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Editorial

461-465 **Plague in the modern world: Ecological persistence, epidemic containment, and the limits of public health preparedness**
Liqin Sun, Haipeng Zhu, Qingsong Qi, Jin Li, Meina Tian, Fuxiang Wang, Jean-Pierre Routy, Jiaye Liu, Hongzhou Lu

466-470 **A lifetime with RNA: John J. Rossi and the questions that science revisits**
Wei Tang, Yoko Fujita-Yamaguchi

471-475 **Matching clinical claims to evidence for the use of artificial intelligence (AI) in medicine**
Kenji Karako

Review

476-492 **Beyond individual virtue: Institutionalizing humanism, agency, and algorithmic governance in health systems**
Peipei Song, Wei Tang

493-507 **Traditional Chinese medicine for diabetic lower extremity arterial disease: A review of recent advances**
Fanghua Qi, Pingping Cai, Lin Zhao, Mo Wang

508-527 **Cholinergic dysfunction in Alzheimer's disease: Mechanistic perspectives and multi-target effects of traditional Chinese medicine**
Yixin Wang, Hongxing Li, Lin Hao, Pai Guo, Bingyan Xu, Meng Cai, Shuo Li

528-543 **The spatiotemporal ecosystem of gallbladder cancer: From inflamed areas to invasive margins — Integrating biomechanical injury, the bile microenvironment, precursor lineages, and spatial niches**
Qingyang Meng, Houbao Liu

Original Article

544-555 **Multidimensional social capital and latent anxiety severity profiles among Chinese medical graduate students: A multicenter crosssectional study**
Wen Zhu, Xin Zheng, Ziwei Wang, Niannian Li, Yue Fang, Zhongliang Bai, Jun Wu, Sanyuan Hao, Ziwen Xu, Jiajie Zhao, Ren Chen, Xiaoli Wu

556-571 **Selectivity-aware dual-target cholinesterase optimization using a novel model SPaR-GNN**
Bocheng Zhang, Shaobo Bian, Yahui Liu, Kenji Karako

572-583 **Clinicopathological characterization and prognostic factors of H3 K27M-altered diffuse midline gliomas: A real-world cohort study**
Chunbo Liu, Changwu Wu, Hao Peng, Weicheng Huang, Zheng Chen, Fushu Luo, Yongye Zhu, Qing Liu, Jun Tan

Editorial

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Plague in the modern world: Ecological persistence, epidemic containment, and the limits of public health preparedness

Liqin Sun[1,§], Haipeng Zhu[2,§], Qingsong Qi[2], Jin Li[2], Meina Tian[2], Fuxiang Wang[1,*], Jean-Pierre Routy[3,4,*], Jiaye Liu[5,*], Hongzhou Lu[1,*]

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SUMMARY: *Yersinia pestis* persists across sylvatic rodent–flea reservoirs globally and is not currently amenable to eradication. Paleogenomic and paleoecological evidence indicates that historical plague pandemics reflected complex interactions among pathogen biology, environmental conditions, and societal vulnerabilities rather than pathogen virulence alone. Drawing a qualified epidemiological comparison to cholera, contemporary public health interventions show that effective disease control does not necessarily require eliminating a pathogen from its natural reservoirs; for plague, modern clinical and public health measures have substantially decoupled zoonotic spillovers from pandemic amplification. Whereas cholera control relies predominantly on water and sanitation barriers alongside clinical, vaccination, and surveillance programs, plague containment depends primarily on zoonotic monitoring, rapid diagnosis, early antimicrobial therapy, and contact management. The October 2026 unexplained fatal pneumonia in a Russian anti-plague laboratory worker illustrates both the capacity and the limitations of outbreak surveillance when targeted assays reported by national authorities are negative but etiology remains unresolved. With 97.7% of the 2646 confirmed human cases reported globally during 2019–2025 concentrated in two endemic countries, sustaining containment requires addressing healthcare inequities, maintaining clinical vigilance, monitoring antimicrobial resistance, and enforcing rigorous biosafety.

Keywords: Yersinia pestis; zoonotic spillover; transmission dynamics; antimicrobial resistance; International Health Regulations

1. Introduction

On October 2, 2026, the fatal acute pneumonia of a 28-year-old laboratory worker at an anti-plague research institute in Irkutsk, Russian Federation, prompted international public health inquiries regarding potential occupational exposure to Yersinia pestis (*1-3*). Local authorities placed approximately 200 healthcare and community contacts under medical observation (*1-3*). In its October 10, 2026 notification, the World Health Organization (WHO) stated that, according to information provided by the Russian authorities to WHO, more than 5500 laboratory tests were performed, with no detection of *Y. pestis* or other investigated high-consequence pathogens; no additional linked cases were reported among contacts during the completed observation period, and the primary illness remained classified as pneumonia of undetermined etiology (*1-3*). However, WHO noted that a key uncertainty remained the absence of a confirmed diagnosis in the deceased worker, leaving the exact cause of death—and whether the contact observation period sufficed to exclude all potential etiologies—independently unverified while public briefings and international news reports noted rapid multilateral queries alongside ongoing questions regarding diagnostic resolution and information transparency (*1-3*).

Rather than confirming that international surveillance and legal compliance mechanisms have fully succeeded, this episode illustrates both the capacity and the limitations of contemporary outbreak surveillance, particularly when initial laboratory findings reported by national authorities are reassuring but the underlying etiology remains unresolved (*1-3*). Negative targeted assays lower the likelihood of an immediate Y. pestis

outbreak but neither establish the cause of death nor validate every aspect of biosafety oversight and international risk communication (*1-3*). Beyond its immediate context, this event highlights a central question in infectious disease governance: how have modern medicine and public health institutions altered the epidemic potential of a pathogen entrenched in wildlife reservoirs, and how can containment capacity be sustained when human disease becomes globally rare yet geographically concentrated (*4*)?

2. The persistence of plague

A foundational reality of plague epidemiology is that Y. pestis is a complex zoonotic pathogen not currently amenable to eradication (*4,5*). Unlike smallpox, an obligate human viral infection eradicated through global vaccination, plague circulates independently of human populations within sylvatic rodent and flea networks across the Eurasian steppes, East African highlands, the Americas, and parts of Asia (*4-6*). Within these natural foci, transmission among mammalian hosts is mediated primarily by ectoparasitic fleas in which bacterial biofilm obstructs the proventriculus, promoting regurgitative transmission during blood meals (*5,6*). Epizootic amplification responds to fluctuations in precipitation, vegetation, and rodent density, although ecological lag phases vary substantially across ecosystems and geographic regions (*5,6*). When epizootic die-offs deplete wild rodents, infectious fleas seek alternative hosts, driving spillover into peridomestic animals and human settlements (*5,6*). Because humans are incidental hosts, curing human cases does not alter wildlife maintenance cycles, requiring public health systems to continuously monitor natural foci and manage the ecological interface between wildlife reservoirs and human communities (*4,5,7*).

3. Revisiting historical pandemics

Three historical plague pandemics have been documented: the First Pandemic (beginning with the Justinianic Plague, 541–750 AD), the Second Pandemic (initiated by the Black Death, 1346–1353), and the Third Pandemic, which spread globally from Asia in the nineteenth century (*5,6*). Ancient DNA analyses of fourteenth-century burial sites demonstrated that medieval Y. pestis strains carried the core virulence plasmids (pCD1, pMT1, and pPCP1) conserved in modern isolates (*8*), while phylogenomic reconstructions trace early Second Pandemic diversification to Central Eurasia around 1338 (*9*). Although paleogenomics provides no evidence for an extinct ancestral "super-strain", the absence of a distinct hypervirulent lineage does not exclude all historical differences in pathogen phenotype, nor does it justify attributing medieval mortality to societal factors alone (*8,9*).

For the First Pandemic, despite ancient genomic confirmations of *Y. pestis* (*10*), palaeoecological and documentary evidence does not support an empire-wide demographic collapse (*11*); for the Second Pandemic, pollen records indicate marked regional heterogeneity in land-use change and agricultural disruption across Europe (*12*). Historical plague mortality reflected the interaction between pathogen biology, ecological conditions, and societal vulnerability, rather than pathogen virulence alone (*5,8-12*).

Dense commensal rodent (such as *Rattus rattus*) and vector flea (such as *Xenopsylla cheopis*) populations, crowded housing, nutritional deficits, and the absence of etiological diagnosis or antimicrobial therapy allowed historical spillovers to amplify into major demographic crises (*5,6*), whereas nineteenth-century steamship trade dispersed the 1.ORI lineage globally and established secondary sylvatic foci before bacteriological control emerged (*5,13*).

4. Why catastrophic transmission has become less likely

Modern societies avoid pandemic amplification primarily because clinical care and public health interventions interrupt transmission chains and suppress the effective reproduction number (R_t) below unity (*4,7,14*). Clarifying how these interventions operate requires distinguishing between clinical forms and transmission pathways (*4,7,14*). Human infection typically presents as bubonic plague following a flea bite, or less commonly as primary septicemic or primary pneumonic plague (*4,7,14*). Untreated bubonic or septicemic infection can disseminate hematogenously to the lungs, causing secondary pneumonic plague; however, primary pneumonic plague also arises directly from inhaling infectious respiratory droplets—or, in high-risk laboratory or procedural settings, aerosols—from infected humans or animals (*4,7,14*). Early antimicrobial treatment may prevent progression from bubonic or septicemic plague to secondary pneumonic disease, while rapid identification, respiratory precautions, and contact management are essential to interrupt transmission from primary or secondary pneumonic cases (*4,7,14*).

Mathematical evaluations of historical primary pneumonic plague outbreaks estimate an average basic reproduction number (R_0) of approximately 1.3 (with variance = 3.1 for the fitted geometric secondary-case distribution, and outbreak-specific average secondary cases per primary case ranging from 0.7 to 3.0) (*15*) and an offspring dispersion parameter (κ) near 1.37 (90% CI: 0.88–3.53) (*16*). Because these estimates are derived from historical outbreak data and depend on study populations, case ascertainment, contact environments, and modeling assumptions, they should not be treated as universal biological constants of modern plague transmission, nor does a moderate

average R_0 preclude superspreading when care is delayed (*15,16*). Nevertheless, the rapid clinical progression of pneumonic plague following a brief incubation period (typically 1-4 days, within the general 1-7-day range for human plague), together with predominantly close-contact respiratory transmission in community settings, underscores the importance of early case recognition and prompt intervention (*4,7,14*).

Within this operational window, the 2021 WHO plague management guidelines and the 2024 WHO operational manual recommend bedside Fraction 1 capsular antigen rapid diagnostic tests (F1-RDT) to accelerate clinical triage and isolation; however, WHO notes that these F1-RDT recommendations rest on very-low-certainty evidence, and imperfect specificity in pneumonic screening necessitates confirmation by bacterial culture or validated molecular assays before an outbreak is officially verified (*7,14*). Prompt effective antimicrobial therapy—with the 2021 WHO guidelines adding fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) to first-line streptomycin and gentamicin for pneumonic or septicemic plague, and to first-line streptomycin, gentamicin, and doxycycline for bubonic plague—reduces mortality and transmission risk; respiratory isolation and droplet precautions remain essential during the initial 48 hours of treatment and until clinical improvement (*7,14*). Concurrently, personal protective equipment (PPE) during post-mortem handling prevents funeral-associated transmission, while seven-day post-exposure prophylaxis with oral doxycycline or ciprofloxacin protects close contacts (*7,14*). Because no plague vaccine is currently WHO-recommended for general use—with older whole-cell and live-attenuated formulations exhibiting notable reactogenicity and limited durability, restricting any use to selected high-risk occupational groups in some countries—and because insecticidal flea control must precede rodent control to prevent displaced fleas from biting humans, operational containment relies heavily on diagnostic, vector-control, and therapeutic readiness (*4-7,14*).

The 2017 urban plague epidemic in Madagascar highlights both the utility of coordinated response and the complexity of outbreak data (*17*). Of 2414 clinically suspected plague cases notified nationally, 1878 were clinically classified as pneumonic plague: 32 (1.7%) were laboratory-confirmed, 386 (20.6%) were probable, and 1460 (77.7%) remained suspected (*17*). While unconfirmed status does not exclude plague among all suspected cases, heightened urban concern likely expanded syndromic reporting of non-plague respiratory illnesses (*17*). The decline in notified cases may have reflected a combination of outbreak-control measures, changes in case ascertainment, and diagnostic reclassification; the relative contribution of each cannot be determined from these data (*17*).

Conceptually, both cholera and plague demonstrate that effective disease control does not necessarily require elimination of the pathogen from its environmental or animal reservoirs; however, the extent of control depends on sustained public health infrastructure, healthcare access, and surveillance capacity (*4,5,7,14*). Rather than representing a rigid dichotomy between passive infrastructure versus active suppression—which oversimplifies cholera programs that also require surveillance, treatment, oral vaccination, and maintenance—they represent distinct primary barrier architectures: cholera control relies predominantly on water, sanitation, and environmental exposure barriers, whereas plague containment depends primarily on zoonotic surveillance, rapid diagnosis, antimicrobial therapy, and contact management (*4,5,7,14*).

5. Remaining vulnerabilities

Global reductions in average plague risk obscure severe geographic disparities (*18*). According to the September 2026 WHO surveillance report covering 2019-2025, 10 countries reported 3860 suspected human plague cases; 6 of these countries reported 2646 confirmed cases and 423 deaths, corresponding to a crude case-fatality ratio of 11.0% using the 3847 suspected cases from those 6 countries as the denominator rather than laboratory-confirmed cases alone (*18*). Notably, the Democratic Republic of the Congo and Madagascar accounted for 97.7% of all reported confirmed cases globally during 2019–2025, although WHO noted that identical suspected and confirmed case counts in some national reports indicate that independent laboratory confirmation cannot be assumed for every reported confirmed case (*18*). These findings highlight the geographic concentration of reported plague cases and raise important questions about how differences in ecological exposure, diagnostic access, case classification, and health-system capacity shape the observed disease burden (*7,18*) (Table 1).

Addressing these vulnerabilities under conditions of global rarity and focal endemicity requires sustained institutional commitment across four interconnected priorities (*4,7,14,18*).

First, closing healthcare delivery gaps in endemic rural foci—by decentralizing F1-RDT stockpiles, strengthening specimen transport networks for culture and molecular confirmation, coordinating sequential flea and rodent control, and guaranteeing uninterrupted access to frontline fluoroquinolones and aminoglycosides at primary health centers—is essential to prevent sporadic bubonic spillovers from progressing to fatal septicemic or secondary pneumonic clusters (*7,14,18*).

Second, countering clinical amnesia in low-incidence regions requires integrating high-consequence zoonoses into continuing medical education and emergency triage algorithms, as unfamiliarity with rare primary pneumonic or septicemic presentations frequently causes delays in initiating effective treatment within the narrow therapeutic window and heightens nosocomial

Table 1. From historical plague pandemics to modern containment: Changing determinants of epidemic risk

Dimension	Historical vulnerability	Modern change and remaining limitation
Ecological reservoirs	Persistent sylvatic rodent–flea maintenance cycles and unmonitored epizootic spillovers (*5,6*)	Natural foci remain permanent; integrated ecological and vector surveillance reduces but cannot eliminate spillover exposure (*4-7*)
Clinical management	Absence of etiological diagnosis or pathogen-directed therapy (*5*)	Prompt aminoglycoside and fluoroquinolone therapy reduces mortality and transmission risk; diagnostic delays and rural access gaps persist (*4,7,14,18*)
Transmission control	Limited diagnostic, respiratory isolation, post-mortem precaution, and contact management capacity (*5*)	Rapid clinical recognition, respiratory precautions, post-mortem PPE, contact management, and post-exposure prophylaxis reduce opportunities for onward transmission; uneven availability persists (*7,14,17*)
Societal conditions	Variable sanitation, commensal rodent density, crowded housing, nutrition, and medical knowledge (*5,8-12*)	Greater health-system capacity globally, yet 97.7% of reported confirmed cases remain concentrated in endemic settings facing resource constraints (*4,7,18*)
Surveillance	Limited pathogen identification and outbreak intelligence (*5*)	Integrated laboratory and international IHR (2005) surveillance; incomplete verification, syndromic misclassification, and unresolved diagnostic uncertainty (*4,7,17,18*)

Note: Numbers in parentheses correspond to the manuscript reference list. IHR (2005), International Health Regulations (2005); PPE, personal protective equipment.

transmission risk (*4,7,14*).

Third, systematic genomic and phenotypic resistance surveillance remains vital following the emergence of transferable IncC plasmid-mediated multidrug resistance (pIP1202) (*19,20*) and chromosomal *rpsL* streptomycin resistance in clinical Y. pestis isolates from Madagascar, including documented person-to-person transmission of streptomycin-resistant pneumonic plague (*21*). Although experimental flea co-infection models suggest that IncC plasmid carriage may incur competitive fitness costs during vector colonization while retaining full virulence and vector-transmission competence in single-strain infections (*20*), laboratory findings offer mechanistic hypotheses rather than guarantees of natural containment, underscoring the necessity of routine susceptibility monitoring and validated therapeutic alternatives (*7,14,20,21*).

