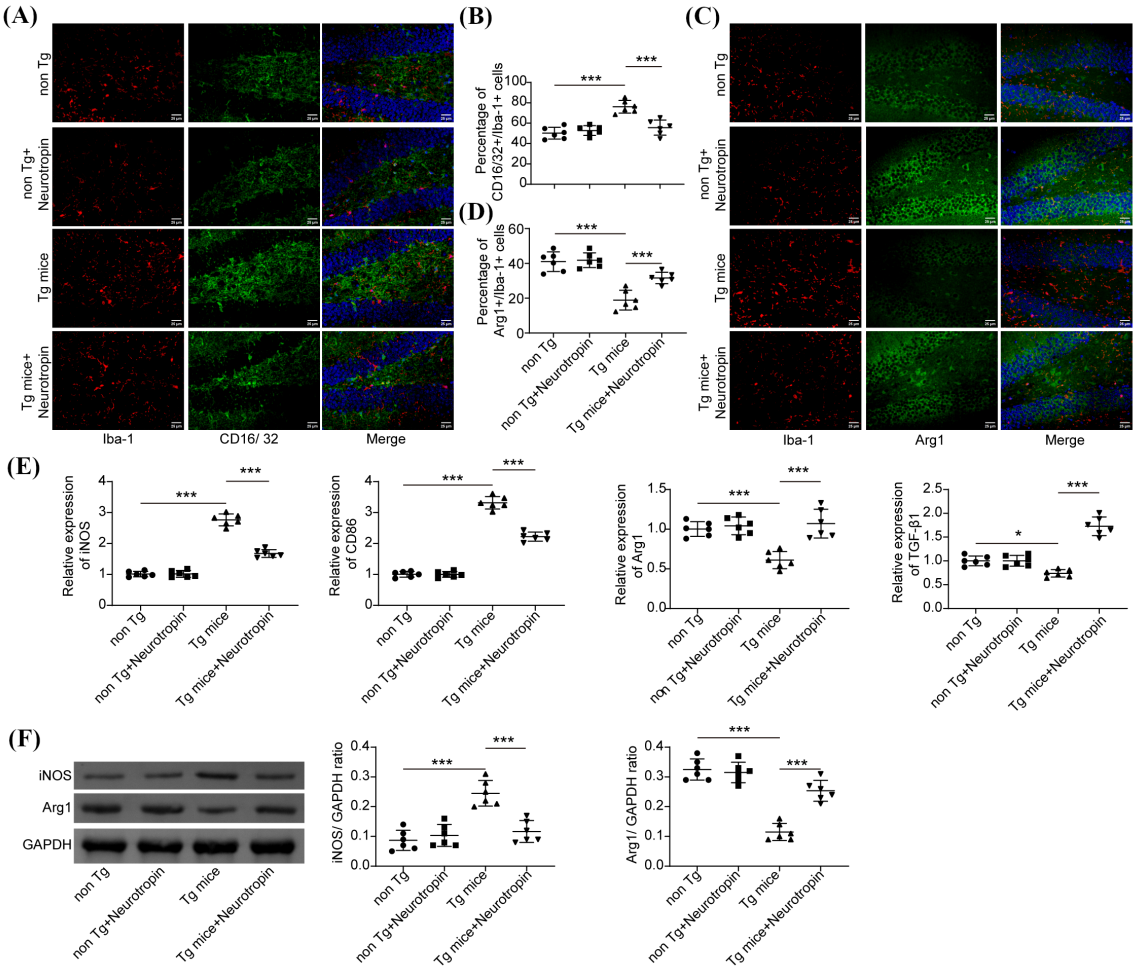

Figure 3. Neurotropin suppressed LPS-induced M1 microglia polarization but promoted microglia polarization to the M2 phenotype of microglia. WT mice (non-Tg) or 5xFAD mice (Tg mice) were treated without or with Neurotropin. Co-expression of Iba1 and the M1 polarization marker, CD16/32, in hippocampal sections was assessed by IF staining **(A)**, and the quantitative results were calculated based on the images **(B)**. Co-expression of Iba1 and the M2 polarization marker, Arg1, in hippocampal sections was assessed by IF staining, and **(C)** the quantitative results were calculated based on the images **(D)**. **(E)** Gene expression of iNOS, CD86, Arg1, and TGFβ1 in hippocampal sections was measured by qRT-PCR assay. **(F)** Protein expression of iNOS and Arg1 in hippocampal sections was measured by Western blot analysis. $n = 6$, $^{*}p < 0.05$, $^{***}p < 0.001$.

non-Tg or Tg mice treated without or with Neurotropin (Figure 4A). However, Calhm2 and Calhm3 expression levels were significantly increased in Tg mice compared to non-Tg mice. Neurotropin treatment in Tg mice significantly attenuated Calhm2 expression but did not significantly alter Calhm3 expression, indicating that Calhm2 may play a specific role in the response to Neurotropin treatment in Tg mice. Western blot analysis confirmed that Calhm2 protein expression was elevated in Tg mice but suppressed following Neurotropin treatment (Figure 4B). Additionally, Calhm2 was found to be colocalized with Iba-1. These findings suggest that Calhm2 may serve as a regulatory node in the therapeutic effect of Neurotropin in Tg mice.

To validate the role of Calhm2 in these processes, Calhm2 was overexpressed in LPS/Neurotropin-treated BV-2 cells. Neurotropin mitigated the promotional effect of LPS treatment on pro-inflammatory cytokine excretion, including IL-1β, IL-6, and TNF-α, in BV-2 cells, while Calhm2 overexpression reversed the effects of Neurotropin treatment (Figure 5A). The LPS-induced mtROS levels and JC-1 expression were suppressed by Neurotropin treatment in BV-2 cells (Figure 5B-5E). However, the overexpression of Calhm2 had the opposite effect. Neurotropin treatment reduced the quantity of $CD16/CD32^{+}$ cells, which were induced by LPS, in BV-2 cells (Figure 5F). Nevertheless, Calhm2 overexpression led to a further increase in $CD16/CD32^{+}$ cells in LPS/Neurotropin-treated BV-2 cells. Additionally, the expression of Calhm2 was enhanced by LPS treatment but suppressed upon Neurotropin treatment (Figure 5G). After Calhm2 overexpression, the expression of Calhm2 was again increased. These results indicate that Calhm2 mediated Neurotropin's anti-inflammatory and mitochondrial protective effects in microglia.

3.5. Neurotropin suppressed FUS-mediated Calhm2 transcription and disrupted Calhm2–EFhd2 interaction

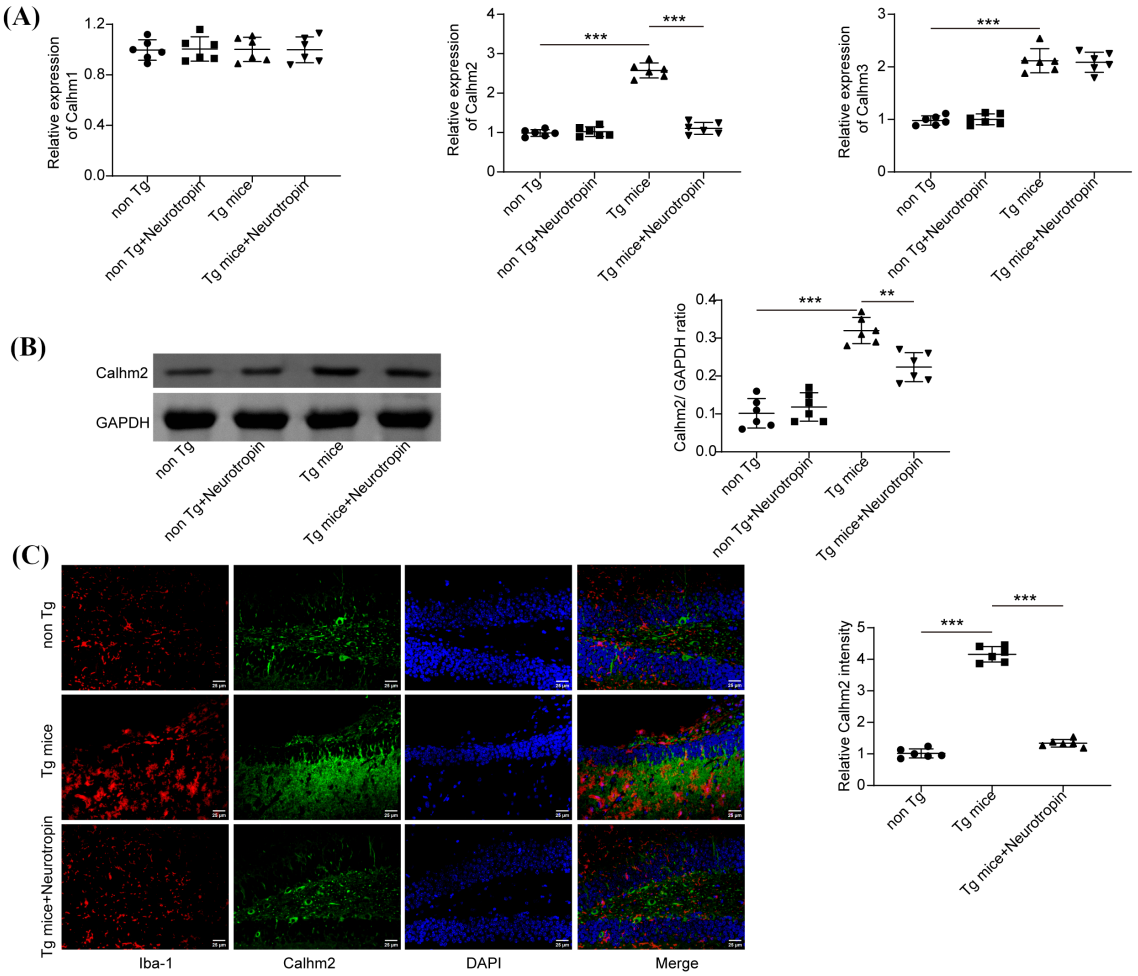

Figure 4. Calhm2 may play a role in Neurotropin treatment. WT mice (non-Tg) or 5xFAD mice (Tg mice) were treated without or with Neurotropin. **(A)** Gene expression of Calhm1, Calhm2, and Calhm3 was detected by qRT-PCR analysis. **(B)** Protein expression of Calhm2 was assessed by Western blot analysis. **(C)** Co-localization of Calhm2 and Iba-1 was examined by immunofluorescence staining. $n = 6$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To explore the upstream regulation of Calhm2, we utilized bioinformatic tools. RNA-society predicted that FUS might potentially bind to Calhm2 (Figure 6A; last accessed October 2023). JASPAR provided the DNA motif for FUS binding (Figure 6B; last accessed October 2023). Calhm2 was significantly enriched when treated with antibodies against FUS (Figure 6C). Additionally, the relative luciferase activity of Calhm2 was markedly increased in cells transfected with si-FUS (Figure 6D). Knockdown of FUS significantly reduced the expression levels of both FUS and Calhm2 (Figure 6E). FUS protein levels were elevated in Tg mice compared to non-Tg and Neurotropin-treated non-Tg mice (Figure 6F). In contrast, Neurotropin treatment led to a decrease in FUS protein levels in Tg mice. Altogether, FUS targeted Calhm2 to mediate its transcription.

Subsequently, to investigate the regulatory mechanism of Calhm2, we performed Co-IP analysis using an immunoprecipitated Flag-Calhm2 antibody from cell lysates to identify potential interacting proteins, specifically EFhd2. Co-IP verified that Calhm2 interacted with EFhd2 in brain tissue from mice (Figure 6G). Additionally, Neurotropin treatment reduced the interaction between Calhm2 and EFhd2. Further analysis revealed that Calhm2 and EFhd2 were colocalized in the cytoplasm of LPS-treated BV-2 cells, and their fluorescence intensities were significantly reduced upon Neurotropin treatment (Figure 6H). Overexpression of Calhm2 did not significantly impact EFhd2 mRNA and protein expression levels (Figure 6I-6J). These data indicate that while Calhm2 interacted with EFhd2, it did not regulate its mRNA and protein expression.

3.6. Neurotropin rescues brain ATP deficiency, mitochondrial dysfunction, and microglia M1 polarization *via* Calhm2 in 5xFAD mice.

To validate the *in vitro* findings, we next investigated the role of Calhm2 *in vivo* using 5xFAD mice. Overexpression of Calhm2 significantly increased amyloid plaque burden in the hippocampus of Neurotropin-treated Tg mice (Figure 7A). Additionally,

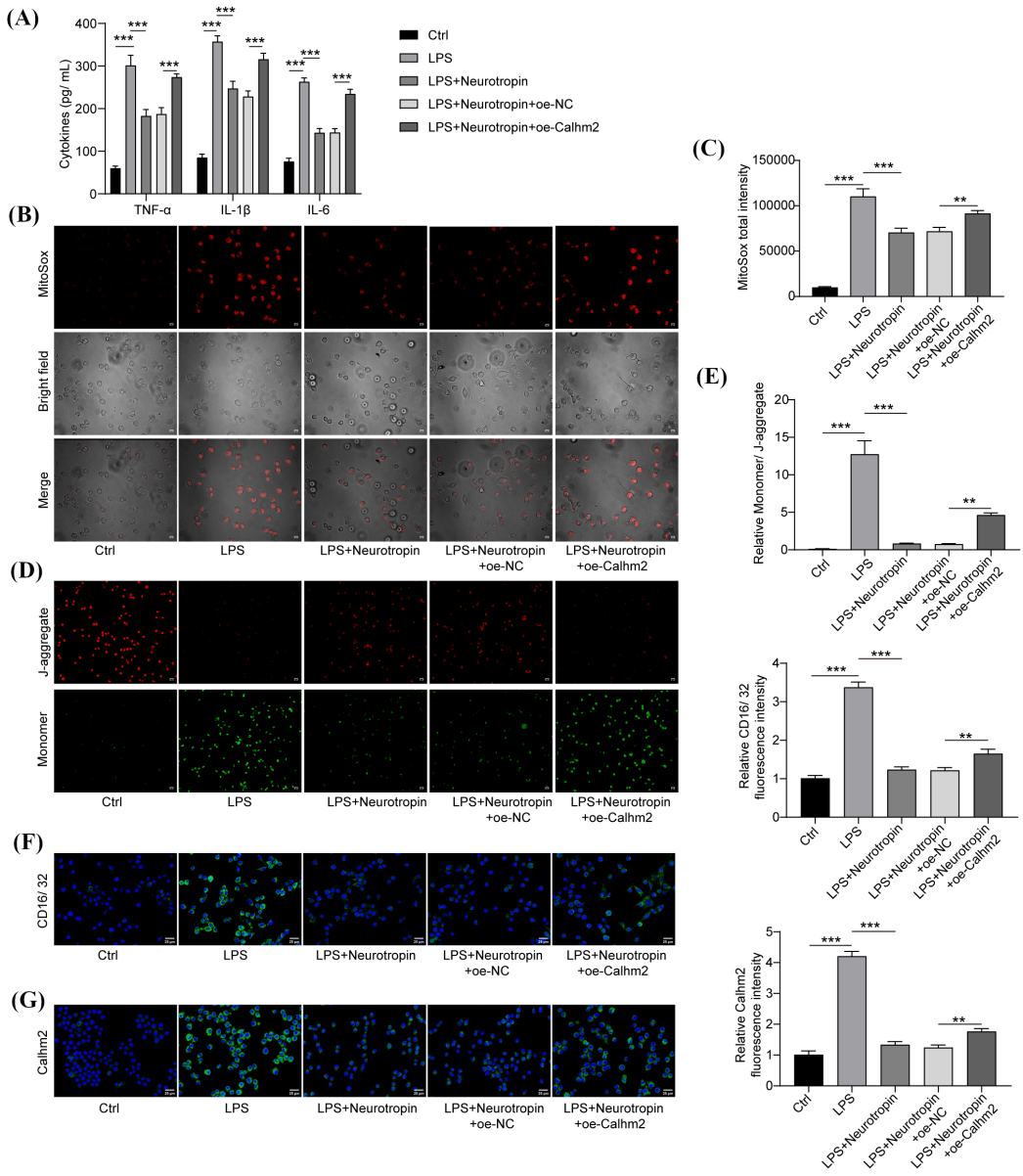

Figure 5. Calhm2 overexpression reversed neurotropin's protective effects against LPS-induced microglial inflammation and mitochondrial stress. Mouse-derived microglial BV-2 cells were treated without (control, ctrl) or with LPS, LPS/Neurotropin/oe-NC, or LPS/Neurotropin/oe-Calhm2. **(A)** ELISA assays were conducted to measure IL-1β, IL-6, and TNF-α. **(B)** Mitochondrial superoxide production was detected, and **(C)** quantitative analysis was performed to assess mitochondrial ROS (MitoSox, red). **(D)** Representative JC-1 IF staining images and **(E)** quantitative analysis. **(F)** Representative CD16/32 IF staining images and quantitative analysis. **(G)** Calhm2 was detected by immunofluorescence staining. $n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Calhm2 was colocalized with Iba-1 in the hippocampus of Neurotropin-treated Tg mice (Figure 7B). The levels of IL-1β, IL-6, and TNF-α in both serum and hippocampal tissues were markedly elevated in Calhm2-overexpressing mice (Figure 7C-7D). Furthermore, Calhm2 overexpression resulted in reduced mitochondrial length-to-mitochondrial ratio, ATP levels, and respiratory chain complex I activity in the hippocampus (Figsure 7E-7F). MDA production, SOD activity, and CD16/32 fluorescent staining intensity were also significantly increased in Calhm2-overexpressing mice (Figure 7G-7H). Taken together, Neurotropin protected against brain ATP deficiency, mitochondrial dysfunction, and microglial polarization *via* Calhm2 in 5xFAD mice.