Fourth, because reference laboratories and research institutes maintain extensive collections of viable Y. pestis strains, stringent biosafety governance must be coupled with transparent etiological investigation and timely notification under the International Health Regulations (2005) of events that may constitute a public health emergency of international concern (PHEIC), including pneumonic plague and clusters of unexplained severe respiratory illness (*1,4,7*).

6. Conclusion

The enduring presence of *Y. pestis* across sylvatic reservoirs underscores a fundamental epidemiological principle: the ecological survival of a zoonotic pathogen does not dictate the scale of human disease (*4–6*). Whereas historical plague pandemics emerged from the convergence of pathogen biology, environmental perturbations, and premodern societal vulnerabilities (*5,8-12*), contemporary diagnostic, antimicrobial, and public health interventions have greatly reduced, but not eliminated, the probability that wildlife spillover will amplify into a large epidemic or pandemic (*4,7,14-17*). Yet this decoupling remains incomplete, neither self-sustaining nor globally equitable; its durability is contingent on sustained surveillance, timely diagnosis and treatment, accessible frontline healthcare in endemic margins, continuous resistance monitoring, and rigorous biosafety governance (*4,7,14,18-21*). As illustrated by the recent unexplained fatal respiratory illness in a laboratory worker, credible outbreak intelligence requires transparent etiological investigation without premature attribution or institutional reassurance (*1-3*). Ultimately, when an entrenched pathogen cannot be eradicated from nature, public health success must be measured by two interconnected benchmarks: sustainably reducing the human disease burden in endemic regions while preserving long-term capacities for risk identification, diagnosis, treatment, and transmission control across the world's most vulnerable health systems (*4,7,14,18*).

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Editorial

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A lifetime with RNA: John J. Rossi and the questions that science revisits

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SUMMARY: Dr. John J. Rossi's scientific career has unfolded alongside the transformation of RNA from a biological intermediary into a programmable therapeutic modality. Beginning with catalytic RNA and anti-HIV ribozymes, his work progressed through RNA interference, expressed siRNA and shRNA, combinatorial RNA gene therapy, and aptamer-guided delivery. This Editorial uses that trajectory to examine a broader feature of modern biomedicine: discovery and delivery must advance together, and scientific progress often depends on returning to unexplained observations with better tools. Rossi's roles at City of Hope and in building the oligonucleotide therapeutics community further show that continuity is sustained not only by experiments, but also by institutions, mentorship, and collaboration. His 2026 reflection on revisiting long-standing biological questions therefore offers a timely vantage on a career spent expanding what RNA can do. The resulting history links changing concepts of RNA with persistent questions of specificity, control, delivery, safety, and clinical translation across biomedicine.

Keywords: RNA biology, RNA interference, ribozyme, RNA therapeutics, scientific continuity

1. A lifetime with RNA

On October 13, 2026, John J. Rossi, Ph.D. was honored with the Lifetime Achievement Award of the Oligonucleotide Therapeutics Society (OTS) at its 22nd Annual Meeting, held October 11–14 at the Colorado Convention Center in Denver, Colorado, USA. This recognition provides a timely opportunity to reflect not simply on a sequence of individual achievements but on a scientific career that has unfolded alongside the transformation of RNA from a biological intermediary into a programmable therapeutic modality. This recognition provides a timely opportunity to reflect not simply on a sequence of individual achievements but on a scientific career that has unfolded alongside the transformation of RNA from a biological intermediary into a programmable therapeutic modality (Figure 1).

Asked shortly before the award what he considered his greatest scientific accomplishment, Rossi pointed to the development of small therapeutics for the treatment of patients with HIV that were eventually tested in clinical trials (personal communication, August 21, 2026). The emphasis is revealing. It places the weight not on a single molecular observation but on the difficult transition from mechanism to engineering and from experimental design to clinical translation—a theme that runs through much of his scientific career.

Within this context, in a recent *BioScience Trends* Editorial Rossi reflected on a deceptively simple feature of scientific progress: new technologies do not merely generate new questions; they also allow old questions to be asked again (*1*). He illustrated this idea with two examples: the re-examination of a *Streptomyces* lectin originally studied in the 1970s using modern analytical techniques, and the investigation of ethanol responses in *Caenorhabditis elegans* using contemporary transcriptomic approaches.

This point is particularly profound when considered in light of Rossi's own scientific career. He received his PhD in microbial genetics from the University of Connecticut in 1976, completed postdoctoral training at Brown University, and began his long association with City of Hope in 1980 (*2*). Over more than four decades, his research moved through catalytic RNA, antisense strategies, RNA interference, small noncoding RNAs, combinatorial gene therapy, and targeted RNA delivery. This was not simply a succession of topics. It paralleled a transformation in biology: RNA came to be understood



Figure 1. John J. Rossi honored with the Oligonucleotide Therapeutics Society Lifetime Achievement Award. John J. Rossi at the 22nd Annual Meeting of the Oligonucleotide Therapeutics Society, held in Denver, Colorado, USA, October 11–14, 2026. *Photo*: Courtesy of OTS Oligo Meeting.

not only as an intermediary in gene expression but also as a catalyst, a regulator, an experimental instrument, and a therapeutic modality.

2. RNA becomes an active molecule

When self-splicing RNA was demonstrated in the early 1980s, it overturned the assumption that biological catalysis was exclusive to proteins (*3*). Catalytic RNA soon became both a fundamental discovery and an engineering opportunity. In 1990, Sarver, Rossi, and colleagues reported that hammerhead ribozymes precisely cleaved HIV-1 RNA in a cell-free system and reduced viral gag RNA and p24 antigen in human cells (*4*). The conceptual move was striking. RNA was no longer only the molecule to be read or measured; it could be engineered to recognize and destroy another RNA.

Feasibility in a model system, however, was not the same as therapy. Ribozymes had to find accessible targets inside cells, remain stable, be expressed at useful levels, and avoid disrupting normal cellular processes. Rossi's later assessment of therapeutic ribozymes emphasized that intracellular localization, RNA metabolism, target accessibility, and delivery were inseparable from catalytic design (*5*). By the late 1990s, anti-HIV ribozyme strategies were being examined clinically. This transition from biochemical principle to human application established a pattern that would recur throughout RNA therapeutics: discovery and delivery had to advance together.

3. From RNA interference to programmable silencing

The discovery that double-stranded RNA could induce potent and sequence-specific gene silencing in *C. elegans* sparked another conceptual chapter (*6*). The identification of short siRNA duplexes that functioned in mammalian cells made the pathway experimentally and therapeutically accessible (*7*). Rossi's group soon explored this topic. In 2002, Lee and colleagues used a mammalian polymerase III promoter to express functional siRNAs in human cells; constructs directed against HIV-1 rev inhibited viral gene expression by up to four orders of magnitude in a cotransfection system (*8*). RNA could now be programmed not only as an enzyme but also as a guide for endogenous silencing machinery.

The power of this approach also exposed new constraints. Viral escape could occur when relying on a single target, unintended seed interactions could alter other transcripts, innate immune pathways could be activated, and high-level shRNA expression could compete with endogenous microRNA processing. Rossi's 2006 review framed these issues as central to clinical development rather than peripheral technicalities (*9*); the same year, dose-dependent toxicity from oversaturation of small-RNA pathways in mice demonstrated how consequential the level of expression could be (*10*). The lesson was sobering but productive: programmable specificity did not eliminate the need for biological control.

4. From molecular recognition to clinical translation

The translational history of anti-HIV RNA therapy therefore became a history of platforms. A randomized phase 2 study of a cell-delivered anti-HIV ribozyme in 74 adults did not meet its primary viral-load endpoint, although the intervention was reported to be safe and biologically active and some secondary measures favored the ribozyme group (*11*). The result was neither a triumph nor a dead end. It clarified the gap between molecular activity and durable clinical effects.

Combination strategies were one response. In 2010, DiGiusto and colleagues transplanted autologous CD34-positive hematopoietic progenitor cells engineered to express three RNA-based anti-HIV agents: a tat/rev shRNA, a TAR decoy, and a CCR5 ribozyme (*12*). Hematopoietic engraftment was achieved in all four patients; gene-modified cells and introduced RNAs were detected, with vector-derived sequences persisting at low levels in multiple blood-cell lineages for up to 24 months. The study was not evidence of an HIV cure. Its importance lay elsewhere, that is, several generations of RNA engineering – ribozyme, decoy, and shRNA – had been assembled into one cell-based clinical platform.

A second response was to make delivery itself molecularly specific. Aptamer-siRNA chimeras combined recognition and silencing in the same RNA architecture. An anti-gp120 aptamer could both interfere with viral entry and carry an siRNA against HIV transcripts (*13*). Chemically synthesized aptamer constructs later enabled interchangeable, multiplexed siRNA delivery *in vivo*, suppressing viral and host targets while addressing the problem of escape (*14*). Subsequent receptor-targeted conjugates extended this strategy to transcriptional silencing and systemic delivery in humanized mice (*15*). Through these studies,

RNA evolved from serving as cargo to an integrated system: a recognition element, therapeutic effector, and delivery vehicle. These studies did not anticipate the exact platforms that now dominate clinical settings, but they addressed the same design problem: coupling potent RNA to a delivery system that determines where it acts (*16*). That logic is now implemented, by distinct mechanisms, in lipid nanoparticle-encapsulated patisiran and GalNAc-conjugated inclisiran (*17,18*).

RNA therapeutics cannot be described as molecular discovery alone. Potent target cleavage or silencing *in vitro* may still fail because of instability, endosomal trapping, off-target tissue uptake, toxic expression, or rapid clearance. Rossi's work repeatedly involved this interface, where an elegant mechanism had to be rebuilt as a usable therapeutic system.

5. A field rather than just experiments

Rossi's career also shows that a field consists of more than just experiments. At City of Hope, he directed the Center for RNA Biology and Therapeutics for more than two decades, while his roles in graduate education, journal editing, biotechnology, and the Oligonucleotide Therapeutics Society helped connect mechanistic biology to therapeutic development (*2,19*). These activities matter to scientific continuity because institutions and communities preserve expertise and allow different disciplines and generations to work on persistent translational problems.

Oligonucleotide therapeutics required synthetic chemistry, RNA structure, computational design, pharmacology, toxicology, vector biology, clinical medicine, and manufacturing to become mutually intelligible. The Oligonucleotide Therapeutics Society provided a forum for this exchange, and Rossi served as its founding president (*2,19*).

The intellectual scope widened as questions regarding HIV - specificity, resistance, innate immune recognition, intracellular trafficking, and selective delivery - became more pointed and more relevant to cancer, genetic disease, and other therapeutic areas. Rossi's later emphasis on RNA medicines and noncoding RNA therefore expanded on the same central problem: how can RNA's informational and structural properties be used without losing control of context, dose, or destination (*2,16*)?

By 2018, the first RNA interference drug had been approved in the US. RNAi had become a clinical modality, although targeting, endosomal escape, durability, manufacturing, and access remained challenges (*16*). Rossi's trajectory thus provides a window into how RNA biology matured through repeated cycles of mechanism, engineering, delivery, and clinical testing.

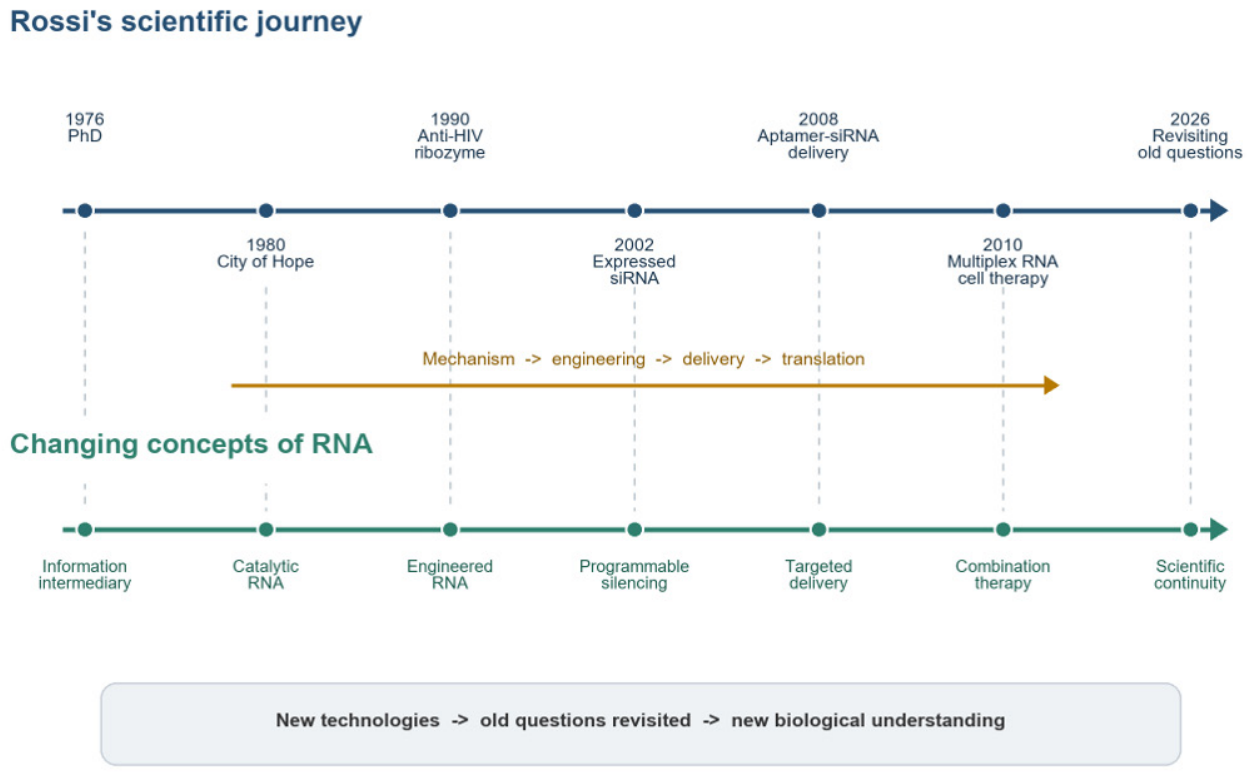


Figure 2. A lifetime with RNA: Scientific milestones and changing roles of RNA. Representative milestones in John J. Rossi's career are aligned with shifts in how RNA has been understood and used. Dotted lines indicate conceptual correspondence rather than single-cause attribution. The central arrow summarizes a recurring translational sequence: mechanism, engineering, delivery, and clinical testing.

6. Changing scientific verbs

Seen in this light, Rossi's career is also a history of changing scientific verbs. Researchers first observed RNA, then sequenced it, cleaved it, silenced it, expressed it, combined it, and attempted to deliver it selectively. Each new verb expanded the range of what could be asked. At the same time, the central questions remained remarkably consistent: How does RNA recognize its target? How is its activity controlled in a cell? How can molecular specificity be translated into clinical benefit? Figure 2 places these changing roles of RNA alongside representative milestones in Rossi's career.

This continuity explains why Rossi's 2026 Editorial looks backward without being nostalgic. The *C. elegans* study he discussed used time-resolved RNA sequencing to separate an ethanol response into early detoxification and redox programs, later DAF-16-associated gene induction, and repression of peroxisome- and development-related signaling (*20*). Earlier experiments noted a lifespan-related phenotype; contemporary transcriptomics have revealed a sequence of pathway-level responses. The aforementioned study did not merely produce more data. It changed the form of the question. The lectin example offers the complementary molecular-scale case: mass spectrometry and expanding sequence databases revealed structural features that earlier methods were unable to (*1*). Taken together, the lectin and *C. elegans* studies show the same principle from protein architecture to system-wide transcription.

This choice of subject is best understood as a vantage point rather than an endpoint. A new technique enables researchers to perform experiments that could not be performed earlier; a later advance can make the past intelligible. Observations that were previously set aside because the sequence, structure, or system-wide response could not be identified may be returned to as productive questions. Rossi's Editorial therefore reads not only as a comment on omics technologies but also as a reflection on the timescale of research itself - on what one generation observes, what it cannot yet explain, and what it leaves for another generation to reexamine.

Good questions often outlast the methods first used to investigate them. Rossi's journey from hammerhead ribozymes to targeted RNA therapeutics shows that progress is neither a straight line nor a continual abandonment of the past. It is a repeated cycle of old observations and new instruments. As technology matures, an unexplained result can be explained, and a question that appears to be settled is given new life.

That may be the most durable lesson of a lifetime with RNA: scientific continuity depends not only on moving forward but also on knowing when the tools are good enough to return to previous topics.

In closing, Rossi left us with these words: "all successful translational aspects of my research stem directly from interpreting sound basic research findings, most of which occurred in lower organisms and plants. Basic research is the foundation of all good science. We must stand on the shoulders of those whose work laid the foundations."