4. Discussion

Neurotropin is extensively utilized as an analgesic for treating intractable neuropathic pain (*22*). Recent research has revealed that Neurotropin can protect the brain from ischemic stroke, enhance remyelination in demyelinating diseases, and reduce memory impairment and neuroinflammation (*8,23*). However, its effects on microglial polarization and mitochondrial dysfunction in AD have yet to be investigated. This study demonstrated that Neurotropin inhibited microglia

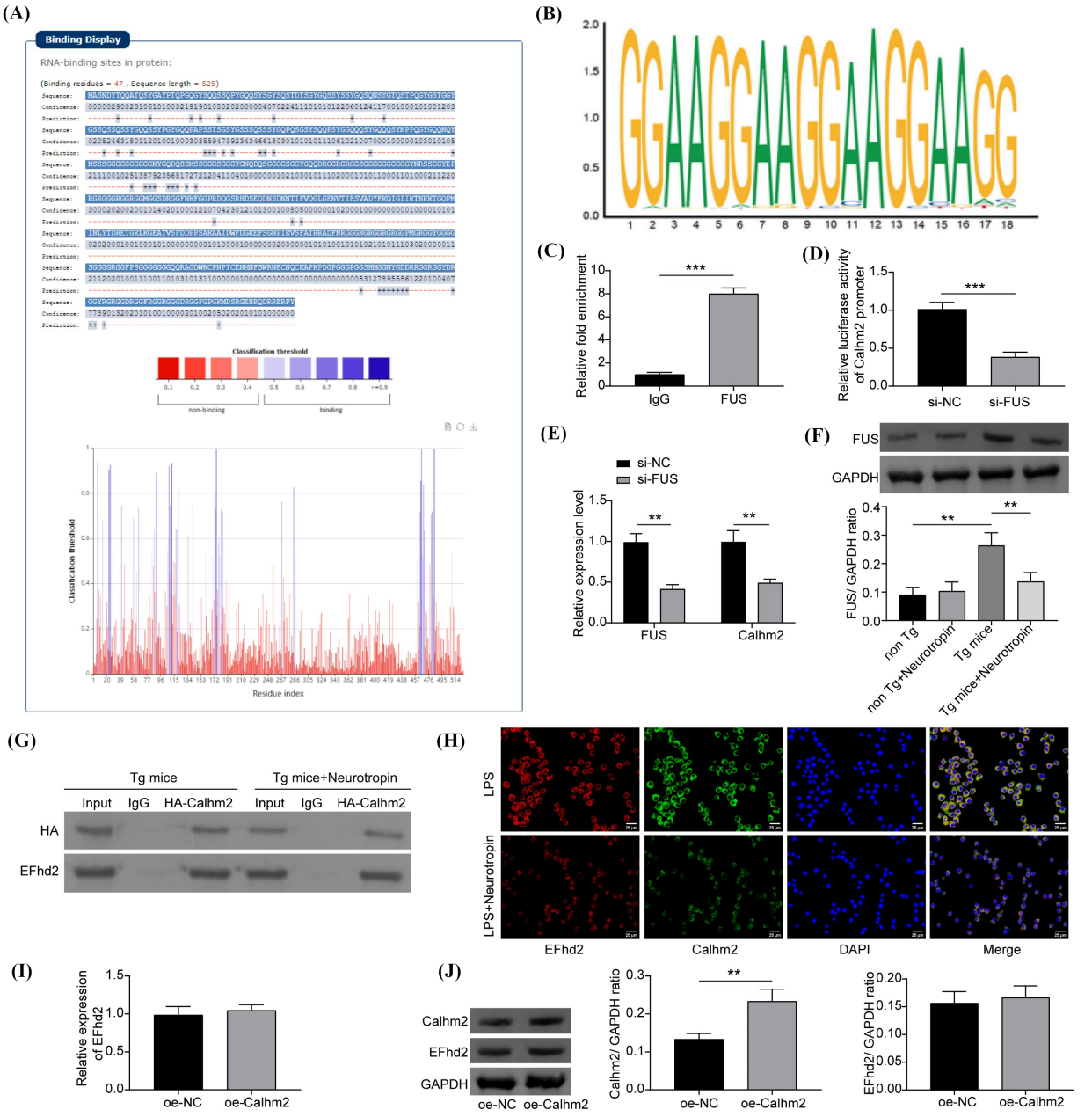

Figure 6. Neurotropin suppressed FUS-mediated Calhm2 transcription and disrupted Calhm2–EFhd2 interaction. (A) The potential binding relationship between FUS and Calhm2 was predicted using bioinformatics. **(B)** The DNA motif of FUS was obtained from JASPAR. The binding of FUS to the Calhm2 promoter was assessed by **(C)** ChIP and **(D)** luciferase reporter assays. **(E)** Calhm2 expression was detected by qRT-PCR. **(F)** *In vivo* levels (n=6) were assessed in WT mice (non-Tg mice), 5xFAD mice (Tg mice), and Tg mice treated with Neurotropin; FUS levels were detected by Western blot. **(G)** The interaction of Calhm2 and EFhd2 in Tg mice and Neurotropin-treated Tg mice was verified by Co-IP, n = 6. **(H)** Co-localization of Calhm2 and EFhd2 by Immunofluorescence Staining in BV-2 Cells treated with LPS, with or without Neurotropin, n=3. The expression of Calhm2 and EFhd2 in Calhm-overexpressed BV-2 cells was assessed *via* **(I)** qRT-PCR and (J) Western blot, n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$.

activation, neuroinflammation, and mitochondrial dysfunction, and reduced Aβ pathology and cognitive decline in 5xFAD mice. We also revealed that Calhm2 was involved in Neurotropin-mediated effects in AD. Calhm2 overexpression attenuated the protective effects of Neurotropin against LPS-induced mitochondrial dysfunction and inflammation in microglial cells. Additionally, we found that FUS targeted Calhm2, which interacts with EFhd2. Eventually, the FUS/Calhm2 regulatory axis was validated using 5xFAD mice. Collectively, our results highlight the critical role of FUS/Calhm2 in Neurotropin-mediated microglial polarization and mitochondrial dysfunction in AD, providing a foundation for using Neurotropin as a treatment method for AD.

Mitochondrial dysfunction and microglial polarization to M1 phenotype are significant contributors to AD pathogenesis (*24*). The interaction with Aβ disrupts mitochondrial electron transfer system activity and induces the activation of M1 microglia, leading to

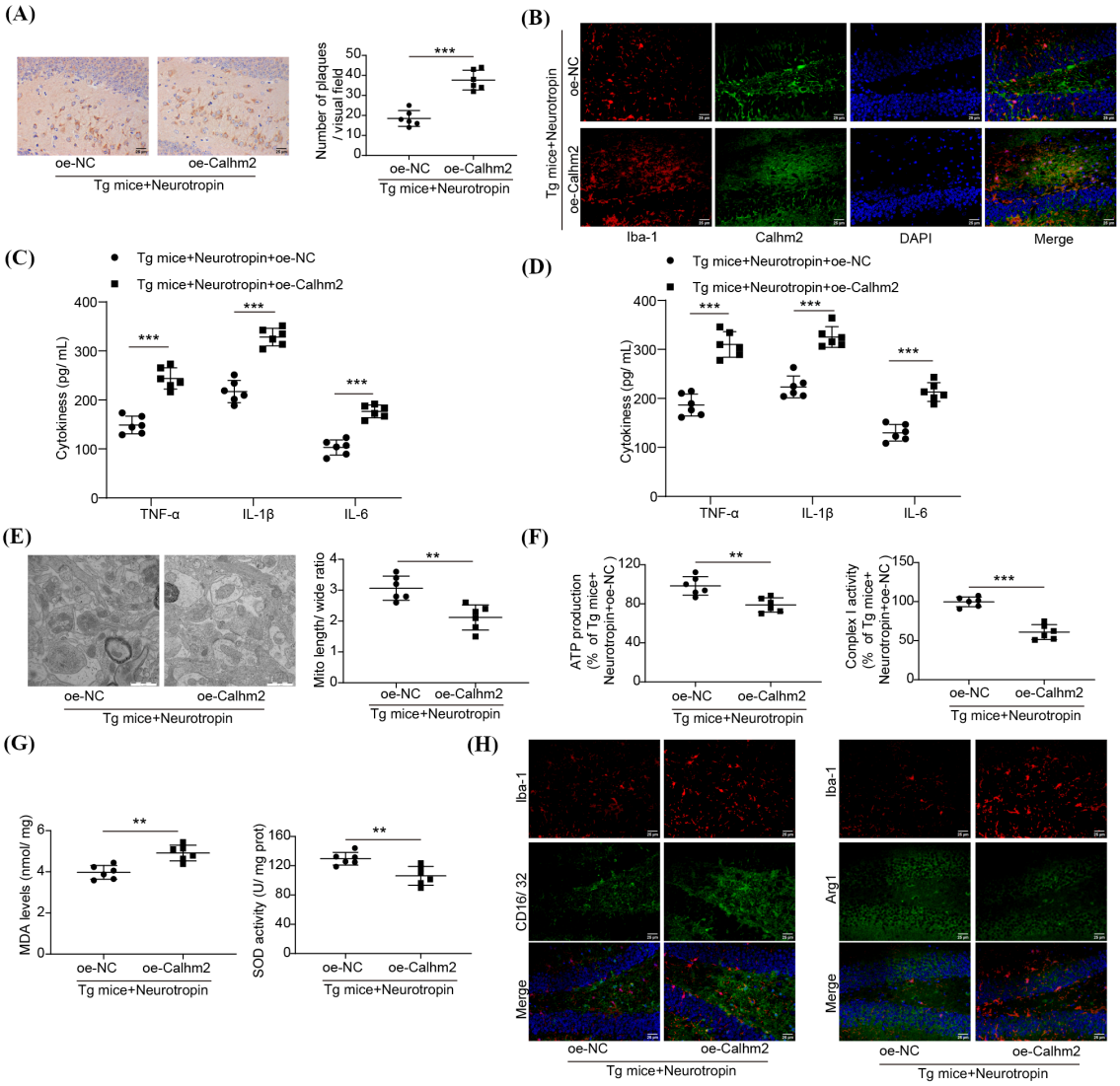

Figure 7. Neurotropin rescues brain ATP deficiency, mitochondrial dysfunction, and microglia M1 polarization *via* Calhm2 in 5xFAD mice. Neurotropin was used to treat Tg mice, and Calhm2 was overexpressed. **(A)** Immunohistochemical staining of 6E10 in the hippocampus of mice, and the number of plaques. **(B)** Co-localization of Calhm2 and Iba-1 was assessed by immunofluorescence staining. ELISA assay for measuring IL-1β, IL-6, and TNF-α in **(C)** serum and **(D)** hippocampal tissues. **(E)** Mitochondrial morphology in the hippocampus was observed by TEM. **(F)** Energy metabolism was estimated based on ATP level and respiratory chain complex I activity in the hippocampus. **(G)** MDA production and SOD activity in the hippocampus were measured. **(H)** Co-expression of Iba1 and M1/M2 polarization markers, CD16/32 for M1 and Arg1 for M2, in the hippocampal sections. $n = 6$. $^{**}p < 0.01$, $^{***}p < 0.001$.