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Editorial

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Matching clinical claims to evidence for the use of artificial intelligence (AI) in medicine

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SUMMARY: Technical performance alone does not establish the clinical value of artificial intelligence (AI). Clinical studies can reveal favorable expert ratings, limited clinician engagement, and uncertain effects on patient outcomes within the same intervention. We present an editorial synthesis with six complementary evidence domains: technical performance, external validity, human-AI interaction, workflow integration, clinical utility and outcomes, and lifecycle performance. These domains identify the questions addressed by a study. Each claim should be assessed with respect to study design, risk of bias, performance or effect estimates, uncertainty, and the evaluated setting. Intended use, autonomy, and potential harm determine the evidence required across domains. Journals should distinguish improvements in intermediate measures from demonstrated patient or health-system benefits and require reassessment when changes potentially invalidate earlier findings. The framework supports evaluation of AI-enabled care as delivered in a clinical system.

Keywords: clinical validation, human factors, workflow integration, patient outcomes, lifecycle evaluation

1. Introduction

The growing volume of medical artificial intelligence (AI) research has not been matched by evidence of benefit in routine care. A 2025 systematic review of 1,263 intensive-care AI studies classified 74% as involving early development or initial validation, 24% as involving external validation, and 25 studies (2%) as involving clinical integration; none met the review's definition of full implementation (*1*). A scoping review drawing on 218 systematic reviews published between September 2023 and September 2024 identified 4,667 primary studies, of which 88.2% were preclinical and 2.4% were randomized trials (*2*). These proportions describe the literature sampled by the reviews, rather than a census of current clinical use. Even among randomized trials, primary endpoints frequently concern diagnostic yield or performance (*3*). In perioperative anesthesia, a review of eight reports of randomized trials found small study populations and substantial heterogeneity for some pooled outcomes (*4*).

Earlier contributions to BioScience Trends described AI-enabled medical support systems and data platforms (*5*), applications from diagnosis to treatment (*6*), and explainability (*7*). These developments provide a basis for asking how AI changes care. Interpretability may help clinicians examine a recommendation, but clinical value also depends on whether they can act on it, the consequences of doing so, and the resources required. Evaluation must therefore consider the model together with its interface, users, workflow, and patients.

The editorial problem is an inferential one. A benchmark characterizes performance with data under specific conditions, whereas a claim about improved care concerns an intervention in a clinical setting. Incomplete information, changing prevalence, local protocols, and patient preferences can affect that intervention. A Nature Medicine editorial has already argued that stronger clinical claims require stronger evidence, from computational performance through clinical usefulness and workflow integration to patient outcomes (*8*). We have developed this principle into a six-domain synthesis that treats external validity and lifecycle performance as distinct domains, that extends assessment to agentic systems, and that specifies claim limits that editors can apply to individual studies, with examples that distinguish measured findings from unestablished benefits.

2. Clinical findings and their limits

In a four-week prospective pilot of the SHAKED decision support system in a tertiary emergency department, expert reviewers rated 99 of 100 sampled

outputs as clinically appropriate. The proportion of intervention-wing encounters in which clinicians opened the SHAKED interface declined from 68% in week 1 to 30% in week 4. No difference in emergency-department length of stay was detected between the wings studied (*9*). The study illustrates that output quality, sustained use, and operational effects require separate assessment.

A trial randomizing 103 clinical officers across 16 Kenyan primary-care facilities included 9,347 patient encounters in its primary analysis. The trial did not establish a reduction in 14-day treatment failure (adjusted odds ratio: 0.77, 95% confidence interval: 0.55-1.08). Secondary assessments favored the quality of clinical documentation. No intervention-related serious adverse events or safety signals were identified during the trial (*10*).

In a single-center quality-improvement study of discharge-date estimates, case managers had lower mean absolute errors than an AI tool at 48 and 24 hours before actual discharge (*11*). These time points were anchored to the observed discharge date. The difference does not establish why human estimates were more accurate: they were visible to care teams and could themselves have influenced the timing of discharge. More broadly, a systematic review found that balanced assessments of patient-relevant benefits and harms remain uncommon (*12*). Together, these examples show why favorable performance, uncertain benefit, and possible harms must retain their distinct meanings.

3. Evidence domains and the claims they support

We organized editorial assessment into six complementary evidence domains (Figure 1 and Table 1). The framework is a conceptual proposal. It builds on existing work on implementation outcomes (*13*) and the lifecycle approach of FUTURE-AI (*14*). Its practical contribution is a set of domain-specific questions and claim limits for editors, illustrated by clinical studies, rather than a new principle of proportional evidence. A study may inform several domains, with different results and different degrees of uncertainty in each.

"Technical performance" concerns discrimination, calibration, task accuracy, robustness, and failure modes under specified conditions. "External validity" concerns whether model performance is sustained across relevant times, sites, populations, and devices. It also concerns whether findings on human-AI interaction, workflow, and clinical outcomes are applicable to other users and care settings; external validation of model performance alone does not establish this. Claims should remain limited to the settings and shifts evaluated. If investigators recalibrate or otherwise adapt a model, the updated version needs to be evaluated with data independent of those used for adaptation. Repeated validation of model performance can enhance knowledge of prediction but does not by itself estimate the effect of using the system on patient care.

"Human-AI interaction" concerns changes in judgment and behavior, including changes in decisions, appropriate overrides, usability, and trust calibration. Simulations and live clinical studies answer different questions, and their settings should be explicit.

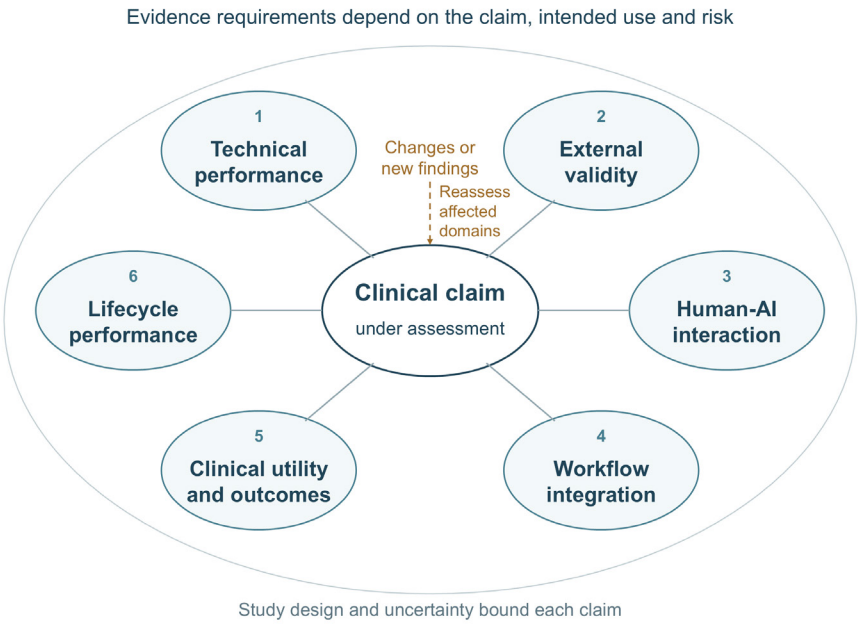


Figure 1. Complementary evidence domains for clinical artificial intelligence (AI) claims. The six domains inform claim-specific assessment; they may overlap and need not all apply to every claim. Solid lines connect domains to the claim being assessed, and the dashed arrow indicates reassessment after changes or new findings. The outer boundary represents shared considerations of the claim, intended use, and risk. Numbers identify domains without implying rank or sequence. Interpretation depends on methodological quality, comparator relevance, estimates, uncertainty, and applicability. This conceptual synthesis is not a validated grading instrument and is informed by references 8, 13, and 14.

Table 1. Evidence domains and editorial assessment of clinical AI claims

Evidence domain	Editorial question	Examples of evidence	Claim scope and limits
Technical performance	How does the model perform under specific conditions?	Discrimination, calibration, task accuracy, robustness, failure analysis.	Performance in terms of tested data and tasks; no inference of patient benefit.
External validity	Which findings are applicable to other settings and users?	Independent model validation; replication of interaction, workflow, or outcome findings across settings and users.	Findings in evaluated settings; model validation alone does not prove that clinical effectiveness transfers.
Human-AI interaction	How does AI affect users and decisions?	Changes in decisions, appropriate overrides, usability, and trust calibration; simulations or live evaluation.	Effects apply to the users and settings studied; safety must be directly assessed.
Workflow integration	How does AI affect the provision of care?	Timeliness, workload, communication, access, alert burden, implementation hazards.	Operational effects in the workflow evaluated; adoption or saved time alone do not establish patient benefit or net clinical utility.
Clinical utility and outcomes	What benefit or harm follows compared to relevant care?	Comparative patient outcomes, safety, quality of life, equity, resource use.	Effects supported by design, estimates and uncertainty; noninferiority requires a justified margin.
Lifecycle performance	What happens during continued use and after a change?	Version-linked outcome monitoring, incident review, postdeployment studies, reassessment.	Performance and harms are observed within the evaluated periods and versions; monitoring arrangements alone do not establish sustained benefit.

The domains are complementary and may overlap within a study. Each claim should be appraised against study design, risk of bias, comparator relevance, performance or effect estimates, uncertainty, and applicability. Intended use, autonomy, potential harm, and reversibility guide evidence requirements. Findings during continued use may prompt reassessment of affected domains. AI, artificial intelligence.

"Workflow integration" concerns timeliness, workload, communication, access, and hazards introduced elsewhere in care. A tool may increase workload while still providing net benefit or save time without improving care. Neither adoption nor reduced task time alone establishes clinical utility.

"Clinical utility and outcomes" concerns the consequences of using AI compared to an appropriate alternative. Relevant outcomes depend on intended use and may include morbidity, symptoms, quality of life, safety, equity, and healthcare system resource use. A claim of patient benefit requires patient-relevant evidence; a claim of efficiency requires a meaningful comparison of resource use while assessing possible diminishment of quality or safety. Noninferiority on prespecified clinical outcomes, assessed against a justified margin, may support efficiency-based adoption when resource benefits are also demonstrated. Study design should follow the causal question. Randomized trials are valuable for estimating intervention effects when feasible. Well-designed quasi-experimental or observational studies may also contribute, provided their assumptions, confounding, and other limitations are addressed. A retrospective model-validation study alone cannot establish improved care. The comparator should reflect feasible contemporary practice, including existing decision-making support and the consequences of replacing an established service.

"Lifecycle performance" concerns performance and harms observed during continued use and after relevant changes. Version control, monitoring, incident investigation, and change management provide the means to collect and respond to evidence; their existence is not evidence of sustained benefit. Claims should specify the populations, period of observation, versions, and outcomes assessed. Plans for monitoring and reassessment should be specified during development, including what would trigger an investigation, renewed evaluation, modification, or discontinuation. An update, new use, or detected deterioration should prompt reassessment in the affected domains.

Across domains, the required evidence depends on intended use, autonomy, potential harm, and reversibility. Methodological appraisal should examine risk of bias, comparator relevance, performance or effect estimates, precision, and applicability. A low-risk administrative application does not require the same outcome trial as an autonomous treatment system. Equally, monitoring a high-risk system does not compensate for inadequate evidence before deployment.

4. Evaluation of agents and changing systems

Agentic systems make the boundaries of the evaluated intervention particularly important. The Mother-Child AI Agent combines a prediction model using longitudinal electronic health records with a tool that retrieves recommendations from medical literature and guidelines

(*15*). Predictive validation and recommendation retrieval should be distinguished from evidence that autonomous clinical action improves outcomes. Systems described as agents differ in their authority to recommend, initiate, or perform actions, and studies should state that authority explicitly.

When a system selects tools or follows multistep plans, its evaluation should address errors across the full sequence, repeated-run variability, tool failures, human escalation, and recovery from a partial failure. The relevant configuration can include the model version, prompts, sources retrieved, available tools, and requirements for human approval. Testing components in isolation may miss unsafe interactions between them. These questions belong within the same evidence domains, with evaluations proportionate to the actions the system can take. World Health Organization guidance on large multimodal models similarly emphasizes stakeholder participation and post-release auditing (*16*).

5. Regulation and evidence over time

Regulatory initiatives reinforce the need to consider evidence over the life of a system. The US Food and Drug Administration's January 2025 draft guidance describes lifecycle management and marketing-submission recommendations for AI-enabled device software functions (*17*). Its August 2025 final guidance on predetermined change control plans addresses planned modifications, the methods for developing and validating them, and impact assessment (*18*). An August 2026 discussion paper seeks feedback on the regulation of generative-AI-enabled medical devices; it does not establish draft or final regulatory expectations (*19*). International Medical Device Regulators Forum principles also address representative data, independent testing, human-AI team performance, and monitoring (*20*).

Japan's Second Basic Plan on Artificial Intelligence provides a national policy context for AI development, deployment, and governance (*21*). The Pharmaceuticals and Medical Devices Agency has separately solicited public comment on aspects for the review of specific categories of software as a medical device (*22*). A national strategy, drafts submitted for comment, and device-specific guidance have different purposes and authority. For journals, their relevance is that system changes and continuing use raise scientific questions that a single predeployment evaluation cannot answer.

6. Conclusion

The clinical claims made for medical AI should match what studies directly measure and the settings in which their findings apply. The six complementary evidence domains indicated here help distinguish technical performance, use in practice, and patient or health-system benefit, while retaining methodological appraisal within each domain. This conceptual synthesis complements established reporting guidelines for AI trials, early clinical evaluations, prediction models, and diagnostic-accuracy studies (*23–27*).

Authors should make the scope and uncertainty of their findings explicit in titles, summaries, and conclusions. Journals should distinguish intermediate improvements from demonstrated clinical benefit, report harms alongside benefits, and welcome informative neutral or negative results. Changes in systems, intended uses, or care settings may require reassessment of affected domains.

The framework requires evaluation before use as a formal editorial instrument, and particularly for consistency between reviewers and its ability to improve the alignment of claims with evidence. For now, it raises a practical question: what did the study directly measure, and how far do its findings extend?

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Review

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Beyond individual virtue: Institutionalizing humanism, agency, and algorithmic governance in health systems

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SUMMARY: Humanistic values are usually treated as qualities of clinicians, although modern care is produced by organizations, financing arrangements, regulatory systems, and digital infrastructures. This conceptual and critical integrative review examines how humanistic tenets became embedded beyond the clinical encounter while drawing a boundary between humanistic tenets and health policy that is merely beneficial or efficient. Landmark scholarship, international frameworks, comparative healthcare-system studies, critical accounts of managerialism, and selected primary legal and governmental sources from the United States, United Kingdom, Japan, and China were synthesized by domains of responsibility rather than a presumed universal chronology. The framework distinguishes six non-interchangeable domains—physician, patient, population, organization, a person's life, and healthcare system—defined by different moral qualities, scales, temporal horizons, and governance mechanisms. Technology governance cuts across all six; technology itself is not a moral quality. The Japanese pathway illustrates the institutionalization of universal coverage before autonomy became comparably explicit, whereas China's recent trajectory is interpreted as compressed institutionalization across coverage, rights, quality, reforms in the provision of care, and aging. A humanistic healthcare system is proposed as an ideal and a directional continuum. Its core tenets are dignity, agency, attention to suffering, and responsiveness to person-defined life goals. Safety, equity, solidarity, participation, and trustworthiness acquire humanistic significance when they uphold those tenets rather than functioning only as performance objectives. Institutionalization can make humane care more reliable, but auditing, targets, and digital systems can also produce a predilection for procedures. Professional judgment, institutional capability, public accountability, and contestable technology must therefore remain mutually corrective.

Keywords: bioethics, organizational ethics, patient-centered care, universal health coverage, patient safety, algorithmic governance

1. Introduction

Modern medicine has acquired powers that earlier generations could scarcely imagine. The ethical paradox is that greater diagnostic precision, therapeutic options, data, and specialization can widen rather than close the gap between what a system can do and what a particular life requires.

The usual response is to ask clinicians for more empathy, communication, professionalism, or exposure to the humanities. Those responses remain indispensable, but they locate the remedy too narrowly. A compassionate clinician cannot, through character alone, fix an unpayable benefit, unsafe staffing, a fragmented referral pathway, an opaque algorithm, or a payment rule that rewards activity over patient-defined benefit. This review therefore advances a broader thesis: humanistic responsibility in modern medicine has expanded from a virtue expected of the physician to a capability expected of the healthcare system. Institutions are not literally compassionate persons. They can, however, make dignity, agency, justice, safety, participation, and attention to the meaning of illness more—or less—possible through law, financing, workflow, service design, and governance.

This review calls this process the layered institutionalization of humanistic values. 'Layered' is essential. The layers do not constitute a universal chronology in which autonomy replaces beneficence, or safety replaces justice. Older obligations persist while newer sites of responsibility are added. Nor does the framework claim that the medical humanities led universal coverage, the patient-safety movement, or integrated care.