reduced ATP production, increased ROS generation, and excretion of inflammatory cytokines (*25*). This eventually leads to aggressive AD progression (*25*). Previous studies indicated that reverse mitochondrial dysfunction and the activation of M1 microglia could attenuate AD in animal models. For instance, mitochondrial function can be restored by the stimulation of insulin signaling in AD (*26*). Another study from An *et al*. showed that exenatide decreased mitochondrial dysfunction and cognitive impairment in the 5×FAD mouse model of Alzheimer's disease (*27*). Additionally, overexpression of MKP-1 induced microglia polarized to the M2 phenotype and reduced the M1 phenotype microglia (*28*). Similar to these studies, we revealed that Neurotropin treatment attenuated the activation of microglia and neuroinflammation in 5xFAD mice and LPS-induced M1 microglial polarization. We also observed that Neurotropon treatment inhibited mitochondrial dysfunction in terms of mitochondrial morphology, mtDNA, and energy metabolism in 5xFAD mice and LPS-induced microglial cells.

Previous studies have shown that calcium homeostasis is closely related to neuroinflammation and microglial activation (*14*). The genetic knockout of calcium channels, along with specific pharmacological inhibition, has shown protective effects against AD pathology and improvements in cognitive function in AD models (*29,30*). Furthermore, blocking calcium channels with Nicardipine significantly diminished microglial activation *in vitro* (*31*). This suggests that the

Calhm family may be a potential therapeutic target for AD. Among the Calhm family members, Calhm1 did not show significant differences in 5xFAD mice and WT mice. Calhm2 and Calhm3 were significantly increased in 5xFAD mice in comparison to WT mice; however, only the expression of Calhm2 was reduced in 5xFAD mice after Neurotropin treatment. Furthermore, Calhm2 was colocalized with microglial cells, suggesting that Calhm2 might play a role in Neurotropin-mediated microglial deactivation and AD. Consistent with our data, Cheng *et al*. also documented that Calhm2 was highly expressed in the AD mouse model (*14*). Knockout of Calhm2 remarkably decreased Aβ deposition and neuroinflammation (*14*). Another recent study by Liao *et al*. demonstrated that the V136 mutation was closely correlated with AD, as it led to the loss of Calhm2 ATP release in astrocytes (*32*). Our study further demonstrated that Calhm2 overexpression reversed the inhibitory effects of Neurotropin on microglial M1 polarization, inflammatory cytokine secretion, and mitochondrial dysfunction in LPS-induced microglia, confirming that Calhm2 is associated with Neurotropin-modulated AD. To the best of our knowledge, this study revealed for the first time that Neurotropin mediates its effects on microglia *via* Calhm2.

FUS, a multifunctional RNA/DNA-binding protein, is involved in numerous cellular processes such as DNA repair, cell proliferation, transcription, and RNA and microRNA processing (*33*). Increasing evidence suggests that FUS may play a role in the pathological mechanisms of neurodegenerative diseases (*33*). The abnormal aggregation of the FUS protein has been observed in several neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and polyglutamine diseases (*34,35*). Regarding AD, it was demonstrated by a recent study that FUS was associated with the catalytic subunit of mitochondrial ATP synthase, known as ATP5B. This interaction disrupted the assembly of ATP synthase complexes and subsequently suppressed the synthesis of ATP in mitochondria (*18*). However, the role of FUS in AD was not fully understood. In our study, we found that FUS expression was increased in 5xFAD mice compared to wild-type mice, and FUS regulated the transcription of Calhm2 in microglia. This is the first report indicating the regulatory role of FUS on Calhm2. Similarly, many studies also suggest that FUS exerts these effects by binding to RNA and DNA or by regulating the transcription of downstream genes (*36*). For instance, FUS regulates critical autophagosome formation genes, including FIP200, ATG16L1, and ATG12, in a mouse neuroblastoma cell line (*35*). Another study reported that FUS modulates the transcription of the manganese superoxide dismutase gene (*37*). These further supported our conclusion that the transcription of Calhm2 was modulated by FUS in microglia.

Furthermore, our research revealed that Calhm2 interacts with EFhd2 in AD. Treatment with Neurotropin significantly reduced the binding between Calhm2 and EFhd2 in 5xFAD mice. This finding suggests that Neurotropin may regulate mitochondrial function, microglial polarization, and neuroinflammation by diminishing the interaction between Calhm2 and EFhd2. Consistently, a previous study from Bo *et al*. also documented that Calhm2 regulated STAT3 signaling in microglia by interacting with EFhd2, which resulted in enhanced microglial activation, contributing to the progression of Parkinson's disease (*38*). In addition, the depletion of EFhd2 remarkably reduced LPS-induced macrophage inflammation (*39*).

In summary, our research demonstrated that Neurotropin mitigates mitochondrial dysfunction and inhibits microglial polarization *via* the FUS/Calhm2/EFhd2 axis. These findings establish a novel theoretical foundation for using Neurotropin as a therapeutic approach for Alzheimer's disease. While our study provides valuable insights into the mechanisms by which Neurotropin alleviates AD pathology, several limitations should be considered. First, the research primarily utilized the 5xFAD mouse model, which may not fully replicate the complexity of human AD. Consequently, further studies involving diverse animal models and human subjects are necessary to validate these findings. Second, past research has shown that under physiological conditions, FUS is primarily located in the nucleus. However, during neurodegenerative diseases, FUS translocates to the cytoplasm. Due to funding restrictions, we did not examine whether FUS translocates to the cytoplasm in LPS-induced microglia. Investigating this phenomenon would be an interesting direction for future research. Finally, although we observed significantly elevated levels of IL-1β, IL-6, and TNF-α in both the hippocampus and serum of Calhm2-overexpressing mice, we did not directly evaluate blood–brain barrier (BBB) integrity, which may contribute to this phenomenon. Direct assessment of BBB function should therefore be included in future work.

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

Ethics approval and consent to participate: This research was conducted in strict accordance with the ARRIVE guidelines. The study protocol was thoroughly evaluated and received approval from the Ethics Committee of The First Affiliated Hospital of Nanchang University (Approval No. CDYFY-IACUC-202401QR002).

Availability of data and material: All data generated or analyzed during this study are included in this published article.

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Original Article

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Compare antiretroviral drug concentrations in hair and plasma across EFV-based regimens in China

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SUMMARY: Effective antiretroviral therapy (ART) depends on adequate drug exposure. Plasma ART concentrations provide a short-term assessment of drug exposure, and hair promises to be an alternative matrix for measuring long-term exposure. We aimed to determine the association between plasma and hair ART concentrations and explore the therapeutic concentrations in hair. A cohort study in which HIV-infected adults receiving tenofovir disoproxil fumarate (TDF) + lamivudine (3TC) + efavirenz (EFV) regimen for at 6 months were recruited and paired hair and plasma samples collected at about 6±1 months of ART. Previously validated liquid chromatography and tandem mass spectrometry methods were used to measure ART concentrations in plasma and hair. Among 74 participants enrolled, 47 used a 400 mg dose of EFV daily and 27 used 600 mg EFV daily. Hair and plasma EFV concentrations were strongly correlated, with particularly strong association observed in the 600 mg EFV group. The hair EFV concentration of female participants was significantly higher than in male participants, which might be the inter-individual variations in the drug metabolism and dissolution and life habits. The concentrations of TDF and 3TC in hair are too low to determine effective threshold and relationship with plasma drugs concentrations. The accumulation and correlation of hair and plasma EFV concentrations promise to determine a therapeutic range in hair. The therapeutic range for EFV in hair needs to be calculated in order to give quantitative results more value within the field of drug exposure assessment.

Keywords: Antiretroviral therapy, Hair analysis, Plasma, Drug concentrations, LC-MS/MS

1. Introduction

Antiretroviral therapy (ART) is a combination of antiretroviral drugs, which block viral spreading and reduces HIV-related mortality (*1-2*). Adequate exposure to ART drugs is key to remaining virologic suppression, preserving immune function and preventing viral resistance (*3-5*). Therefore, measuring drug exposure would benefit forecasting treatment outcomes (*6-9*). Conventionally, drug exposure is measured by determining concentrations of parent drugs or metabolites in plasma, however, plasma concentrations typically offer only a short-term assessment of drug exposure and is easily affected by external conditions (*10*).

More recently, hair has been considered as an alternative matrix for measuring drug exposure, showing some advantages. Drug concentrations in hair provide a window of detection up to weeks or months (*11-12*). Hair collection is easier than blood sampling since it is non-invasive, and the samples can be stored at room temperature (*13*). Therefore, hair analysis provides a valuable advantage by enabling assessment of long-term medication adherence and estimating average drug exposure over extended periods (*14-16*). Analysis of drug concentration in hair is now routinely used in following scenarios including doping control, diagnosis of anti-psychotic drug abuse and chronic intoxication, criminal assaults, and detection of excessive alcohol abuse (*17-18*). In recent years, liquid chromatography and tandem mass spectrometry (LC-MS/MS) method has been developed for quantitative determination of some conventional ART drugs in plasma or hair among people living with HIV (*19-24*). Some studies suggested the application of LC/MS/MS have shown the relationship between ART levels in hair and virologic outcomes, which can predict virologic failure, medication compliance and drug

resistance (*20,23-25*).