These developments have distinct political, economic, professional, and social histories. The more modest and defensible claim is that they institutionalized values that overlap with the moral concerns of medicine and made those values less dependent on the discretion of a single clinician.

The argument is conceptual and historically informed rather than systematic. Landmark ethical texts, policy frameworks, critical counter-literatures, and comparative health-system scholarship are used to identify shifts in the location and machinery of responsibility. The United States, United Kingdom, Japan, and China serve as pathway cases rather than civilizational types. Their value lies in showing how comparable tenets may be upheld by different institutional means and emerge in different orders. Figure 1 summarizes the revised architecture: six responsibility domains extend from physician to healthcare system, while technology governance cuts across them rather than forming a final historical stage.

The problem has been described through suffering, illness narrative, narrative competence, the scope of medical humanities, patient-centered quality, people-centered services, high-quality healthcare systems, and AI governance (*1-12*). The respective literature is rarely read as a single institutional history. The central question of this review is therefore not whether medicine has become more or less humane in the abstract, but through what pathways humanistic values have become enshrined in law, organizations, financing, provision of care, and technology governance.

The term humanistic is used here in a deliberately restricted sense. Its core concerns are the moral standing and lived experience of persons: dignity and agency, attention to suffering, and responsiveness to the goals through which a life has meaning. Other values—including solidarity, equity, safety, participation, and trustworthiness—are not automatically humanistic merely because they are desirable. They enter this framework when they protect persons from domination, abandonment, avoidable harm, or exclusion. Institutionalization denotes the conversion of those tenets into durable rights, routines, resources, capabilities, and routes of accountability.

2. Conceptual and critical integrative review: Design and methods

This article is a conceptual and critical integrative review. Integrative reviews are appropriate when a question spans empirical, theoretical, historical, and policy literature and when the purpose is theory-building

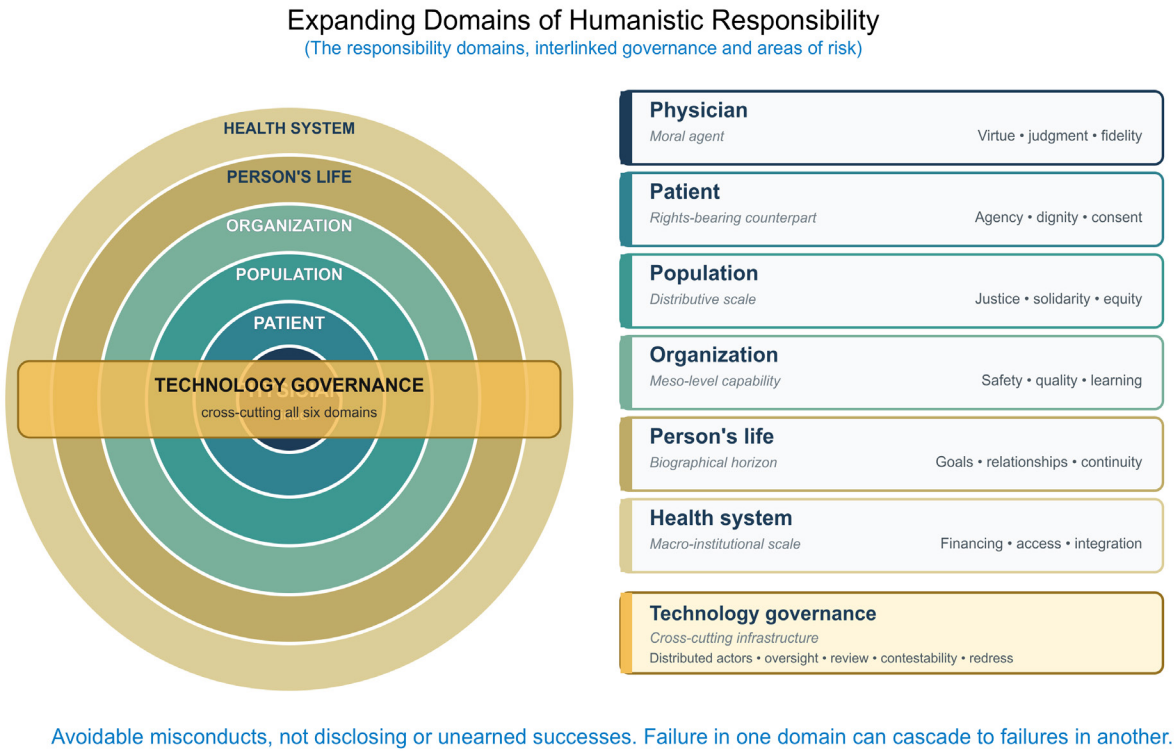


Figure 1. Expanding domains of humanistic responsibility in modern medicine. Six concentric domains depict analytically cumulative responsibilities, not temporal stages: physician and patient are relational loci; population is distributive; organization is meso-institutional; a person's life is a biographical and temporal horizon; and healthcare system is macro-institutional. The horizontal band represents technology governance as a cross-cutting layer that alters every domain. Technology is not an additional moral quality; responsibility remains with the distributed actors who design, procure, deploy, regulate, use, contest, and repair technical systems. Countries may institutionalize the domains in different orders.

and conceptual synthesis rather than estimation of a single effect (*13,14*). Review typologies distinguish this approach from systematic effectiveness reviews and scoping inventories (*15,16*). The review was designed to reconstruct concepts, institutional actors, tensions, and contrasting national pathways; it does not claim exhaustive retrieval or systematic-review status.

Targeted searches were conducted through August 12, 2026, using PubMed, Google Scholar, the World Health Organization (WHO) Institutional Repository, national legal and policy portals, and citation chaining. Search concepts combined humanism, medical humanities, professionalism, patient rights, informed consent, justice, solidarity, universal health coverage, patient safety, organizational ethics, shared decision-making, advance care planning (ACP), goal-oriented care, integrated or people-centered services, artificial intelligence governance, and the names of the four countries. To test rather than merely confirm the proposed framework, supplementary searches addressed managerialism, bureaucracy, auditing culture, ill-defined recipients of care, standardization, digital dehumanization, street-level implementation, community accountability, and decolonization. English-language conceptual and empirical scholarship was prioritized; no language restriction was applied to primary Japanese- and Chinese-language government sources. Searches were updated iteratively when comparison exposed a missing institutional actor, counterexample, or key convention.

Evidence was structured around eight areas: medical professionalism and ethics; patient rights and informed consent; justice, solidarity, and universal coverage; patient safety, improved quality of care, and organizational ethics; shared decision-making, ACP, and goal-oriented care; integrated and people-centered health services; comparative institutional development in the United States, United Kingdom, Japan, and China; and governance of AI for health purposes. The four countries were selected as cases of contrasting pathways: a plural market-oriented system, a tax-funded national service, a social-insurance system with unrestricted access, and a rapidly reforming large middle-income system. They are analytically informative rather than statistically representative.

Selection was purposive and iterative. Sources were retained when they (a) established a landmark ethical or institutional development; (b) provided a conceptual bridge between clinical and system responsibility; (c) documented a national policy pathway; or (d) challenged a simple sequential model. Milestones were included when a change in national legislation, financing, regulation, or provision of care made a relevant value more durable or contestable; dates denote formal events rather than uniform implementation or causal sufficiency. The final corpus privileges conceptual significance and primary documentation over bibliometric representativeness. Because this is not a systematic review, no pooled estimates, formal risk-of-bias assessment, or claim of literature saturation is made.

Analysis proceeded through constant comparison. For each source, the synthesis recorded the value at stake, bearer of responsibility, institutional actor, scale or temporal horizon, and characteristic failure mode. A domain was retained only if failure at that level could independently preclude humane care even when adjacent levels functioned well. Candidate categories were merged when they differed only in terminology and revised when national cases contradicted a linear sequence. Figure 1 presents the resulting architecture: six responsibility domains plus a cross-cutting technology-governance layer. It is a heuristic synthesis intended for empirical testing, not a deductively complete taxonomy.

3. Drawing the boundary: Core tenets, compatible goods, and exclusions

A framework that redescribes every beneficial reform as humanistic explains almost nothing. Its boundary must therefore be normative and institutional at once.

At the core are tenets applicable to persons as persons: recognition of equal dignity; preservation of meaningful agency; attention to suffering and vulnerability; and responsiveness to the relationships, projects, and goals that give a life its shape. These tenets distinguish humanistic evaluation from a generic assessment of technical performance. Equity, solidarity, safety, participation, and trustworthiness are compatible and often indispensable values, but their humanistic status is relational. A safety program is humanistic when it protects people from avoidable harm, listens after injury, discloses error, and deals with consequences; it is not made humanistic by a lower event rate alone. Universal coverage carries humanistic meaning when equal moral standing and protection from abandonment govern designed benefits, not simply when enrollment rises. The same distinction applies to participation that redefines participants rather than engaging in consultation, and to trustworthy technology that preserves agency rather than merely obtaining regulatory approval.

The exclusion test is intentionally demanding. A policy justified solely by throughput, financial solvency, institutional reputation, surveillance, or aggregate output is not humanistic, even if it is efficient. Nor does a mission statement frequently referencing dignity qualify when those affected cannot understand decisions, contest them, or seek remedy. Humanistic institutionalization requires both a person-regarding purpose and a credible mechanism through which that purpose constrains action.

The six domains are not interchangeable categories arranged for rhetorical symmetry. The physician is a moral agent whose judgment and character operate within a professional relationship. The patient is a counterpart imbued with rights whose agency can be respected or

overridden. Population identifies the distributive scale at which access, burdens, and benefits are allocated. Organization identifies the meso-level capacity that coordinates people, information, workflow, and learning. A person's life is a temporal and biographical horizon: it asks whether care comes with a cumulative burden and has relationships and goals rather than whether one isolated choice was offered. Healthcare system identifies the macro-institutional mechanisms—financing, eligibility, workforce, regulation, integration, and public accountability—that structure all local encounters. Their distinctness is revealed by dissociation: an autonomous choice may occur in an unjust coverage system; universal entitlement may coexist with an unsafe hospital; a high-performing organization may still provide care that conflicts with a person's priorities. Collapsing patient into a person's life would erase the difference between choosing and living with the consequences. Collapsing organization into healthcare system would erase the gap between national rules and local capabilities.

Technology does not add a seventh moral quality. Data systems, devices, and algorithms alter what can be seen, predicted, standardized, and contested in every domain. Their governance is therefore represented as a cross-cutting layer with accountable actors that include developers, data stewards, vendors, procurers, organizations, professionals, regulators, and members of the public who are affected.

4. Humanistic values before systems: Virtue, profession, and attention

For much of medical history, ethical responsibility had a visible center: the physician.

The Hippocratic tradition framed medicine through restraint, confidentiality, loyalty, and conduct; later virtue-based accounts located good care in character and practical wisdom. This physician-centered moral grammar should not be dismissed as paternalism in disguise. Clinical work still requires qualities that rules cannot supply. Suffering is not identical to disease, and recognizing it demands interpretive attention to the person whose world has been disrupted. Narrative medicine renewed this insight by treating attention, representation, and affiliation as clinical capacities rather than ornamental sensitivities. Medical humanities supplied a language with which to express meaning, mortality, embodiment, uncertainty, and asymmetry. Their enduring strength is moral perception. Their institutional weakness is contingency: humane care becomes fragile when workload, hierarchy, incentives, or fragmented information systematically oppose even well-considered judgment.

Its limitation was not excessive concern for character but insufficient control over the conditions in which character operated. A moral framework centered on the good physician can identify betrayal, cruelty, indifference, and incompetence. It is less able, by itself, to guarantee that patients can refuse treatment, that limited care is fairly distributed, or that an institution learns from recurrent harm. Reliance on virtue also creates fragility: humane care becomes contingent on the clinician who happens to be present. The subsequent history of modern medicine can therefore be read as an effort to retain professional virtue while adding institutional features that make respect and protection more reliable.

The classical and modern literature locates responsibility in the practitioner, the profession, or the moral relationship of care (*17–27*). This locus remains foundational: no organizational protocol can supply courage, practical wisdom, fidelity, or the interpretive attention required to recognize suffering. Its weakness is contingency. A system that depends on unusually virtuous clinicians cannot guarantee humane treatment when workload, hierarchy, incentives, and fragmented information systematically oppose it.

Professionalism also links the individual and the institution. A profession claims social authority in exchange for competence, service, integrity, and accountability. And yet professional self-regulation can protect patients or shield occupational power. The historical move beyond virtue did not make professionalism irrelevant; it subjected professional authority to external rights, public standards, and organizational oversight.

5. The patient imbued with rights

A decisive expansion occurred when the patient was redefined as an entity imbued with rights instead of a recipient of beneficence.

Research ethics supplied prominent institutional landmarks, including the Nuremberg Code, the Declaration of Helsinki, and the Belmont Report. These arose from different abuses and do not form one seamless genealogy with clinical consent. Alongside legal developments in bodily self-determination and patient-rights movements, however, they changed what professional good intention could justify. The ethical shift was more pronounced than the addition of a rubric. Beneficence asks whether a clinician has chosen wisely for the patient; a rights-based model adds a prior question—who is entitled to decide? Katz exposed silence as a structural feature of professional authority, while shared deliberation later treated patients and professionals as contributors of different knowledge. Autonomy did not displace beneficence, non-maleficence, or justice. It restricted the conditions under which professional judgment could substitute for the person's own agency.

Rights require institutional actors. Meaningful informed consent depends on information practices, documentation, interpreters, assessment of capacity, privacy protections, consultations on ethics, complaint processes, and avenues for redress. Research ethics

likewise requires scientifically and socially valuable protocols, fair selection, independent review, and continuing respect—not merely a signature. International human-rights instruments further connected autonomy to dignity, vulnerability, equality, and social responsibility. Humanistic tenets thus became partially procedural and legal. This produced its own risks: procedures can become rigid, and formal choice can obscure unequal power or constrained options. Nevertheless, the patient imbued with rights marks an enduring shift from trusting that clinicians will act humanely to requiring institutions to protect the concept of self-determination.

Research codes, court decisions, patient-rights instruments, and principlist bioethics collectively transformed consent from professional courtesy into an institutional requirement (*28–41*). Their histories differ and should not be collapsed: research protections responded to experimentation and abuse, while clinical consent developed through tort law, rights movements, and changes in professional practice. The shared institutional effect was to make benevolent intention insufficient justification for overriding a capable person's choices.

Rights can nonetheless become thin proceduralism. A signed form does not establish comprehension, voluntariness, feasible alternatives, or freedom from structural coercion. Relational and trust-based critiques show why agency depends on information, social position, language, accommodation of disability, time, and the credibility of institutions. Institutionalized autonomy must therefore include routes for supported decision-making, complaint, review, and redress—not documentation alone.

6. From individual ethics to social justice

Clinical ethics readily imagines one clinician and one patient. A healthcare system must also decide who is able to intercede in that encounter.

Universal health coverage (UHC) should not be redescribed simplistically as medical humanities. Its causes lie in political settlement, fiscal capacity, labor institutions, social movements, and state formation. But UHC can be understood as an institutional expression of several humanistic values: equal moral worth, a shared burden of financing, access to needed services, and protection from financial ruin. The WHO's financing framework makes clear that coverage involves the distribution of pooled resources and trade-offs among population coverage, services, and cost sharing. It is therefore not only an actuarial arrangement but a recurring decision about whose needs matter.

Coverage also illustrates why formal eligibility and actual access must be distinguished. A benefit may exist in law while distance, waiting time, disability, language, discrimination, informal payment, or poor-quality services make it unusable. Conversely, rapid access to technically available care may still expose households to impoverishment or offer interventions that do not satisfy needs. A humanistic interpretation of UHC must therefore attend to effective coverage: whether people can obtain acceptable, competent care without financial hardship. This moves the ethical analysis beyond the presence of an insurance card and towards the institutional circumstances under which equal status is an actionable capability. It also connects justice to quality. Offering disadvantaged populations nominal access to unsafe, disrespectful, or clinically ineffective services does not encourage solidarity; it distributes a diminished form of care.

Justice also complicates a purely autonomy-centered account of humane care. Unlimited responsiveness to one person's preferences can reduce opportunities for others when resources are finite. Daniels links fair access to the protection of normal opportunity, while Powers and Faden place health within a broader account of social justice and well-being. Public health ethics makes collective action, proportionality, transparency, and justification central because population policies distribute both benefits and burdens. The relevant moral quality is no longer only the choosing patient but the citizen or resident whose access depends on pooled institutions. Humanistic concern at this layer involves not closeness during the encounter, but whether anyone can intercede in the encounter.

The normative field widened when access to care, which became a public obligation rather than a private transaction. Constitutional tenets, human-rights instruments, the founding of the NHS, UHC policy, theories of health justice, public-health ethics, capabilities, and the Sustainable Development Goals all locate moral responsibility at the population and governmental levels (*42–54*). Universal coverage is not synonymous with humanistic care, but it can institutionalize equal moral worth, solidarity through the pooling of risk, access according to need, and protection from financial ruin.

Effective coverage is the relevant ethical standard. Formal eligibility can coexist with distance, waiting, discrimination, language barriers, catastrophic co-payment, or poor-quality care. Conversely, rapid technical access may deliver interventions that are unsafe or unresponsive to need. Justice therefore joins coverage to quality and makes resource allocation ethically apparent. Every system constrains care; the question is whether constraints are necessary, proportionate, transparent, revisable, and attentive to those who bear their burdens.

7. When humanistic reliability became an organizational responsibility

Patient safety moved responsibility from the purview of the individual to reliance on an organized effort.

Traditional responses to error often searched for the careless practitioner. Systems thinking instead examined how equipment, staffing, hierarchy, workflow,

communication, and organizational culture made harm possible or failed to prevent it. The shift did not absolve individuals; it recognized that exhortations to 'be more careful' cannot control hazards designed into care. Crossing the Quality Chasm then linked moral aspiration to system design through six aims—safe, effective, patient-centered, timely, efficient, and equitable—defined as expected aspects of care rather than optional virtues of exceptional clinicians. Donabedian's structure-process-outcome model offered a durable way to connect arrangements with performance. Quality improvement created an applied repertoire for embedding values in routine, even as measurement introduced new ethical problems of its own.

At this point, the consistent incorporation of humanistic values increasingly became an organizational effort. Safety depended on reporting systems and team communication; respect depended on privacy, access, and complaint procedures; equity depended on data and accountability; patient-centeredness depended on whether schedules, records, payment, and care pathways could respond to lives rather than isolated episodes. Accordingly, research on organizational ethics expanded beyond bedside consultation to examine an institution's mission, resource allocation, business conduct, leadership, and policies.

That institutional pivot also changed what counts as an ethical failure. Lack of an explanation may reflect an individual lapse, but it may also reflect appointment lengths, productivity targets, a poorly designed interpreter workflow, or records that ignore the patient's stated priorities. Repeated disrespect is rarely adequately explained by a collection of unrelated bad attitudes. It can be produced by architecture, queue management, visiting rules, billing practices, or the lack of accommodation for a disability. Once such patterns are evident, asking clinicians to compensate through goodwill alone transfers responsibility downward. Organizational ethics is therefore not simply clinical ethics conducted by a committee. It examines how policies distribute attention, discretion, burdens, and standing, and whether leaders create the material conditions under which professional obligations can be fulfilled.

The practical significance is evident when institutional redesign prevents harm at scale. Pronovost and his colleagues' work on catheter-related infections combined evidence, checklists, supply access, team norms, and measurement—demonstrating how safer care requires more than individual vigilance. Berwick's account of patient-centeredness similarly demanded that systems place the patient's needs at the center even when this challenges professional or organizational convenience. WHO's global patient-safety plan now treats safety as coordinated action across governments, organizations, clinicians, patients, and communities. Once harm is structurally produced, humane intent without organizational capability is ethically insufficient.

Patient safety, improvement of quality, organizational ethics, safety culture, learning-health-system ethics, resilience research, and the Francis Inquiry shifted analysis from isolated error to the structures that shape action (*55–69*). An institution can fail ethically even when no individual intends harm: appointment systems can curtail explanations, rigid procurement requirements can result in an unsafe baseline, productivity rules can crowd out deliberation, and fragmented records can obscure the achievement of goals. The Mid Staffordshire inquiry made this problem unusually evident by showing how a penchant for targets, poor listening, and failed governance could coexist with formal structures of care, and it prompted enhanced arrangements for inspections, openness, and candor in England. Repeated disrespect is rarely explained by a collection of unrelated attitudes.

This shift changes accountability. It neither excuses individual negligence nor treats organizations as persons. It recognizes distributed agency: leaders, boards, payers, regulators, designers, and clinical teams control different aspects of care. Organizational ethics is thus more than a hospital committee. It examines how policies distribute time, attention, discretion, risk, and standing, and whether the institution enables professionals to meet their obligations.

Table 1 summarizes the six responsibility domains and the cross-cutting technological layer, their normative emphases, institutional actors, and characteristic failure modes.

8. From patient choice to the life of the person

A patient may have rights and still be forced to choose among disparate clinical options.

Attention to the person's life changes the perspective from which care is evaluated. Patient-centered care includes a biopsychosocial view, recognition of the patient as a person, shared power, and a therapeutic alliance. Shared decision-making operationalizes part of this agenda by combining evidence about options with knowledge of what matters in a particular life. ACP adds time. Contemporary definitions emphasize an ongoing process of identifying values, discussing goals and preferences, communicating with trusted others, and revisiting choices as illness and circumstance change. Success is therefore not gauged by the filling out of documents. The more stringent test is whether care remains concordant with a person's goals when illness limits one's input, increases dependence, or makes trade-offs unavoidable.

Multimorbidity and population aging expose the limits of disease-centered performance. A guideline-consistent plan for each disease can produce a collectively burdensome regimen that serves no coherent life goal. Goal-oriented and patient-priorities approaches therefore ask what outcomes are preferable or to be avoided and then align interventions across conditions. This does not turn medicine into satisfying preferences; clinicians still

Table 1. Domains in the expanding architecture of humanistic responsibility

Domain	Normative emphasis	Institutional actors	Characteristic failure
Physician	Virtue, fidelity, compassion	Professional formation, codes, peer standards	Humane care depends on exceptional individuals
Patient	Autonomy, dignity, rights	Consent law, privacy, ethics review, complaint and redress	Procedural signatures without agency
Population	Justice, solidarity, equity	Pooling, eligibility, benefit design, public-health policy	Formal coverage without effective access
Organization	Safety, quality, accountability	Reporting, teamwork, measurement, leadership, learning systems	Blame, silence, a predilection for procedures
A person's life	Goals, relationships, continuity	SDM, ACP, multimorbidity and palliative-care pathways	Checklist completion without concordance
Healthcare system	Access, integration, governance	Primary care, financing, allocation, coordination, community participation	Fragmentation or unjust constraint
Technology governance (cross-cutting)	Distributed accountability across all domains	Developers, data stewards, vendors, procurement, institutions, professionals, regulators, affected publics	Moral off-loading, deskilling, surveillance, and scalable bias

judge feasibility, safety, and proportionality. It changes where moral attention is directed from an isolated decision to a life unfolding through care.

Japan provides a useful institutional illustration. The Ministry of Health, Labour and Welfare's revised guidance for decision-making in the final stage of life emphasizes the person's decision, repeated discussion, multidisciplinary care, relief of suffering, and reconsideration as wishes change. The vocabulary is often framed as ACP, decision-making support, or end-of-life care rather than 'medical humanities.' That difference is analytically important: humanistic values may be most consequential when they are incorporated into ordinary rules and expectations. The question is not whether a system frequently touts humanistic ideals but whether it provides continuity, time, and legitimate roles for persons and families to articulate what matters.

Institutionalization can nevertheless hollow out the practice it intends to protect. Consent can become a signature, shared decision-making a checklist, and advance care planning a document target—a form of box-ticking bioethics in which evidence that a task occurred substitutes for evidence that a person was heard. Defensive medicine may convert communication into liability management, while the burden of indicators can displace the unhurried listening on which interpretation depends. Standardization remains necessary for coordination and accountability, but documentation should facilitate the preservation of records, continuity, and deliberation rather than stand in for them. Measures should therefore examine comprehension, the opportunity to disagree, elicitation of goals, continuity across settings, and concordance between care and the person's priorities. Institutions are a necessary precursor but not sufficient as the ultimate result; repeated implementation in clinical settings, reflection, and revision keep routines connected to humanistic purposes.

Patient-centered care, shared decision-making, ACP, decision aids, evidence-based choice, goal-oriented care, multimorbidity practice, and palliative-care policy expand the focus of attention to a life rather than an isolated decision (*70–84*). The critical shift is one from only asking which option the patient selects to asking why that option matters within the context of relationships, burdens, identity, and future goals.

9. Different national pathways to institutionalization

Comparative analysis should resist treating national systems as moral embodiments. Labels such as market, Beveridge, Bismarck, or social insurance identify financing and governance patterns, not complete ethical cultures. Each country consists of multiple payers, providers, professions, laws, and local variations. Moreover, cultural explanations easily become circular: autonomy is attributed to individualism because the system emphasizes choice, and individualism is then inferred from the emphasis on choice. Law, political coalitions, professional power, payment, administrative capacity, and demographic change are more tractable pathway mechanisms.

In the United States, rights, professionalism, research oversight, litigation, assessment of quality, and consumer choice became prominent institutional factors. And yet a plural insurance system links access to public eligibility, employment, private coverage, and network rules. Comparative evidence continues to show the tension between high spending and differences in access and outcomes. The pathway therefore has a strong penchant for formal autonomy and organizational innovation

but is continually contested at the level of distributive justice. Market coordination does not eliminate the state; Medicare, Medicaid, regulation, tax subsidies, and public research are central. Nor does consumer choice guarantee meaningful agency when prices are opaque or coverage constrains options.

The United Kingdom institutionalized universalism through a tax-funded national service and made primary care an organizing entry point. The ethical significance of gatekeeping is ambiguous but not inherently anti-humanistic. It can limit the immediate choice of a specialist, and yet it can also provide continuity, prioritization by clinical need, and stewardship of shared resources. The NHS pathway joins individual dignity and involvement to a public ethic in which everyone counts and finite resources should be used fairly. Its recurrent problems—waiting, workforce strain, and capacity—show that humane entitlement to care must be matched by operational capability.

Japan demonstrates why the framework must be layered rather than sequential. Universal health insurance was achieved in 1961, before autonomy and informed consent became highly evident in Japanese bioethics and clinical policy. The system preserves comparatively broad patient choice and lacks a compulsory British-style GP gatekeeper, while national fee scheduling and price revision regulate costs and incentives for care providers. Its trajectory therefore placed social coverage and administrative price control before the later formalization of some patient-rights and shared-decision-making practices. Aging, hospital dependence, long-term care, and community-based integrated care have subsequently shifted responsibility in the direction of continuity and the person's life.

China illustrates compressed institutionalization. Expansion of basic insurance, patient-rights legislation, public-hospital reform, quality and safety regulation, strategic purchasing, enhancement of primary care, integrated provision of care, and aging-related care have advanced within a few decades. These layers do not move at the same speed. Broad coverage can coexist with regional inequality; legal rights with communication gaps; hospital capability with weak continuity; payment reform with incentives that remain difficult to align. The appropriate question is not whether China has imported a Western system but how multiple values are being simultaneously incorporated within a vast mixed system.

Across all four cases, common values are achieved *via* different institutional means. Autonomy may be upheld by courts, professional guidance, or statute; solidarity among the public by taxes or social insurance; safety by accreditation, reporting, or administrative regulation; and personhood by primary care, ACP, community integration, or long-term-care policy. Institutions can also conflict: free access may weaken coordination; gatekeeping may delay needed care; a uniform fee schedule may suppress prices while encouraging volume; market competition may mobilize investment while fragmenting accountability. A humanistic assessment must therefore ask not which national model is morally superior but how its arrangements convert values into reliable capabilities and how its trade-offs are justified.

Every system manages scarcity, even when rationing is not named explicitly. The relevant differences concern where constraints appear and who has the authority to impose them: price at the point of use, coverage exclusions, referral gates, waiting lists, workforce distribution, designed benefits, technology assessment, or payment schedules. These mechanisms have distinct ethical profiles. A visible queue can be politically contentious but publicly reviewable; a high deductible can preserve nominal choice while silently deterring care; free provider choice can respect preference while weakening continuity; and strict regional planning can improve distribution while narrowing local responsiveness. Comparative ethics should elucidate these constraints. The question is not whether a system limits choice, cost, or supply but whether the limitation is necessary, proportionate, transparent, revisable, and attentive to those who carry its burdens.

Comparative research on healthcare systems shows why national models should not be treated as moral embodiments (*85–97*). The United States, United Kingdom, Japan, and China combine financing, law, professional authority, public administration, and market mechanisms differently. Each model varies internally. Those examples are used here to identify institutional actors and ordering effects, not to rank cultures.

Primary sources support a selective milestone comparison. The United States pathway includes a clinical-rights milestone—Canterbury v Spence in 1972—alongside research ethics and quality-system events (*5,28,30,38,55,108*). In the United Kingdom, the 2013 Francis Inquiry marks a system-level reckoning with an organizational culture that allowed targets and institutional self-protection to displace listening, safety, and compassion (*69*). Japan's Ministry of Health, Labour and Welfare instituted universal insurance in 1961 and it publicly assumed the risk of long-term care through the 2000 Long-Term Care Insurance system and the 2002 Comprehensive Measures for the Promotion of Medical Safety (Iryō Anzen Suishin Sōgō Taisaku). The 2002 measures explicitly reframed safety from dependence on individual effort to a system-wide responsibility and required hospitals and clinics with beds to establish safety policies, internal reporting, committees, and staff training; designated hospitals also required safety managers, organizational elements, and consultation services. Later milestones include the 2007 end-of-life decision-making process guideline, its revisions, and health/long-term-care integration (*98–102*). Chinese primary sources document the 2009 reform framework, the 2015 hierarchical-care policy, the 2016 contracted family-doctor policy and Healthy China 2030, the 2018 regulation on

medical disputes, and statutory protection of dignity, privacy, and coordinated care in the Basic Healthcare and Health Promotion Law (*97,103–107*). The timeline additionally draws on the Common Rule and the three United Kingdom statutes governing capacity and service integration (*108–111*). These nodes are illustrative, not exhaustive.

Japan is the clearest reason to reject a universal sequence: social justice through UHC was institutionalized before autonomy gained some traction in national bioethical discourse. This review describes China's recent trajectory as 'compressed institutionalization': expansion of coverage, patient-rights legislation, safety regulations, payment reforms, restructuring of primary care, integration, and aging-related policies proceeded in overlapping decades. This is an interpretive concept proposed by the present review, not an established country classification. In both instances, formal rules can precede capability and national policy can coexist with regional and organizational variation.

Table 2 compares the institutional actors and tensions in the four pathways; Figure 2 displays selected milestones without implying a causal sequence.

10. Laying the groundwork for a humanistic healthcare system

The architecture yields a working definition but not a

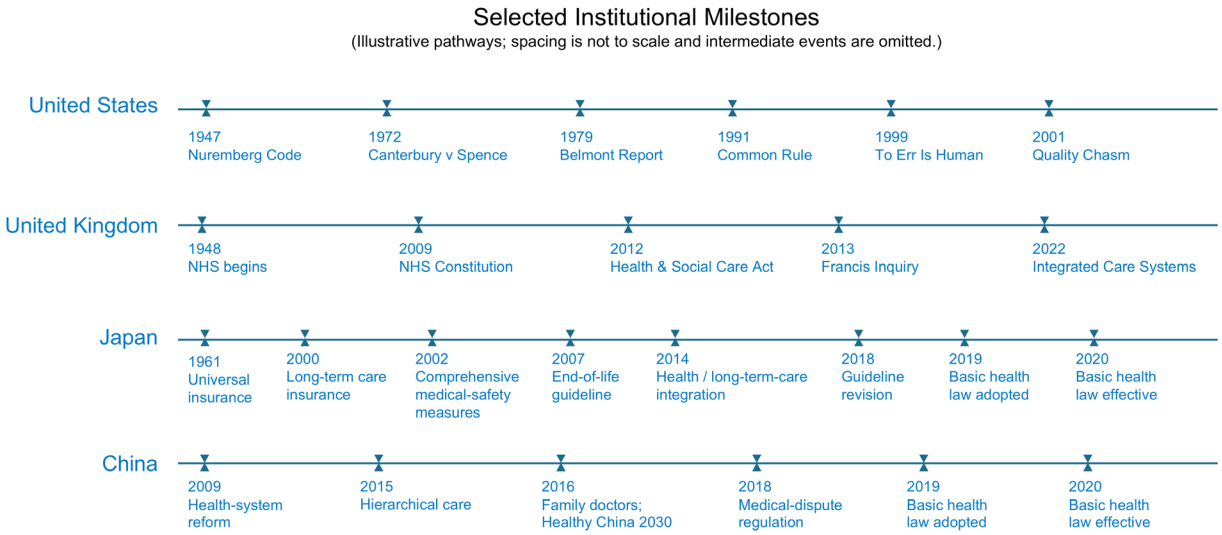


Figure 2. Selected institutional milestones in four national pathways. The timeline is illustrative and deliberately selective. The 1999 label denotes the release of To Err Is Human, cited in the 2000 National Academies Press edition. It is neither a stage model nor a claim of linear or sufficient causation. Dates identify formal policy or legal events rather than first local adoption, complete implementation, or demonstrated effects. The Japanese 2002 milestone is the Ministry of Health, Labour and Welfare's Comprehensive Measures for the Promotion of Medical Safety, which framed safety as a system-wide responsibility and specified organizational infrastructure. Intermediate revisions, including Japan's 2015 end-of-life guidance revision, are omitted. Japanese and Chinese milestones were checked against publicly accessible government materials in the original languages. Sources for Figure 2: (*5,28,30,38,45,46,55,69,89,90,97–111*).