The global scale-up of ART necessitates the development of multifactorial evaluation paradigms to adequately measure therapeutic effectiveness. The predominantly recommended regimens in China include two nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI), encompassing tenofovir disoproxil fumarate (TDF) + lamivudine (3TC) + efavirenz (EFV). An EFV-based regimen (with a 600 mg dose of EFV, known as EFV600) was preferred as first-line treatment for HIV-1 infection by World Health Organization until June 2018, after which clinical treatment gradually transitioned to 400 mg of EFV (*26*). In plasma, a number of studies have determined pharmacokinetic pattern and therapeutic range of some antiretroviral drugs, but little research exists to assess ART concentration level in hair (*21-22,24*). Hence, in the present study we aimed to determine the association between plasma and hair ART concentrations across different EFV-based regimens and explore the ART concentration level in hair.

2. Materials and Methods

2.1. Study design and population

We conducted a cohort survey to recruit HIV-positive people from March 2019 and February 2020 in a designated ART hospital located in Wenshan prefecture, Yunnan province, China. Eligible participants were HIV adults (aged ≥ 18 years old), infected through heterosexual transmission, and had received ART for 6 months with viral loads < 50 copies/mL. The participants used a 300 mg dose of TDF daily, a 300 mg dose of 3TC daily and EFV (with a 400 or 600 mg dose of EFV daily) as a regimen throughout the study.

The baseline demographic characteristics, laboratory results and clinical characteristics were extracted from their clinical records, including age, gender, ART regimens, CD4 cell counts and so on. Hair and blood samples were collected from participants at about 6±1 months of ART. Then, hair samples were assayed for drug concentration among patients whose plasma HIV-1 RNA levels were lower than 50 copies/mL.

2.2. Sample collection and testing

We drew 5 mL of whole blood for HIV-1 viral load using COBAS Amp liPrep/COBAS Taq Man HBV test, v2.0 (Roche Diagnostics, Germany), a fully-automated system that employs real-time PCR technology with a limit of detection of 50 copies/mL. An additional 5 mL whole blood sample was drawn for probing the plasma ART concentrations (in the time window between 12±1 hour after self-reported medication intake). For the determination of plasma ART concentrations, blood samples were centrifuged at 3500 rpm for 10 minutes. Plasma was transferred into labelled cryovials that were frozen at -80°C until analysis.

All participants provided at least 30 strands of 1cm hair close to the scalp as possible in the posterior vertex region, since hair grows at an average of 1 cm/month. The hair samples were placed in small plastic bags and stored at room temperature in the dark to avoid excessive exposure to moisture until analysis, using methods previously published (*19,27*). We washed and dried the hair segments with acetone, and than transferred the hair samples into a frozen grinder for full grinding (-30°C, 4 minutes) to obtain hair powder. Accurately weighed 10 mg of hair powder, added the extraction solution containing internal standard (TDF-D6, 3TC-$^{15}N_2$, EFV-^{13}C) for vortex extraction (37°C, 1h). Followed by centrifugation, we transferred the supernatant into a 96-hole plate, dried the extract with nitrogen, and mixed with deionized water. The mixture was whirled and used for analysis. The validation was done under a solution of standards spiked with blank hair matrices that were the hair strands 1 cm away from the scalp from a healthy female adult.

2.3. Liquid chromatography and tandem mass spectrometry

LC-MS/MS is utilized as the primary method for detecting the concentrations of the ART drugs because of high sensitivity, specificity and less analysis time. Hair and Plasma concentrations are measured by using LC-MS/MS; hair concentrations with an assay range of TDF (0.04-8 ng/mg), 3TC (0.15-30 ng/mg), and EFV (0.4-80 ng/mg); plasma concentrations with an assay range of TDF (4-800 ng/mL), 3TC (15-3000 ng/mL), and EFV (4–8000 ng/mL).

The LC-MS/MS system consists of Applied Biosystems Sciex Triplequad 4500MD triple quadrupole tandem mass spectrometer, SciexExionLC controller, auto-diagnostics (AD) liquid phase pump, AD column heater and AD autosampler. The high performance liquid chromatography (HPLC) conditions are as follows: the column is a Phenomenex Luna Omega; the mobile phase A is composed of methanoic acid and HPLC-grade water; the mobile phase B is composed of methanoic acid and methanol. The column oven temperature is maintained at 40°C and test is 6 min. Data processing was performed using Analyst 1.6.3 software. All drug concentration tests were conducted by Calibra Lab at DIAN Diagnostics (Hangzhou, Zhejiang, China).

2.4. Statistical analysis

Descriptive data were summarized using median and interquartile range (IQR) for continuous data and percentages for categorical data. The X^2 test was used

to compare proportions, and the two-tailed *t*-test (for normal variables) or the Mann-Whitney test (for skewed variables) was used to compare continuous variables. Statistical Analysis System (SAS 9.4, SAS Institute Inc., Cary, NC, USA) and SPSS Statistics 26 (SPSS Inc., USA) were used for statistical analysis, including Spearman's correlation. Multivariate regression modeling was performed in a forward stepwise manner with predictor variables being added to the model if they demonstrated *P* values > 0.05.

2.5. Ethics statement

The present study obtained ethical approvals from the institutional review board (IRB) at the National Center for AIDS/STD Control and Prevention of the China Center for Disease Control and Prevention (NCAIDS, China CDC) and the approval number was X190111540. Each participant provided written informed consent.

3. Results

3.1. Demographics of participants

A total of 74 participants receiving the 3TC+TDF+EFV regimen were enrolled in the survey. Among them, 47 participants used a 400 mg dose of EFV daily and the rest used a 600 mg dose of EFV daily. The average and standard deviation (SD) age in years of the participants was 44.8±11.1 years. Most participants (68.9%) were between the ages of 18 and 50 years, and fifty-four percent were male. Thirty-five percent of the participants had baseline CD4 counts below 200 cells/μL. There was no statistically significant difference in baseline characteristics between the two groups of participants. The demographic and clinical characteristics of all participants are listed in Table 1.

3.2. Stratified analysis of drug concentrations in plasma and hair

Distribution of plasma drug median concentrations are shown in Table 2. In the 400 mg EFV group, plasma TDF, 3TC and EFV median and interquartile range (IQR) concentrations were 73.75 (53.31-95.47) ng/mL, 220.73 (146.95-361.95) ng/mL and 1457.68 (990.17-2000.84) ng/mL, respectively; plasma TDF, 3TC and EFV median and IQR concentrations were 74.22 (60.32-114.71) ng/mL, 272.03 (131.62-334.22) ng/mL and 2349.73 (1702.76-2832.02) ng/mL in the 600 mg EFV group.

Table 3 summarizes the distribution of hair drug median concentrations. In the 400 mg EFV group, hair TDF, 3TC and EFV median and IQR concentrations were 0.05 (0.01-0.08) ng/mg, 0.87 (0.55-1.23) ng/mg and 2.36 (1.84-3.46) ng/mg, respectively; hair TDF, 3TC and EFV median and IQR concentrations were 0.01 (0.01-0.06) ng/mg, 0.61 (0.35-0.89) ng/mg and 3.39 (2.14-5.43) ng/mg in the 600 mg EFV group. In the 600 mg EFV group, hair EFV median (IQR) concentrations of males was 2.18 (1.95-2.41) ng/mg, and the concentrations of females was 5.06 (4.11-8.10) ng/mg. Hair EFV median concentration was significantly higher in females than males in the 600 mg EFV group ($p < 0.05$).

Neither age nor baseline CD4 count showed a significant difference between the median ART concentrations in hair and plasma in both groups (all $p > 0.05$). Plasma EFV median concentration was significantly higher in the 600 mg EFV group than in the 400 mg EFV group ($p < 0.05$), as was hair concentration ($p < 0.05$).

3.3. Scatterplots of the correlation between hair and plasma drug concentrations

Scatterplots of the correlation between hair and plasma drug concentrations are presented in Figure 1. Spearman's correlation coefficients were used to assess the relationship between the concentrations of ART drugs in the two matrices. The results indicated that hair and plasma EFV concentrations were correlated

Table 1. Demographics of participants (*n* = 74)

Characteristics	Overall, *n* (%)	400 mg EFV Regimen, *n* (%)	600 mg EFV Regimen, *n* (%)	*P* value
Overall	74 (100.0)	47 (63.5)	27 (36.5)	
Sex				
Male	40 (54.1)	27 (57.5)	13 (48.2)	0.44
Female	34 (45.9)	20 (42.5)	14 (51.8)	
Age, years				
average (SD; Range)	44.8 (11.1; 22-74)	45.4 (11.3; 22-74)	44.3 (10.5; 26-74)	0.54
18-50	52 (70.3)	30 (63.8)	21 (77.8)	0.21
> 50	22 (29.7)	17 (36.2)	6 (22.2)	
Baseline CD4 Count (cells/μL)				
Median count; IQR	302 (169, 387)	312 (172, 405)	252 (163, 356)	0.57
≤ 200	25 (33.8)	16 (34.1)	10 (37.0)	0.79
> 200	49 (66.2)	31 (65.9)	17 (63.0)	

Abbreviation: SD, Standard Deviation; IQR, Interquartile Range; EFV, efavirenz.

Table 2. The stratified analysis of drug concentrations in plasma

Variable	400 mg EFV Regimen (n = 47)			600 mg EFV Regimen (n = 27)		
	TDF	3TC	EFV	TDF	3TC	EFV
Total	73.75 (53.31-95.47)	220.73 (146.95-361.95)	1457.68[a] (990.17-2000.84)	74.22 (60.32-114.71)	272.03 (131.62-334.22)	2349.73[a] (1702.76-2832.02)
Sex						
Male	72.18 (53.31-93.55)	231.21 (161.24-360.17)	1434.26 (1106.14-2138.85)	69.66 (61.04-113.32)	248.55 (155.68-339.21)	2289.75 (1640.17-2988.86)
Female	76.21 (55.19-113.17)	214.24 (104.88-457.88)	1583.98 (924.84-1888.72)	84.69 (48.98-115.42)	279.84 (83.41-356.54)	2395.53 (1904.86-3406.07)
P value	0.52	0.70	0.48	0.85	0.96	0.62
Age, years						
18-50	71.88 (57.55-95.17)	211.86 (120.20-324.02)	1424.78 (982.22-2021.98)	69.66 (57.89-113.16)	272.03 (137.97-322.11)	2377.73 (1810.24-2988.85)
> 50	85.51 (53.20-98.12)	231.21 (163.98-440.90)	1504.97 (1000.01-2085.06)	97.08 (51.46-132.53)	283.31 (98.71-422.22)	2265.41 (1267.88-3605.53)
P value	0.58	0.35	0.86	0.36	0.79	0.52
Baseline CD4 Count (cells/uL)						
≤ 200	79.27 (54.74-101.60)	198.86 (161.26-327.93)	1481.33 (966.23-1881.94)	89.85 (71.21-103.43)	201.83 (83.41-356.54)	2182.40 (1904.86-2456.92)
> 200	72.17 (53.09-93.75)	252.77 (111.47-378.19)	1434.26 (1010.23-2085.37)	66.49 (52.04-114.87)	276.24 (175.69-334.22)	2672.59 (1640.17-3431.22)
P value	0.50	0.62	0.91	0.23	0.53	0.29

Abbreviation: DC, drug-concentration; SD, standard deviation; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; EFV, efavirenz; [a]Mann-Whitney *U* test: Plasma EFV median concentrations were significantly higher in the 600mg EFV group than those in the 400mg EFV group ($p < 0.05$).

Table 3. The stratified analysis of drug concentrations in hair

Variable	400 mg EFV Regimen (n = 47)			600 mg EFV Regimen (n = 27)		
	TDF	3TC	EFV	TDF	3TC	EFV
Total	0.05 (0.01-0.08)	0.87 (0.55-1.23)	2.36[b] (1.84-3.46)	0.01 (0.01-0.06)	0.61 (0.35-0.89)	3.39[b] (2.14-5.43)
Sex						
Male	0.01 (0.01-0.08)	0.89 (0.54-1.41)	2.37 (1.72-3.15)	0.01 (0.01-0.07)	0.76 (0.44-1.23)	2.18 (1.95-2.41)
Female	0.01 (0.06-0.09)	0.85 (0.62-1.21)	2.36 (2.01-4.36)	0.01 (0.01-0.06)	0.53 (0.31-0.83)	5.06 (4.11-8.10)
P value	0.45	0.59	0.25	0.82	0.16	< 0.01
Age, years						
18-50	0.01 (0.01-0.07)	0.87 (0.54-1.19)	2.34 (1.73-3.28)	0.01 (0.01-0.07)	0.61 (0.35-0.89)	2.41 (2.05-5.72)
> 50	0.07 (0.01-0.09)	0.87 (0.56-1.51)	2.36 (1.91-4.05)	0.01 (0.01-0.02)	0.65 (0.44-1.07)	3.87 (3.09-7.74)
P value	0.05	0.74	0.56	0.25	0.68	0.29
Baseline CD4 Count (cells/uL)						
≤ 200	0.07 (0.01-0.09)	0.88 (0.76-1.61)	2.27 (1.86-3.37)	0.01 (0.01-0.06)	0.53 (0.32-0.67)	3.76 (2.22-4.58)
> 200	0.01 (0.01-0.07)	0.84 (0.51-1.16)	2.36 (1.71-3.86)	0.01 (0.01-0.07)	0.76 (0.37-1.15)	2.41 (2.12-7.70)
P value	0.14	0.17	0.59	0.64	0.13	0.96

Abbreviation: DC, drug-concentration; SD, standard deviation; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; EFV, efavirenz; [b]Mann-Whitney *U* test: Hair EFV median concentrations were significantly higher in the 600mg EFV group than those in the 400mg EFV group ($p < 0.05$).

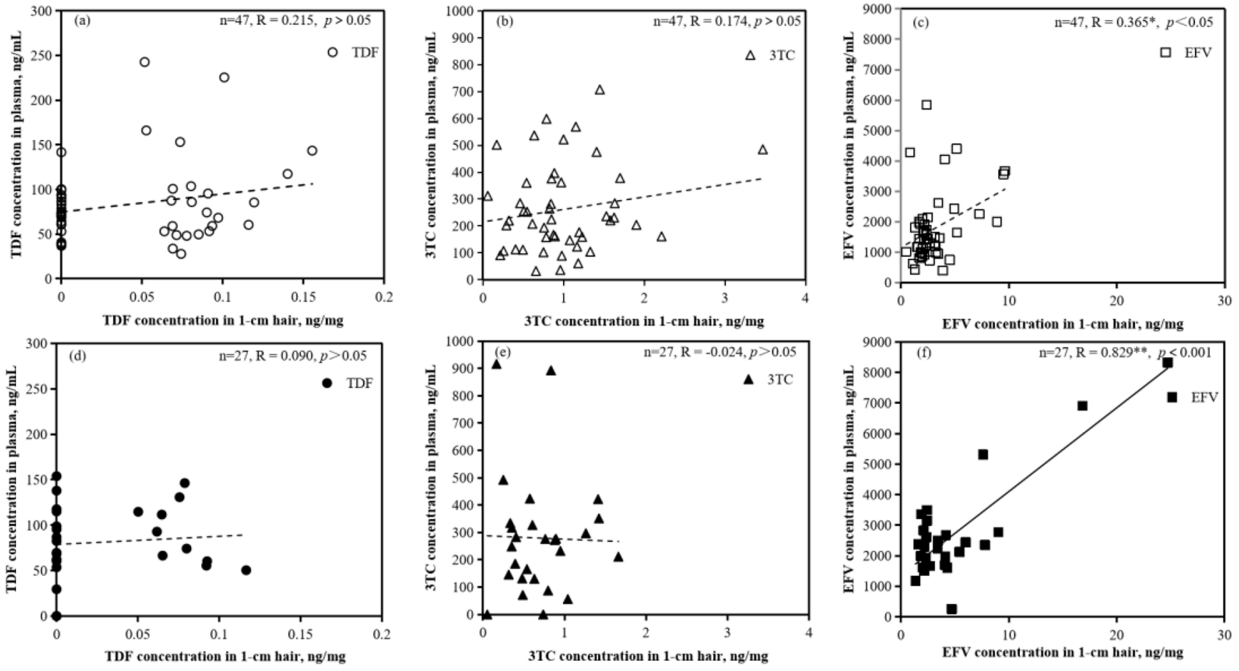

Figure 1. Scatterplots showing the correlation between plasma and hair TDF(a), 3TC(b), EFV(c) concentrations in 400mg EFV group; TDF(d), 3TC(e), EFV(f) in 600mg EFV group. Spearman's correlation coefficients used to assess the relationship between drug concentrations in hair and plasma are shown.

in 400 and 600 mg groups (all $p < 0.05$). Hair and plasma EFV concentrations were strongly correlated in the 600 mg group (correlation coefficients, 0.829; $p < 0.001$). In contrast, TDF and 3TC showed no significant correlation between plasma concentrations and hair concentrations in both groups (all $p > 0.05$).

4. Discussion

Our study first revealed the distribution and association of TDF, 3TC and EFV in plasma and hair among HIV patients of virologic suppression across different EFV-based regimens. Strong correlation was observed between plasma and hair EFV concentrations. The concentrations of 3TC and TDF in hair are too low to be reliably detected, making them difficult to apply in clinical practice. Hair concentrations could be a substitute for plasma testing, provided that corresponding concentration relationships are established through further research.

In the study cohort, hair and plasma EFV concentrations showed correlation, with particularly strong association observed in the 600 mg dose group. Nevertheless, no statistically significant correlation was observed between plasma and hair concentrations for TDF, nor was 3TC. So this phenomenon was mainly caused by differences in the physiochemical properties of ART drugs. Lipophilic molecules can easily penetrate membranes and diffuse into hair (*28*). EFV has the highest lipophilicity and the lowest solubility in the blood, which is easier to incorporate into hair than the other drugs, resulting in a higher correlation (*29*). Therefore, it is expected to find a therapeutic concentration of EFV in hair through accurate predictive models. In contrast, 3TC and TDF contains multiple polar groups which accelerate its dissolution, absorption and transport in the blood, but are not conducive to absorption into hair (*30*). Data from the study showed 3TC and TDF hair concentrations of some participants fell below the detection limits of LC-MS/MS. In this case, we were unable to determine effective hair concentrations threshold for the two ART and relationship between hair ART concentrations and a measure of treatment response. Therefore, we believe that not all ART drugs are suitable for measuring concentrations in hair as an alternative to plasma testing. Under conditions of high adherence, one appropriate drug can be selected as a substitute for the entire regimen.