Table 2. Illustrative national pathways for institutionalizing humanistic values

Pathway	United States	United Kingdom	Japan	China
Rights and agency	Canterbury, consent, research regulation	Statutory rights, NHS Constitution, capacity law	Professional and policy guidance; later explicit SDM/ACP	Statutory dignity, privacy, consent; uneven implementation
Justice and access	Plural public-private coverage	Tax-funded NHS universalism	1961 universal social insurance	Rapid expansion of basic insurance after 2009
Safety and quality	IOM-led systems movement; accreditation and measurement	Francis Inquiry; quality, safety, and candor in institutions	2002 Comprehensive Measures for Medical Safety; organizational duties	2018 dispute-prevention regulation; quality reform
Continuity and life goals	SDM/ACP initiatives within fragmented delivery	Primary-care gatekeeping and personalized care	2000 LTCI; end-of-life guidance; community integration	Hierarchical care, family-doctor contracting, integrated county systems
Dominant tension	Choice and innovation versus cost and inequity	Universalism and stewardship versus capacity and waiting	Free access versus coordination and hospital dependence	Broad coverage versus regional variation and hospital centrality

new label for every well-run service.

The WHO's framework on integrated, people-centered health services supplies the clearest policy analogue. It calls for a shift from systems designed around diseases and institutions to systems designed around people and communities, requiring changes in how services are funded, managed, and delivered. Subsequent guidance on continuity and coordination translates that orientation into practical relationships across time, providers, and settings. The Declaration of Astana similarly positions primary health care as a foundation for universal, integrated, accessible services and empowered communities. These are not simply communication reforms; they alter the architecture of care.

Integration must nevertheless be judged normatively. A highly integrated system could be efficient but coercive, coordinated but inattentive to dissent, or data-rich but discriminatory. Multilevel integration frameworks clarify the clinical, professional, organizational, and system dimensions involved, while quality research on healthcare systems insists that coverage without competent, respectful care is insufficient. Primary care reform likewise requires financing, workforce allocation, information, accountability, and ties to the community, not merely renaming a first-contact service. Humanistic design therefore combines capabilities with safeguards.

The framework suggests six evaluative questions. First, dignity: do routines recognize persons as more than illnesses, transactions, or risk scores? Second, agency: can people understand, influence, refuse, and reconsider decisions? Third, justice: are access, burdens, and benefits distributed fairly? Fourth, safety: does the organization anticipate, learn from, and redress harm? Fifth, life alignment: can care respond to goals, relationships, and a cumulative treatment burden? Sixth, accountability: can institutions explain how financing, prioritization, and technology shape outcomes, and can the people affected contest those decisions? These questions deliberately cross levels. A respectful encounter inside an exclusionary system is not enough; neither is universal access to unsafe or depersonalized care.

The practical implication is a shift from exhortation to design. Humanities education may encourage more profound insights and deliberate judgment, but organizations must give clinicians time, continuity, interpreters, functional teams, trustworthy data, and incentives compatible with humane care. Patient rights must be exercisable by people with limited literacy or power. A commitment to equity must appear in benefit design, geographic allocation, and quality measurement. ACP must be linked to records and emergency pathways. Complaints must produce learning. The test of a humanistic system is not whether every conflict disappears, but whether its structures make humane action more probable, evident, and accountable.

This definition also provides a starting point for research. The layers can be studied as formal tenets, organizational capabilities, and experienced outcomes. Researchers might examine whether legal rights are exercisable, whether safety training reaches the bedside, whether financing facilitates continuity, whether patient-reported priorities alter plans, and whether communities participate before decisions on allocation are reached. Comparative work should trace institutional actors and countervailing mechanisms rather than scoring countries on a single cultural scale. Longitudinal work could ask when a value is incorporated, when it is displaced by competing incentives, and how crises expose the difference between symbolic and operational tenets. Such inquiry would help prevent 'humanistic' from becoming a flattering label and instead make it a verifiable claim regarding system performance.

Continuity, primary care, multilevel integration, and quality system design provide the closest established policy foundations for the proposed concept (*6,7,112–116*). A humanistic healthcare system is one in which dignity, agency, equity, safety, participation, and person-defined goals are embedded not only in encounters but in organizational design, financing, resource allocation, provision of care, and governance. The term is developed here as a systematic analytical framework rather than attributed to the WHO, offered as a newly coined phrase, or presented as settled terminology.

The model identifies six cumulative responsibility domains—physician, patient, population, organization, a person's life, and healthcare system—plus technology governance as a cross-cutting layer. The domains are not ontologically identical. Physician and patient are relational loci; population is distributive; organization is meso-institutional; a person's life is a temporal-biographical horizon; and healthcare system is macro-institutional. The concentric arrangement in Figure 1 depicts analytical scope and accumulation, not chronology, stage progression, or a simple micro-to-macro ladder. A person's life is a clinical-biographical re-entry that reconnects institutional capability to the trajectory of someone's life. Technical systems run through every domain, reshaping bedside judgment, organizational workflow, financing, allocation, and public governance.

The concept also requires a boundary without creating a binary label. A humanistic healthcare system is an ideal and a directional continuum: real systems combine humanistic and dehumanizing features and must negotiate legitimate cost, capacity, public health interests, and distributive constraints. Dignity, agency, and person-defined life goals function as normative floors that cannot be overridden solely by efficiency, throughput, aggregate utility, or institutional revenue. A humanistic system is not one without trade-offs; it is one in which trade-offs are bounded by these normative floors and governed through transparent, proportionate, contestable, and revisable procedures. When values conflict, a more humanistic system makes reasons public, includes the

individuals affected, provides proportionate safeguards and compensation, permits contestation and appeal, remedies harm, and revises policy. Efficiency may serve humanistic ends when it frees up time or resources while respecting these floors; technically competent performance is not sufficient.

The framework also leads to operational questions and measurable constructs. Agency can be assessed with patient-reported shared-decision-making instruments such as the SDM-Q-9, comprehension checks, supported-choice availability, and exercisable appeals (*72,81*). Life alignment can be examined through documented goals, patient-reported outcomes and treatment burden, continuity, and concordance between the care delivered and the priorities expressed (*76,77*). Safety can be examined through a non-punitive reporting culture, learning cycles, disclosure, imposition of a duty of candor, and remediation (*55–69*). Measures should be interpreted together: a high rate of documentation without comprehension, trust, or concordance may indicate formalistic rather than humanistic performance. No single indicator should be treated as a performance target in isolation; potential measures need to be triangulated with qualitative experience, patient narratives, and a reflexive review of their behavioral effects.

Table 3 translates the ideal into evaluable domains and potential health-services indicators.

11. Algorithms and cross-cutting technology governance

Artificial intelligence makes distributed responsibility unusually apparent.

An algorithm may appear in one consultation, but its training data, included variables, interface, procurement, validation, monitoring, and update process are dispersed among developers, vendors, hospitals, payers, regulators, and governments. WHO guidance accordingly assigns duties across this network and emphasizes autonomy, well-being, safety, transparency, accountability, inclusiveness, equity, responsiveness, and sustainability. The central danger is moral off-loading: a technically mediated recommendation is treated as if no accountable person or institution selected its proxy, threshold, workflow, or acceptable error. The problem is not hypothetical. A widely used population-health algorithm reproduced racial inequality because expenditure stood in for health need. Black-box performance also creates a tension between predictive accuracy and reasons attributable to patients and professionals. Digital systems can therefore expand capabilities while limiting transparency, standing, or discretion.

A humanistic healthcare system therefore requires algorithmic accountability as a cross-cutting governance layer, not a specialized add-on. It should define which decisions may be delegated, preserve meaningful human review, test performance across affected groups, document intended use, monitor drift and downstream effects, provide routes for contestation and redress, and disclose when automation materially shapes care. Most importantly, it should ask whether the objective being optimized results in human benefit. Efficiency, utilization, revenue, and predicted risk are not self-justifying goals. The relevant question is whose life is improved, who has standing but is ignored, and who

Table 3. Proposed domains for evaluating a humanistic healthcare system

Domain	Core question	Illustrative indicators
Humanistic boundary	Are trade-offs constrained by normative floors and open to correction?	Published reasons; affected-party participation; equity impact assessment; appeal completion; remediation time
Dignity	Are persons recognized beyond diagnosis or transaction?	Respectful-care experience; privacy; discrimination; accommodation
Agency	Can people understand, influence, refuse, and revisit decisions?	SDM-Q-9 or CollaboRATE; teach-back comprehension; decision-aid reach; supported choice; appeal
Equity	Are access, burdens, and benefits distributed fairly?	Effective coverage; financial protection; geographic and group gaps
Safety	Does the system anticipate, learn from, and repair harm?	Non-punitive reporting climate; closed learning cycles; candor compliance; disclosure and remediation
Life alignment	Does care respond to goals, relationships, and a cumulative burden?	Goal-concordant care; patient-reported outcomes; continuity; treatment-burden measures
Participation	Do patients and communities shape design and allocation?	Co-design; governance representation; public justification
Accountability	Can institutional and algorithmic decisions be explained and contested?	Transparency; audit; human review; redress; monitoring

Note: Candidate measures should be triangulated with qualitative experience, patient narratives, and reflexive review of behavioral effects. No single indicator should be used as an isolated performance target.

bears the brunt of an error when the system scales.

Technology does not have moral qualities or automatically bear responsibility. The technological ecosystem denotes the distributed governance network of developers, data stewards, vendors, procurers, healthcare system leaders, professionals, regulators, and the members of the public that are affected that selects objectives and controls development, deployment, monitoring, and redress (*8,117–124*). Its responsibilities cut across all six domains rather than appearing only after them.

Three requirements are central. First, algorithmic objectives encode values: expenditure, utilization, throughput, predicted risk, and revenue are not self-justifying proxies for human benefit. Second, responsibility must not be off-loaded to a model when institutions selected the data, targets, workflow, and thresholds. Third, non-delegable decisions, meaningful human review, subgroup validation, monitoring, contestability, and redress must be specified. These requirements connect technological accountability to clinical judgment and system governance without turning AI ethics into a separate area of medicine.

12. From theory to system capability: Implementation challenges and strategic priorities

The synthesis can be stated as three propositions. Proposition 1—expansion: responsibility for humane care has extended beyond the individual clinician into relational, distributive, organizational, biographical, institutional, and technological domains. Proposition 2—institutionalization: humanistic values are more reliable when translated into rights, capabilities, financing arrangements, and accountability mechanisms, but institutionalization can also hollow them out. Proposition 3—plural pathways: there is no universal sequence or single model of a humanistic healthcare system; countries institutionalize overlapping values *via* different means and in different orders.

Some accounts of declining humanism at the bedside may overlook a change in visibility: obligations once described mainly as professional character have been translated into rights, organizational routines, coverage, safety systems, care pathways, and governance. This does not make dehumanization a category error. Institutionalized tenets and dehumanizing experiences can coexist, and modern institutions can produce new forms of distance, exclusion, surveillance, and moral harm. The framework therefore asks where responsibility is located and how reliably values are incorporated rather than inferring humane experience from the existence of policy.

Institutionalization also has deleterious effects that a humanistic framework must confront rather than treat as implementation noise. Auditing can convert complex care into what is documentable in a reporting system, while targets can redirect attention toward the measured proxy and away from the person whose need led to it. Standardization is similarly ambivalent: protocols encourage coordination and reduce unwarranted variation, and yet their local enactment redistributes discretion and may suppress contextual knowledge. Foucauldian analyses of targets has revealed the disciplinary effects of apparently neutral performance regimes; ethnography of Indian primary care has shown how unrealistic targets and punitive accountability can produce 'outward performance'—documentation that portends to describe achievement while organizational capability deteriorates (*125–128*). These critiques do not invalidate measurement. They establish a stronger design requirement: indicators must remain answerable to experience, encourage learning rather than theatrical compliance, and be withdrawable when their behavioral effects defeat their stated purpose.

The four pathway cases also place limits on the framework's global claim. High-capacity states can formalize rights, reporting, and appeal through legal and administrative infrastructures that are absent, plural, or donor-tailored elsewhere. Research on street-level bureaucracy in low- and middle-income countries has shown how frontline discretion mediates policy under scarcity; work in Nigeria has located people-centered governance across operational, collective, and constitutional levels; studies of community accountability caution that nominal participation does not reliably shift power; and decolonial scholarship asks who defines the values, evidence, and institutional model being exported (*129–132*). A humanistic healthcare system cannot therefore be a high-income template applied at lower cost. In settings where communities, informal providers, local governments, and external funders share authority, the relevant architecture may be polycentric. The framework's durable question is narrower and more portable: where does consequential power sit, how does it affect persons and populations, and what are the avenues for achieving standing, making revisions, and obtaining redress?

The framework also clarifies recurring false opposition. Gatekeeping is not inherently inhumane; it may facilitate continuity and stewardship or become an access barrier. Patient choice is not inherently humane; nominal freedom may coexist with unaffordable or fragmented options. Standardization can depersonalize, but it can also result in safety independent of records. Efficiency can crowd out attention, and yet waste can itself be unjust. Ethical evaluation must examine the design, justification, distributional consequences, and correctability of each mechanism.

The implication for education is not to replace humanities teaching with systems science. Clinicians need narrative, historical, ethical, and interpretive capacities precisely because institutions cannot anticipate every case. But education that asks individual clinicians to compensate for structurally unsustainable workloads or incoherent incentives results in a shifting of moral culpability. Professional dictates and institutional reform

should be treated as complements.

The relationship is also bidirectional. Supportive institutions safeguard time, standing, continuity, and psychological safety, making ordinary humane conduct more feasible and less dependent on heroism. And yet institutions can become unjust, rigid, or captured by financial and political interests. In such settings, moral courage, conscientious objection within professional limits, whistleblowing, collective advocacy, and solidarity with patients may become the final safeguards of humanistic value. Individual virtue does not replace system capability, but it can diagnose institutional failure, resist the normalization of harm, and prompt reform. System capacity and professional agency should therefore be understood as a recursive relationship: institutions cultivate or suppress virtue, while moral practitioners maintain, contest, and redesign institutions.

Trust is not an eighth domain. It is a cross-cutting relational outcome—and a fallible indicator—of successful institutionalization. Virtue may encourage trust in clinicians; exercisable rights and transparent redress may encourage trust in institutions; safety training may encourage organizational trustworthiness; solidarity may encourage confidence in fair pooling; and contestable technology may encourage warranted reliance. Trust should not be demanded from patients or the public, however. Systems must earn it through competence, honesty, inclusion, and redress, while remaining subject to scrutiny and appeal (*40,41,65,66*).

In research, the layers can be examined as formal tenets, organizational capabilities, and experienced outcomes. Future work should study whether rights are exercisable, safety training reaches the bedside, payment provides continuity, priorities alter plans, and communities participate before decisions om allocation are reached. Comparative work should track actors and countervailing mechanisms rather than score countries on a single cultural scale.

13. Limitations

This review is integrative and seeks to lay out a theory, but it is not exhaustive. Targeted searches, purposeful selection, and citation chaining cannot provide the coverage guarantees of a systematic review, and the corpus was not screened to estimate effects. Landmark English-language scholarship may privilege Anglo-American categories even though Japanese and Chinese primary policy documents and critical literature from lower-resource settings were consulted. The United States, United Kingdom, Japan, and China are contrasting pathway cases, not a representative global sample; scholarship from Africa and South Asia was used to challenge the model but cannot substitute for additional country histories. Figure 2 omits intermediate events—including Japan's 2015 revision of end-of-life decision-making guidance—and its dates do not document initial local adoption, uniform implementation, or causal effects. Institutionalization can occur locally before national policy or remain symbolic after legal enactment. Finally, the six-domain architecture and cross-cutting technology layer require validation with patients, families, clinicians, managers, communities, and policymakers, and particularly where governance is polycentric or resources are severely constrained.

14. Conclusion

Modern medicine has not simply transitioned from humanistic physicians to humanistic systems; responsibility for humane care has been distributed among clinicians, patients, institutions, financing arrangements, governance structures, and technological infrastructures. Professional virtue remains necessary, but it is joined by patient rights, social justice, organizational reliability, attention to a person's life, system governance, and accountable technology. These domains appear in different orders and through different national means; common values do not require a single model of a healthcare system.

A humanistic healthcare system is therefore an ideal and a directional continuum, not a binary designation. It protects normative floors of dignity, agency, and life alignment from being overridden solely by efficiency or revenue, while making unavoidable trade-offs transparent, proportionate, contestable, remediable, and revisable. Institutions are necessary but not sufficient: without humane implementation in clinical settings, they can degenerate into formalism; without institutional support, humane practice is vulnerable. The practical task is to align professional agency, organizational capability, public accountability, and technology governance so that care can be made humane by design and corrected when it is not.