EFV can be used as a representative drug to observe its distribution differences in hair. Our findings reveal a notable disparity in the distribution of EFV concentrations within hair samples when stratified by gender. The concentration of EFV in the hair of female participants was significantly higher than that observed in male participants. The might be the inter-individual variations in the drug metabolism of HIV patients resulting from their physiological characteristics (*i.e.*, weight) and in the drug incorporation into the hair shaft and the drug dissolution out of hair resulting from the

irradiation of sunlight and life habits (*e.g.*, hair washing and cutting frequency) (*31-32*). Further, previous study has demonstrated that CYP2B6 enzyme polymorphisms significantly influence efavirenz pharmacokinetics, which may partially account for the observed interindividual variability even within the same gender (*33*). Therefore, the life habits of participants need to be controlled before they provide hair samples, including less frequency shampooing, especially when detecting low concentrations of ART drugs. Further analysis could include performing the comparisons separately by gender, or considering gender as one of the confounders in multivariate analysis. Genotyping of clinically relevant CYP2B6 polymorphisms may be performed when necessary to evaluate efavirenz metabolic status.

ART concentrations in hair as a substitute for plasma ART concentrations in HIV patients has consistently presented several challenges. Previous studies have used hair drug concentration as a tool for measuring ART exposure. However, the choice of the multivariate statistical models involving hair concentration as the outcome variable in these studies have not rigorously determined ART drugs concentration thresholds in hair and lacked a large enough sample size to validate hair drug concentration in relation to amount in plasma (*16,17,21,24*). On the other hand, current studies lack a standardized protocol for sampling, collection, and processing hair. The hair sampling sites and length have a great impact on measured concentration. The way of collecting hair samples was mostly cutting or grinding 1cm hair segment closest to the scalp into a fine powder (*18,22,23,34*). Though the drug contents in the 1cm hair segment closest to the scalp can probably reflect drug usage during the past month (*35-36*), hair growth rate actually depends on scalp region, age, gender, ethnicity and inter-individual variability. Hair concentrations testing as a substitute for ART exposure measuring have a long way to go before addressing these challenges.

The limitations of our study were: first, the sample size was limited and the types of data collected were relatively simple. This limited the extent to which data could be analyzed and did not allow for certain associations to be investigated. Second, the observational study mainly relied on extraction of routine clinical records. High quality exposure data are needed to further study exposure-response relations and establish joint pharmacokinetic modelling of plasma and hair drug concentrations. Moreover, we did not strictly control physiological characteristics and life habits (*e.g.*, hair washing and cutting frequency) of participants before collecting their hair samples. Finally, since there is no standard protocol for measuring drug levels in hair, our results may not represent the actual drug concentration.

5. Conclusion

In conclusion, we have shown the distribution and correlation of three antiretroviral drugs exhibited pronounced variability in hair. EFV has the high accumulation resulting in a strong correlation between EFV concentrations in hair and plasma. It is crucial to perform follow-up work in which the therapeutic range for EFV in hair needs to be calculated through accurate predictive models and a standard protocol for the sampling, collection and processing of hair should be established.

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Exosome-based liquid biopsy: A new frontier in early cancer diagnosis

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SUMMARY: Early-stage diagnosis offers the greatest survival advantage in oncology, and yet conventional liquid-biopsy markers — circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) — depend on cell death or mechanical shedding and therefore appear late in disease progression. Exosomes, 40–160 nm lipid-bilayer vesicles secreted by viable cells, emerge earlier, outnumber CTCs by several orders of magnitude, and preserve multi-omic cargo that mirrors intratumor heterogeneity. Rapid advances in enabling technologies are driving continual breakthroughs in exosome-based liquid biopsy, laying a solid foundation for its accelerated translation into clinical practice. Key hurdles remain: standardizing exosome isolation, defining quantitative cut-offs that separate malignant from inflammatory EV surges, and building probabilistic multi-omic models to pinpoint tissue origin. Eliminating these obstacles could advance detection by months and shift care from late salvage to true early interception.

Keywords: exosomes, liquid biopsy, early cancer detection

Early detection remains the most powerful lever for improving cancer survival. Contemporary Surveillance, Epidemiology, and End Results statistics show that the five year relative survival for common solid tumors exceeds 80% in stage I yet falls below 20% in stage IV (*1*). Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) have improved monitoring in advanced disease, but both markers depend on cell death or physical shedding and therefore rise late (*2*). Exosomes, which are lipid bilayer vesicles 40 to 160 nanometers in diameter and secreted by viable cells, provide an earlier signal. They outnumber CTCs by several orders of magnitude, enclose intact RNA, DNA, protein, and lipid cargo, and capture intratumor heterogeneity (*3*) (Figure 1). In recent years, with the increasing depth of exosome research, exosome-based liquid biopsy has shown great promise as a novel strategy for the early diagnosis of cancer.

Technical barriers that once limited vesicle work have narrowed. Acoustic microfluidic fractionation now isolates exosomes directly from whole blood within minutes without labels and preserves their functional integrity (*4*). Aptamer functionalized microbead sensors detect CD63- or EpCAM-positive vesicles in two microliters of plasma with a 30-minute turnaround suited to community screening (*5*). Single vesicle imaging flow cytometry and nanoplasmonic readers routinely phenotype particles below 100 nanometers, enabling multiplex counts of PD L1, EpCAM, or glypican 1 positive subpopulations in unprocessed fluids (*6*). Cargo loading into exosomes is an active, energy-dependent process, meaning that the vesicular miRNA profile reflects real-time transcriptional programs in living tumor cells. In contrast, cfDNA fragments derive mainly from apoptosis and necrosis and therefore provide a static snapshot of historical genomic alterations. Integrating both read-outs brings genotype and dynamic pathway activity together in one test (*7,8*).

Exosomal miRNAs travel inside 40–160-nm lipid-bilayer vesicles, a structure that keeps them intact for days in plasma and even allows them to cross barriers such as the blood–brain barrier, whereas protein-bound cell-free miRNAs are exposed to RNases and typically decay within hours (*9*). Exosomes still wear the "name tags" (surface proteins) of the cells that made them. Scientists can use antibodies or nano-flow cytometry to grab vesicles with a specific tag — say, EpCAM — to isolate those coming from epithelial tumors. Cell-free miRNAs and cfDNA float naked in the blood with no membrane or protein tags, so the same trick cannot reveal their tissue of origin (*10*). Clinical data illustrate this complementarity. In a multicenter study of 292 participants, a 13-marker panel containing eight exosomal and five cell-free miRNAs was created; the composite signature detected stage I–II pancreatic ductal adenocarcinoma with an area under the curve

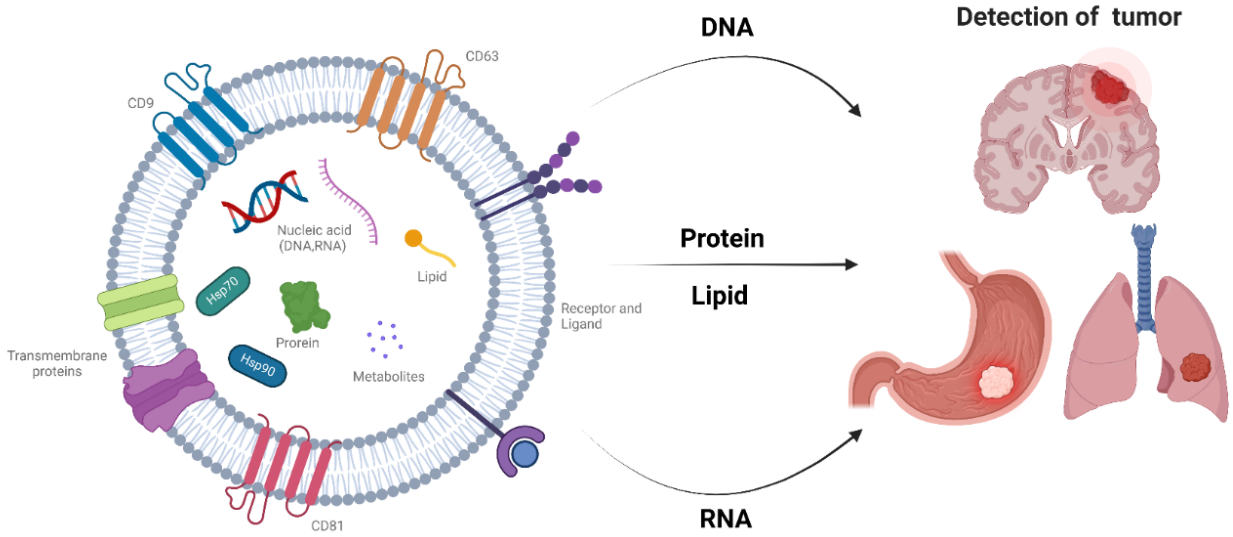

Figure 1. Schematic illustration of exosome-based tumor diagnosis. Tumor cells actively secrete large quantities of exosomes into bodily fluids such as blood, urine, and saliva. These exosomes carry tumor-specific biomarkers, including proteins, nucleic acids (DNA, mRNA, and non-coding RNAs), and lipids, which reflect the molecular and genetic characteristics of the parent tumor.

(AUC) of 0.96, outperforming either subset alone (*11*). In non-small-cell lung cancer, adding exosomal RNA to ctDNA increased the number of EGFR-activating-mutation copies almost 10-fold and raised detection sensitivity in early (M0/M1a) disease from 26% to 74%, an improvement that derives directly from combining the two analyte classes (*12*). Taken together, exosomal miRNAs offer stability, selective enrichment and live-cell transcriptomic context, while cf-miRNAs and cfDNA provide convenient access to circulating molecules and hard genomic endpoints. Capitalizing on their complementary strengths in unified, multi-analyte assays yields markedly higher sensitivity and specificity than relying on any single liquid-biopsy component.

Clinical validation is gaining pace. Glypican-1 positive exosomes distinguish pancreatic ductal adenocarcinoma from benign pancreatic disease with a sensitivity of 100% and a specificity of 100%, and their levels correlate with tumor burden and patient survival after surgical resection (*13,14*). A single-center deep-sequencing study of serum exosomes from nine stage II–IV colorectal-cancer patients and three healthy controls identified a 12-microRNA signature; six miRNAs were significantly up-regulated — four let-7 family members (let-7a-5p, let-7c-5p, let-7f-5p, and let-7d-3p) together with miR-423-5p and miR-3184-5p — and all six peaked in stage II before declining in stages III and IV, while qRT-PCR validation confirmed a > 2-fold elevation of the two representative candidates miR-423-5p and miR-3184-5p with $P < 0.05$, underscoring their promise as minimally invasive early-stage biomarkers (*15*). In a multicenter study, a fucosylated-extracellular-vesicle five-miRNA signature had a sensitivity of 90% and a specificity of 92% for hepatocellular carcinoma in a 606-subject cohort (194 with HCC, 412 with non-HCC) and significantly outperformed both α-fetoprotein and des-γ-carboxy prothrombin (*16*). The iExoDisc, developed by Zhao and colleagues, is an automated centrifugal microfluidic platform for efficiently isolating exosomes from blood samples and performing glycan analysis. The benefits of this platform include: completing exosome isolation within 45 minutes, saving significant time compared to conventional methods like ultracentrifugation; improving exosome purity by 3 to 6 times and achieving a recovery rate of 74.7%; additionally, iExoDisc can identify potential diagnostic markers, such as galactosylation and sialylation, from plasma samples of patients with triple-negative breast cancer (TNBC). This technology provides a new solution for early cancer diagnosis and liquid biopsy (*17*). Meta analyses that pool data from several tumor types assign exosomal microRNA diagnostics a mean AUC of close to 0.84 with a balanced sensitivity and specificity in early disease (*18*). Ge *et al*. identified bile-derived exosomal miR-483-5p and miR-126-3p as biomarkers for distinguishing malignant from benign biliary obstructions (*19*). RNA sequencing in 82 patients showed significant elevation of both miRNAs in malignant cases. miR-483-5p had an AUC of 0.81 (sensitivity of 81.1% and specificity of 81.1%), while miR-126-3p had an AUC of 0.74 (sensitivity of 73.0% and specificity of 86.5%), outperforming CA19-9. These findings highlight the potential of miR-483-5p and miR-126-3p as effective, non-invasive diagnostic tools for malignant biliary obstructions. Clinical translation of engineered exosome therapeutics is steadily advancing; notably, a first-in-human study (NCT03608631) of a Good-Manufacturing-Practice batch of mesenchymal-stromal-cell–derived exosomes carrying KRASG12D-targeting siRNA (iExosomes) is presently assessing safety and feasibility

in patients with pancreatic cancer (*20*). Preclinical studies demonstrate that exoIL-12, an engineered exosome that displays fully active interleukin-12, achieves pronounced tumor retention (roughly a 10-fold increase in intratumoral exposure over recombinant IL-12), drives sustained local IFN-γ production for up to 48 h, and elicits no detectable systemic cytokine release in mice and non-human primates; these pharmacological advantages underpin a forthcoming first-in-human dose-escalation trial in solid tumors (*21*), while exoASO-STAT6 is being evaluated for hepatocellular and colorectal cancers as the first exosomal antisense platform to re-educate tumor-associated macrophages to an M1 phenotype (*22*). Crucially, these precision vesicles dovetail with advances in exosome-based liquid biopsy: circulating exosomal PD-L1 and multi-omic EV panels can now be quantified in microliter plasma samples, enabling ultra-early disease detection, real-time pharmacodynamic monitoring, and rapid identification of resistance pathways (*23,24*). By integrating timely, minimally invasive diagnostics with targeted exosomal payloads, oncology is moving towards a closed-loop paradigm in which early interception, individualized dosing, and adaptive response assessment converge to maximize therapeutic benefit while minimizing collateral toxicity.

Standardization now constitutes the principal bottleneck. The International Society for Extracellular Vesicles published MISEV2023, which lists essential controls for purity, quantification, and function, and yet many diagnostic studies still omit basic purity controls, complicating inter-laboratory comparisons and regulatory review (*25*). Regulatory agencies are debating whether vesicle diagnostics should remain laboratory-developed tests or migrate to full *in vitro* diagnostic oversight, a decision that will shape timelines and post-marketing obligations (*26*). A recent Markov decision analysis in *Gastroenterology* indicated that a triennial blood-based screening test that merely meets the Centers for Medicare & Medicaid Services minimum analytic threshold (sensitivity of 74% and specificity of 90% for colorectal cancer) is overshadowed by annual fecal immunochemical testing (FIT) — that is, it yields fewer quality-adjusted life-years while incurring higher costs, irrespective of how low the per-test price is set. In contrast, modelling shows that a higher-performance assay combining a sensitivity for cancer of at least 90% with a sensitivity for advanced precancerous lesions of 70–80% while maintaining a specificity of 90% could be cost-competitive with FIT, provided its price falls to approximately US $120–140 per test (*27*). Recent state-of-the-field analyses indicated that exosome-oriented technologies — including liquid biopsy platforms — are poised for commercial growth: a pharmaceutical review cited "significant market growth projections" for exosome therapy and reported that the Vesiclepedia database has more than doubled its extracellular vesicle entries since 2019, with 204 exosome-related clinical trials currently registered, collectively signaling rising academic and industrial investment (*28*). Key obstacles remain. Tissue of origin assignment is difficult when multiple organs shed vesicles and machine learning-based deconvolution is only partly effective (*29*). Biological noise from platelet or stromal vesicles increases during infection, trauma, or even vigorous exercise, and threshold definitions that distinguish malignant changes from inflammation are still evolving. Functional validation will clarify whether vesicle markers merely track disease or partly drive progression, a question relevant to eventual therapeutic targeting (*30,31*).

Surface marker capture with antibodies against putative tissue antigens such as L1CAM for the brain, EPCAM for the epithelium, or ASGR1 for the liver is still the most familiar route to enriching vesicles, and yet even carefully optimized protocols show that only about one-half of the immunopurified particles display coherent neuronal co-markers; the yield drops sharply in inflammatory or elderly cohorts, illustrating how marker promiscuity and proteolytic shedding limit specificity (*32,33*). To move beyond single-epitope bias, multiplex single-particle proteomics platforms such as the proximity barcoding assay now read hundreds of surface proteins on individual vesicles and cluster them by origin, but the workflow demands bespoke DNA-antibody libraries, deep sequencing, and days of computation, while still sampling far fewer than a millionth of the vesicles present in a milliliter of plasma (*34*). Bulk RNA approaches instead try to infer provenance computationally: a recent head-to-head comparison of 11 deconvolution algorithms showed that DWLS and CIBERSORTx best explain EV mixtures, and yet even under ideal cell-line conditions they recover only about half of the true variance, and accuracy collapses once platelet and leukocyte vesicles dominate the background (*35,36*). Droplet-level analyses such as SEVtras mine single-cell RNA-seq to score secretion activity directly, offering a clever orthogonal read-out, but performance is tied to the completeness of the underlying tissue atlas and cannot yet quantify the degree of cross-tissue admixture that characterizes plasma (*37*). Extracellular vesicle DNA provides epigenomic barcodes that mirror copy-number and methylation landscapes of donor nuclei; proof-of-principle methylome maps now exhibit a striking concordance with paired tumors, and yet evDNA yields are orders of magnitude lower than cfDNA and bisulphite chemistry erodes what little material is present, making routine multi-omic integration impractical (*38*). Label-free physical sensors provide a reagent-independent route; in a study by Liu *et al*. (*39*), a capillary-phase liquid SERS platform paired with a Bayesian-optimized support vector machine correctly classified plasma extracellular vesicles from stage I–II lung cancer patients versus healthy donors

with an accuracy of 91.5% (95.4% when a convolutional neural network was applied). Although the investigators mitigated matrix artefacts through rigorous baseline correction and by acquiring each spectrum from a fresh aliquot, they still noted that residual lipoproteins can obscure vesicle Raman fingerprints and emphasized the need for validation in larger, more heterogeneous cohorts. Reproducibility also depends on maintaining uniform gold-nanoparticle substrates, the enhancement factors of which can vary between production batches, and on expanding well-annotated spectral libraries for robust machine-learning training. These practical issues — rather than the core sensing principle — remain the principal hurdles to clinical translation of capillary-phase SERS-EV diagnostics. Collectively these findings show that no single modality can yet deliver an absolute, quantitative evaluation of vesicle provenance once multiple organs are shedding vesicles simultaneously; progress will hinge on harmonizing multi-omic reference atlases, standardizing isolation benchmarks across laboratories, and building probabilistic frameworks that integrate orthogonal surface, RNA, lipid and methylation signatures into a unified call for each individual particle.

Exosome research is transitioning from proof of concept experiments to demonstrations of clinical utility. These vesicles combine high abundance, diverse molecular cargo, and direct biological relevance in a single analyte, while recent advances in microfluidic and nanotechnology platforms now enable their routine isolation and analysis. The next critical steps are widespread adoption of MISEV compliant protocols in multicenter trials, establishment of transparent regulatory pathways, and pricing strategies that satisfy health economic models. Achieving these goals could advance cancer diagnosis by months and shift oncology from late stage salvage to true early interception medicine.

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