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Review

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Traditional Chinese medicine for diabetic lower extremity arterial disease: A review of recent advances

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SUMMARY: Diabetic lower-extremity arterial disease (LEAD) is a prevalent macrovascular complication of diabetes and a critical pathological foundation for diabetic foot lesions. Its pathogenesis involves glycolipid metabolic disturbance, excessive oxidative stress, persistent vascular inflammation, and endothelial dysfunction, leading to limb ischemia, refractory foot ulcers, and increased amputation risk. Current standard treatments, including glycemic and lipid control, antiplatelet therapy, and endovascular revascularization, have improved clinical outcomes but still face challenges in fully ameliorating microcirculatory impairment and preventing long-term restenosis. Traditional Chinese medicine (TCM) exerts multi-component and multi-target protective effects against diabetic LEAD. This review summarizes pharmacological advances in representative TCM agents, including *Salvia miltiorrhiza*, *Astragali Radix*, and their bioactive constituents (*e.g.*, tanshinone IIA sulfonate, salvianolic acid B, astragaloside IV, and Astragalus polysaccharide), as well as classical formulas and patent medicines such as Huangqi Guizhi Wuwu Decoction, Tongxinluo capsule, Tangbi capsule, and Danggui Sini Decoction. Their mechanisms involve protecting vascular endothelium, regulating glycolipid metabolism, suppressing vascular inflammation *via* NF-κB and NLRP3 pathways, and activating Nrf2-mediated antioxidant defense, HIF-1α/VEGF, PI3K-AKT, and Ang-1/Tie-2 signaling to restore microcirculation and promote angiogenesis. Clinically, TCM-assisted endovascular intervention reduces postoperative restenosis and improves lower-limb perfusion; external preparations promote diabetic foot ulcer healing; acupuncture, moxibustion, and mind-body exercises improve microcirculation and long-term management. TCM is a promising adjunctive strategy for diabetic LEAD, and further mechanistic and clinical studies are needed to advance its standardized application and improve patient prognosis.

Keywords: Diabetic lower-extremity arterial disease (LEAD), Traditional Chinese medicine (TCM), endothelial dysfunction, oxidative stress, vascular inflammation, angiogenesis

1. Introduction

Lower extremity arterial disease (LEAD) is a prevalent macrovascular complication of diabetes and serves as a critical pathological foundation for diabetic foot lesions (*1*). Multiple risk factors, including prolonged diabetic duration, suboptimal glycemic control, hypertension, and dyslipidemia, are strongly correlated with development of LEAD, the predominant lower-extremity manifestation of peripheral arterial disease (PAD). Its pathophysiological progression involves glycolipid metabolic disturbance, excessive oxidative stress, persistent vascular inflammation, and endothelial dysfunction (Figure 1) (*2*). In recent years, the rising incidence, disability rate, and mortality of LEAD among diabetic patients have imposed a substantial socioeconomic burden on patients' families and society (*3*). Despite its high prevalence in the Chinese diabetic population, LEAD remains substantially underdiagnosed and undertreated. A national multicenter cross-sectional study demonstrated that LEAD prevalence among high-risk patients with type 2 diabetes reached 21.2%, whereas only 10.6% of affected individuals received a formal diagnosis before study enrollment (*4*). In addition, pharmacological management targeting glycemic, blood pressure, and lipid control in this patient population frequently fails to meet guideline-recommended targets.

Current mainstream therapeutic strategies for diabetic LEAD consist of glycemic regulation, lipid-modifying therapy, antiplatelet agents, and vascular revascularization (*5*). Nevertheless, these conventional interventions exhibit limited efficacy in ameliorating microcirculatory impairment and chronic vascular inflammation (*6*). Furthermore, long-term pharmacotherapy may induce diverse adverse reactions, and revascularization remains hampered by a high incidence of long-term

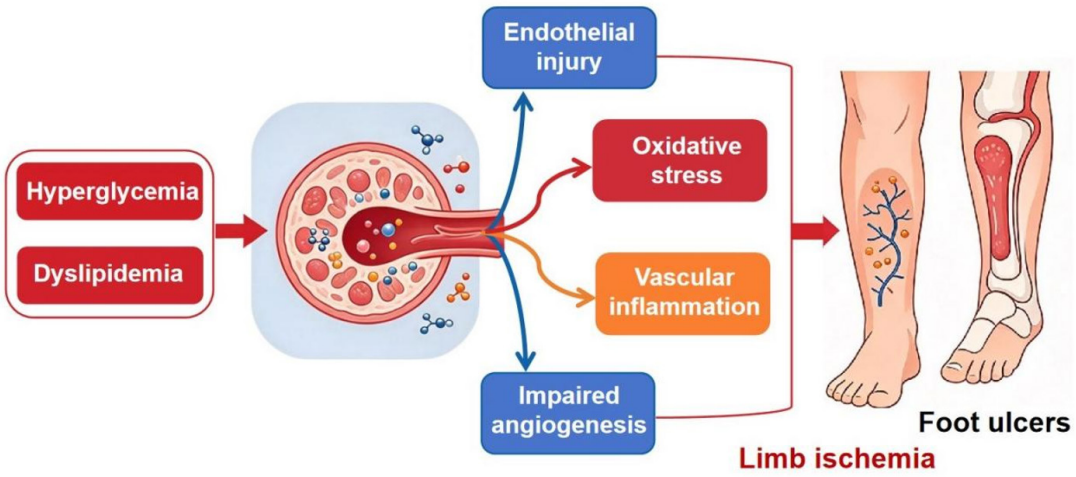


Figure 1. Pathogenesis of diabetic lower-extremity arterial disease (LEAD). Hyperglycemia and dyslipidemia induce endothelial injury, oxidative stress, vascular inflammation, and impaired angiogenesis, ultimately causing limb ischemia and diabetic foot ulcers.

restenosis, which ultimately limits comprehensive and effective management of diabetic LEAD (*7*). Therefore, identifying adjunctive or alternative therapies that can effectively improve microcirculation, suppress vascular inflammation, and delay progression of atherosclerosis has become a research priority.

Characterized by the holistic concept and syndrome differentiation and treatment, traditional Chinese medicine (TCM) has been widely applied in prevention and intervention of diabetes and its related vascular complications for thousands of years (*8*). It exerts multi-target and multi-pathway regulatory effects, exhibiting prominent advantages in ameliorating lower limb blood perfusion, alleviating vascular inflammatory response and preventing development of arteriosclerotic lesions (*9*). A Bayesian network meta-analysis revealed that Chinese herbal injections, such as Dan Shen injection, Danhong injection, and Chuanxiongqin injection, in combination with conventional therapy, significantly improved the ankle-brachial index (ABI), dorsalis pedis artery blood flow, and hemorheological indices, indicating that TCM could be a valuable adjunctive treatment for diabetic LEAD (*10*). Furthermore, modern pharmacology has demonstrated that some Chinese herbal medicines, such as *Salvia miltiorrhiza* (Danshen), and *Astragali Radix* (Huangqi), can exert anti-atherosclerotic actions *via* improving hemorheology, suppressing platelet aggregation, modulating oxidative stress, reducing lipid levels and ameliorating lipid metabolism, and protecting both the endothelium and basement membrane (*11*). These findings provide scientific evidence for use of TCM in treatment of diabetic LEAD.

In recent years, although clinical evidence and molecular mechanistic studies on TCM for diabetic LEAD have accumulated rapidly, a systematic synthesis of these findings is still lacking. In this review, we therefore aim to comprehensively summarize recent advances regarding the pharmacological mechanisms and clinical applications of bioactive constituents, classical TCM formulas, and patent medicines for the prevention and treatment of diabetic LEAD. By integrating these data, we hope to provide a solid scientific basis for optimizing clinical therapeutic regimens and ultimately improving long-term patient outcomes.

2. Modern pharmacological mechanisms of TCM in the treatment of diabetic LEAD

Diabetic LEAD represents a multifactorial vascular complication. Chronic hyperglycemia, dyslipidemia, inflammation, and oxidative stress, together with impaired angiogenesis, synergistically trigger endothelial injury, arterial stiffening and limb ischemia. TCM bioactive constituents, classical formulas, and patent medicines exert promising vascular-protective effects against diabetic LEAD through multi-component and multi-target regulatory mechanisms (Table 1 and Table 2). This section reviews recent advances in modern pharmacological research on TCM against diabetic LEAD, focusing on four interrelated domains: vascular endothelial protection, modulation of glucose and lipid metabolism, suppression of vascular inflammatory responses, and improvement of microcirculation coupled with pro-angiogenic effects (Figure 2). Collectively, these mechanisms support TCM as a potential adjunctive therapy for diabetic LEAD and lay a foundation for subsequent mechanistic exploration and clinical investigation.

2.1. Protection of vascular endothelial function

Vascular endothelial dysfunction is widely recognized as the initiating step in pathogenesis of diabetic LEAD (*12*). Under chronic hyperglycemic conditions, the endothelium undergoes a series of pathological alterations, including reduced nitric oxide (NO) bio-

Table 1. Pharmacological mechanisms of bioactive constituents from traditional Chinese medicine (TCM) in the treatment of diabetic lower-extremity arterial disease (LEAD)

Bioactive constituent	Source	Target/signaling pathway	Main mechanism	Protective effect	*Ref.*
Sodium Tanshinone IIA sulfonate	*Salvia miltiorrhiza* (Danshen)	miR-133a↓/GCH1↑/NO↑	Enhancing NO bioavailability and reducing oxidative injury	Protecting vascular endothelium and improving perfusion in diabetic ischemia	(*16*)
Salvianolic acid B	*Salvia miltiorrhiza* (Danshen)	BMP4↓/ROS↓/NO↑	(i) Inhibiting apoptosis/mitophagy; (ii) Enhancing NO bioavailability	(i) Protecting diabetic endothelial function; (ii) Accelerating wound healing	(*18-20*)
Curcumin+Baicalein	*Curcuma longa* (Jianghuang); *Scutellaria baicalensis* (Huangqin)	Nrf2/HO-1↑; p38/JNK MAPK↓	Mitigating oxidative-mediated endothelial damage	Enhancing endothelial viability and ameliorating diabetic angiopathy	(*23*)
Astragaloside IV	*Astragali Radix* (Huangqi)	AMPK/PI3K/Akt↑; METTL3/m6A/SIRT1↑	(i) Correcting glucose-lipid homeostasis; (ii) Triggering epigenetic modulation	(i) Ameliorating glycolipid disorders and IR; (ii) Attenuating peripheral arterial stiffness and AGE-triggered vascular injury	(*30-33*)
Astragalus polysaccharide	*Astragali Radix* (Huangqi)	miR-721↓/PPAR-γ↑/PI3K/Akt↑; ROS↓	Regulating glycolipid metabolism and scavenging ROS	Restoring insulin sensitivity and promoting angiogenesis	(*35-37*)
		M1↓/M2↑, TNF-α↓, IL-1β↓, IL-6↓, IL-10↑	Regulating M1 to M2 macrophage polarization and reducing pro-inflammatory cytokines	Alleviating local inflammatory responses in diabetic wounds	(*45*)
		VEGF/VEGFR↑, Ang-1/Tie-2↑	Activating VEGF/VEGFR and Ang-1/Tie-2 signaling pathways	Enhancing angiogenesis in diabetic hindlimb ischemia	(*54*)
Resveratrol	Grapevine, berries, and other plants	NF-κB↓, TNF-α↓, IL-1β↓, IL-6↓, ICAM-1↓, MCP-1↓	(i) Inhibiting NF-κB nuclear translocation; (ii) Down-regulating pro-inflammatory cytokines and adhesion molecules	Attenuating vascular inflammatory injury in type 2 diabetes	(*47*)
		PI3K/AKT/Nrf2↑, Ferroptosis↓	Inhibiting high-glucose-induced ferroptosis by activating the PI3K-AKT-Nrf2 signaling pathway	Promoting angiogenesis and accelerating diabetic wound healing	(*55*)
Pseudostellaria heterophylla polysaccharides	*Pseudostellaria heterophylla* (Taizishen)	NLRP3↓, PANoptosis↓	Inhibiting NLRP3 inflammasome assembly and PANoptosis	Improving blood perfusion and microvascular density in diabetic lower limb ischemia	(*48*)
Heterophyllin B	*Pseudostellaria heterophylla* (Taizishen)	SMOX↓, Nrf2↑, TNF-α↓, IL-6↓, IL-1β↓, TGF-β↓	Inhibiting SMOX to reinforce Nrf2-dependent antioxidant defense and suppress pro-inflammatory signaling	Rescuing high-glucose-induced endothelial dysfunction and promoting angiogenesis	(*56*)

Note: Abbreviations: GCH1, GTP cyclohydrolase 1; NO, nitric oxide; BMP4, bone morphogenetic protein 4; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; METTL3, methyltransferase-like 3; m6A, N6-methyladenosine; SIRT1, sirtuin 1; GNG, hepatic gluconeogenesis; AGE, advanced glycation end-product; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; Ang-1, angiopoietin-1; Tie-2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2; SMOX, spermine oxidase.

Table 2. Pharmacological mechanisms of classical formulas and patent medicines from traditional Chinese medicine (TCM) in the treatment of diabetic lower-extremity arterial disease (LEAD)

Classification	Common name	Target/signaling pathway	Main mechanism	Protective effect	Ref.
Classical formula	Huanggi Guizhi Wuwu Decoction	HIF-1α/VEGF↑, TNF-α↓, IL-16↓, ET-1↓, NO↑	Regulating HIF-1α/VEGF signaling and suppressing vascular inflammation	Ameliorating arterial inflammation and endothelial function	(*42*)
Patent medicine	Tongxinluo capsule	ROS/NLRP3/Caspase-1/GSDMD↓, IL-1β↓, IL-18↓; Jagged-1/Notch-1/ephrin-B2↑	Inhibiting endothelial pyroptosis and enhancing endothelial-smooth muscle cell interactions	(i) Restoring hindlimb blood flow; (ii) Protecting endothelial barrier and suppressing vascular inflammation	(*43,44*)
Patent medicine	Danhong injection	VEGF-A/VEGFR-2↑, PPAR-δ↑	Activating VEGF-A/VEGFR-2 and PPAR-δ signaling axes to enhance EPC-driven endothelial repair	Improving peripheral arterial repair and angiogenesis; lowering limb necrosis risk	(*52*)
Classical formula	Danggui Sini Decoction	VEGF/PI3K/Akt↑, ET-1↓, eNOS↑	Activating VEGF/PI3K/Akt-driven angiogenesis and rebalancing the ET-1/eNOS vasomotor axis	Restoring hindlimb perfusion and functional recovery in diabetic ischemia	(*53*)

Note: Abbreviations: HIF-1α, hypoxia-inducible factor-1α; ET-1, endothelin-1; NO, nitric oxide; ROS, reactive oxygen species; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; GSDMD, Gasdermin D; EPC, endothelial progenitor cell; eNOS, endothelial nitric oxide synthase.

availability, excessive oxidative stress, over activated inflammation, and endothelial-to-mesenchymal transition (EndMT), which collectively disrupt vascular homeostasis and predispose the arterial wall to atherosclerotic lesion formation. In the context of lower extremity arterial disease, impaired endothelial function exacerbates local ischemia, thereby contributing to progression of limb-threatening conditions and increasing risk of amputation, disability, and mortality in diabetic patients (*13*). Therefore, protecting the vascular endothelium and preserving its functional integrity may represent a fundamental therapeutic strategy for prevention and treatment of diabetic LEAD.

Salvia miltiorrhiza (Danshen) is a highly valued herb used in TCM. According to ancient Chinese medical documents, *Salvia miltiorrhiza* was believed to be highly potent in dispersing stasis and activating circulation. It is commonly used to treat various circulatory disturbance-related diseases, owing to its pharmacological actions, including vasodilation, anti-coagulation, anti-inflammatory effects, and free radical scavenging activity (*14*).

Tanshinone IIA, a major lipophilic bioactive constituent of *Salvia miltiorrhiza*, exerts a vascular-protective effect. However, its low aqueous-solubility restricts oral bioavailability and hinders clinical application. To overcome this, sodium Tanshinone IIA sulfonate (STS) was developed with greatly improved solubility. STS retains multiple pharmacological activities (anti-oxidant, anti-inflammatory, anti-apoptotic) and has been approved by the China State Food and Drug Administration (CFDA) for treating cardiovascular diseases (*15*). STS protects vascular endothelium under hyperglycemic ischemia. In diabetic mice with hindlimb ischemia, STS improved perfusion recovery, increased capillary density, and decreased reactive oxygen species (ROS) levels and miR-133a expression (*16*). It also reduced malondialdehyde (MDA) content while elevating GTP cyclohydrolase 1 (GCH1) and cyclic guanine monophosphate (cGMP) levels in ischemic muscle, indicating an enhanced anti-oxidative and vascular-protective status. To validate direct endothelial effects, primary endothelial cells were cultured under high-glucose and hypoxic conditions to mimic the diabetic ischemic micro-environment. STS markedly promoted tube formation and increased nitric oxide (NO) production, while also lowering intracellular ROS and miR-133a. Collectively, these findings demonstrate that STS exerts pro-angiogenic effects in diabetic ischemia by suppressing miR-133a and up-regulating GCH1, thereby enhancing NO bio-availability and reducing oxidative injury.

Salvianolic acid B (SalB) is another pivotal bioactive constituent of *Salvia miltiorrhiza* and possesses a vascular-protective effect. It has an extensive range of pharmacological properties, including antioxidant, anti-inflammatory, anti-thrombotic, anti-apoptotic, anti-aging,

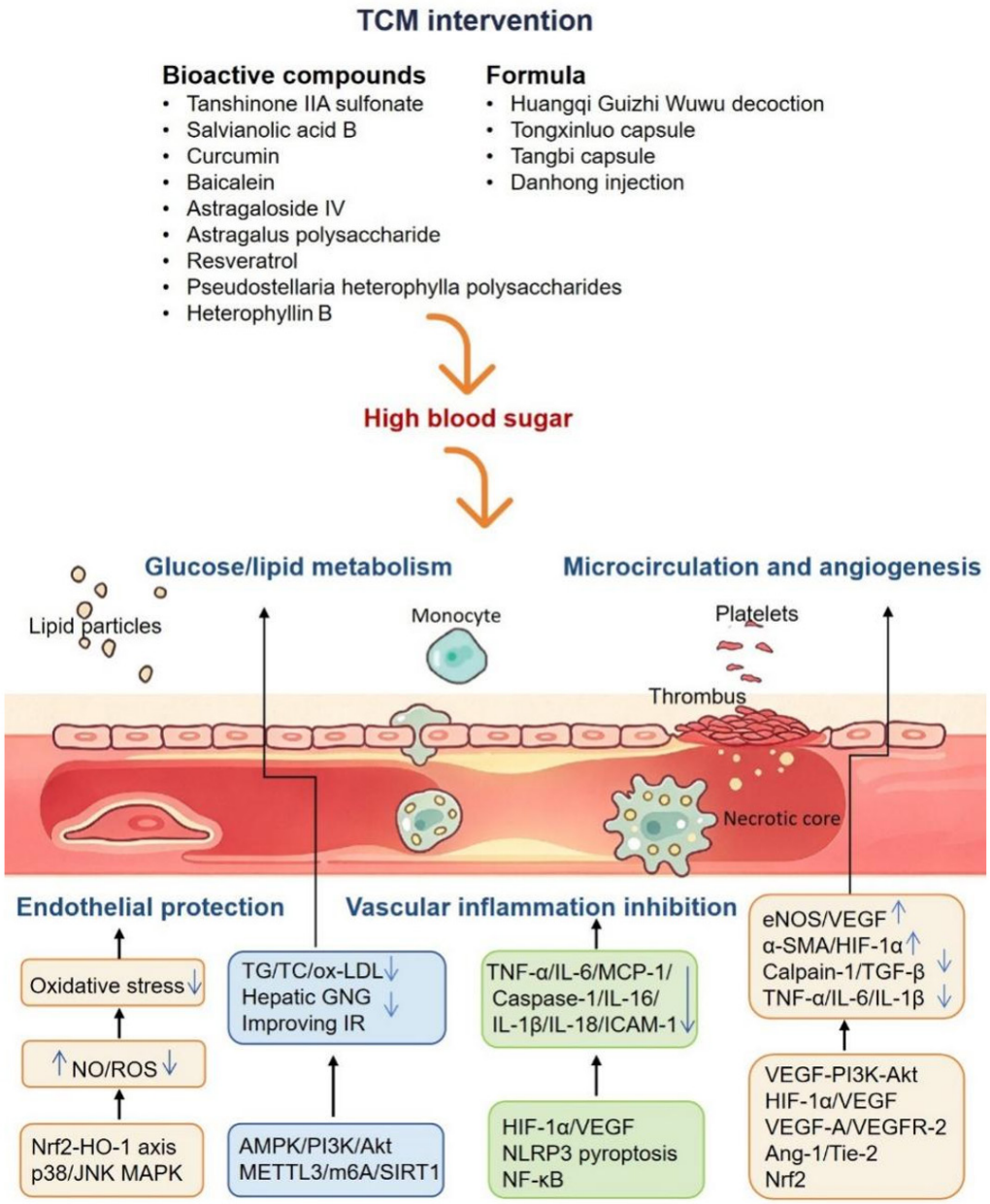


Figure 2. Molecular mechanisms of traditional Chinese medicine (TCM) for diabetic lower-extremity arterial disease (LEAD). Bioactive constituents, classical formulas, and patent medicines of TCM ameliorate hyperglycemia-induced vascular lesions *via* endothelial protection, glycolipid metabolism regulation, anti-inflammation, and promotion of microcirculation and angiogenesis, by modulating multiple intracellular signaling cascades. (Note: Abbreviations: IR, insulin resistance; TG, triglyceride; TC, total cholesterol; ox-LDL, oxidized low-density lipoprotein; GNG, gluconeogenesis; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; METTL3, methyltransferase-like 3; m6A, N6-methyladenosine; SIRT1, sirtuin 1; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; HIF-1α, hypoxia-inducible factor-1α; VEGFR-2, vascular endothelial growth factor receptor 2; eNOS, endothelial nitric oxide synthase; α-SMA, α-smooth muscle actin; Ang-1, angiopoietin-1; Tie-2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2.)

and vascular and mitochondrial protection (*17*). Sal B exhibited a potential to ameliorate diabetes-induced endothelial and mitochondrial dysfunction through inhibition of endothelial-cell apoptosis and excessive mitophagy (*18*). It rescues mitochondrial membrane potential, curtails ROS accumulation and improves NO bio-availability, hence maintaining vascular endothelial homeostasis under hyperglycaemia conditions, as confirmed in high-glucose-stimulated endothelial cells and diabetic mice. Moreover, Sal B ameliorates vascular endothelial dysfunction in diabetic mice by disrupting the bone morphogenetic protein 4 (BMP4)-ROS vicious cycle (*19*). It inhibits BMP4 over-expression, suppresses ROS production, restores NO bio-availability, and improves endothelium dependent aortic relaxation, thereby protecting against hyperglycemia-induced endothelial injury in diabetic mice. In addition, Building on Sal B's endothelial benefits, a metal-polyphenol

nanocomposite hydrogel loaded with Sal B was engineered for diabetic foot ulcers (*20*). It reprogrammed the metabolic microenvironment *via* ROS scavenging, angiogenesis promotion, M2 macrophage polarization, and glucose depletion, collectively accelerating wound healing in diabetic models.

Curcuma longa (Jianghuang) and *Scutellaria baicalensis* (Huangqin) are commonly used herbal medicines in TCM. Both herbs have been investigated for treatment of diabetes and its vascular complications in modern TCM pharmacological research (*21,22*). Curcumin and Baicalein, their respective main bioactive components, possess vascular protective properties. One study has demonstrated synergistic vascular protection conferred by combined curcumin-baicalein treatment (*23*). In H_2O_2-damaged EA.hy926 endothelial cells and Goto-Kakizaki diabetic rats, this combination enhanced endothelial viability, decreased fasting blood glucose and serum lipid levels, preserved aortic structural integrity, and diminished vascular TNF-α. Mechanistically, the Curcumin-Baicalein mixture activated the Nrf2-HO-1 antioxidant axis and inhibited p38/JNK MAPK phosphorylation, thereby mitigating oxidative-mediated endothelial damage. Collectively, this herbal-derived combination represents a promising multi-target strategy for managing diabetic angiopathy.

In summary, vascular endothelial dysfunction is the initiating event in pathogenesis of diabetic LEAD, and TCM-derived bioactive constituents exert robust endothelial protection. These include STS and SalB from *Salvia miltiorrhiza*, as well as the Curcumin–Baicalein combination from *Curcuma longa* and *Scutellaria baicalensis*. They preserve vascular integrity by attenuating oxidative stress and apoptosis, enhancing NO bioavailability and angiogenic capacity, and remodeling the metabolic microenvironment. These multi-target endothelial-protective properties position TCM-derived bioactive constituents as promising therapeutic candidates for management of diabetic vascular complications, warranting further investigation through rigorously designed clinical trials and mechanistic studies.

2.2. Regulation of glucose and lipid metabolism

Glucose and lipid metabolic disorders are well-established core risk factors driving occurrence and progression of diabetic LEAD (*24*). In the diabetic milieu, chronic hyperglycemia, insulin resistance (IR), and aberrant lipid profiles, characterized by elevated free fatty acids, oxidized low-density lipoprotein (ox-LDL), and triglyceride (TG)-rich remnants, collectively instigate a cascade of metabolic insults (*25*). These include enhanced polyol and hexosamine pathway flux, increased advanced glycation end-product (AGE) formation, and impaired lipoprotein lipase activity, which together promote intracellular lipid accumulation, mitochondrial dysfunction, and endoplasmic reticulum stress in vascular cells (*26*). Such metabolic disturbances not only exacerbate systemic inflammatory and oxidative stress but also directly fuel atherosclerotic plaque formation, plaque vulnerability, and micro-vascular rarefaction in the lower extremities. In the context of LEAD, uncontrolled glucose and lipid metabolism aggravates peripheral arterial stiffness, reduces collateral circulation, and accelerates ischemic tissue damage, thereby heightening risk of critical limb ischemia and adverse cardiovascular events (*27*). Therefore, restoring metabolic homeostasis through pharmacological regulation of glycemia and lipidemia may constitute an indispensable adjunctive strategy to halt or retard the natural history of diabetic LEAD, complementing revascularization and anti-thrombotic approaches.

Astragali Radix (Huangqi) is a classical traditional Chinese medicinal herb with inherent tonic properties, which has been widely used for treatment of diabetes, cardiovascular disorders, and refractory wounds in China and Southeast Asia over thousands of years. Accumulating pharmacological evidence demonstrates that *Astragali Radix* exerts multiple therapeutic bioactivities, including anti-inflammatory, antioxidant, and immunomodulatory effects (*28*). To date, more than 100 chemical constituents, primarily saponins, flavonoids, and polysaccharides, have been isolated and identified from this herb.

Among these bioactive constituents, Astragaloside IV (AS-IV) is regarded as a principal active ingredient responsible for its anti-diabetic efficacy (*29*). Consistent with its metabolic regulatory function, accumulating preclinical studies have validated that AS-IV effectively ameliorates diabetic glycolipid disorders by activating the AMPK/PI3K/Akt signaling cascade, suppressing hepatic gluconeogenesis (GNG), improving IR, and reducing circulating levels of TG, total cholesterol (TC), and ox-LDL (*30,31*). By inhibiting excessive lipid deposition and alleviating metabolic stress in vascular endothelial cells, AS-IV attenuates hyperglycemia and dyslipidemia induced peripheral arterial stiffness and atherosclerotic lesions, thereby retarding diabetic LEAD progression (*32*). Beyond classical metabolic pathways, AS-IV also exerts protective effects against diabetic vascular complications *via* epigenetic modulation. Mechanistically, AS-IV up-regulates methyltransferase-like 3 (METTL3) mediated N6-methyladenosine (m6A) modification to enhance Sirtuin 1 (SIRT1) mRNA stability and expression (*33*). The elevated SIRT1 further modulates the FOXO1/AMPK axis to mitigate AGE-triggered cellular injury, inhibit local inflammation, and facilitate vascular and tissue repair in diabetic lower extremity lesions. Comprehensively, AS-IV exerts a multi-level protective effect on diabetic vascular lesions not only by correcting glucose-lipid homeostasis but also by triggering epigenetic modification-dependent vascular protective effects.

Astragalus polysaccharide (APS), a major bioactive polysaccharide isolated from *Astragali Radix*, has been shown to exert anti-inflammatory, antioxidant, and hypoglycemic effects (*34*). Notably, growing evidence indicates that APS ameliorates type 2 diabetes mellitus (T2DM) by promoting glucose transport and modulating lipogenesis. In a high-fat-diet-induced T2DM rat model, APS treatment reverses aberrant hepatic expression of sweet-taste receptors and downstream glycolipid-metabolism genes, including glucose transporters and lipogenic enzymes, thereby restoring hepatic insulin sensitivity and correcting systemic hyperglycaemia and dyslipidaemia (*35*). These metabolic-restoring effects may reduce lipotoxic injury to peripheral arterial walls and retard progression of diabetic LEAD. At the cellular level, adipocyte-based experiments demonstrate APS counteracts TNF-α provoked IR by inhibiting miR-721 expression, which subsequently activates PPAR-γ and the PI3K/AKT signal pathway to facilitate glucose uptake in adipose tissue (*36*). Improved insulin sensitivity within adipose tissue may further mitigate chronic metabolic-related inflammation and foster a favourable systemic micro-environment, which lays a foundation for vascular-protective actions. Beyond its systemic metabolic regulation, biomaterial-based local delivery of APS provides an alternative strategy targeting diabetic peripheral ischemic complications. An APS-loaded electroconductive hydrogel composed of carboxymethyl chitosan and sodium alginate can sustain local release of APS to scavenge reactive oxygen species, suppress inflammatory responses and facilitate angiogenesis in diabetic-injured tissue. Notably, this conductive dressing enables real-time electromyographic monitoring of surrounding muscle function, offering a theranostic approach for the management of ischemic-related lower-extremity lesions secondary to diabetic LEAD (*37*).

In summary, glucose and lipid metabolic disorders are well-established drivers of diabetic LEAD, promoting IR, vascular oxidative stress, and atherosclerotic progression. *Astragali Radix* and its bioactive constituents, AS-IV and APS, exert potent glycolipid-regulating and vascular-protective effects through multiple mechanisms, including activation of the AMPK/PI3K/Akt pathway, suppression of hepatic GNG, modulation of epigenetic modifications such as METTL3/m6A/SIRT1 signaling, and regulation of glucose transport and lipogenesis. These multi-target metabolic and vascular protective properties position *Astragali Radix*-derived components as promising therapeutic candidates for ameliorating metabolic dysfunction and delaying diabetic peripheral vascular complications, warranting further investigation through rigorously designed clinical trials and mechanistic studies.

2.3. Inhibition of vascular inflammatory responses

Chronic low-grade inflammation is a central driver in the pathogenesis and progression of diabetic LEAD (*38*). Under sustained hyperglycemic conditions, the arterial wall is continuously exposed to metabolic stressors that activate multiple pro-inflammatory signaling cascades, most notably the nuclear factor-κB (NF-κB) pathway and the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome. Aberrant activation of NF-κB promotes overexpression of inflammatory cytokines, adhesion molecules, and chemokines, thereby inducing endothelial dysfunction, lipid deposition in the vascular wall, and atherosclerotic plaque formation (*39*). Concurrently, the NLRP3 inflammasome, which is significantly upregulated in vascular cells of diabetic patients with lower extremity arterial disease, particularly in vascular smooth muscle cells, mediates maturation and release of IL-1β and IL-18, further amplifying the inflammatory milieu (*40*). Elevated circulating levels of TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1) have been consistently documented in diabetic patients with LEAD, and these inflammatory mediators correlate with disease severity and adverse limb outcomes (*41*). Thus, pharmacological suppression of vascular inflammatory responses represents a promising therapeutic avenue for mitigating progression of diabetic LEAD. Recently, emerging evidence indicates that TCM bioactive constituents, classical formulas, and patent medicines exert substantial anti-inflammatory effects in the context of diabetic LEAD through multi-target regulation of these inflammatory pathways.

Huangqi Guizhi Wuwu Decoction is a classic traditional Chinese herbal formula documented in the "Synopsis of the Golden Chamber" (*28*). It consists of five herbal components including *Radix Astragali* (Huangqi), *Ramulus Cinnamomi* (Guizhi), *Radix Paeoniae Alba* (Baishao), *Rhizoma Zingiberis Recens* (Shengjiang), and *Fructus Jujubae* (Dazao). It is widely prescribed in China for ischemic stroke and diabetic complications. A recent study by Wang *et al.* demonstrated that Huangqi Guizhi Wuwu Decoction mitigates arterial inflammation in rats with diabetic LEAD (*42*). This decoction markedly decreased serum TNF-α, IL-16 and endothelin-1 (ET-1), and increased NO levels. Mechanistically, such protective actions were linked to regulation of the HIF-1α/VEGF signaling pathway. Collectively, these results indicate that Huangqi Guizhi Wuwu Decoction can suppress vascular inflammation and ameliorate endothelial function, which renders it a promising therapeutic candidate for diabetic LEAD.

Tongxinluo capsule is a Chinese herbal formula composed of 12 herbs containing *Ginseng Radix* (Renshen), *Hirudo* (Shuizhi), *Scorpio* (Quanxie), *Scolopendra* (Wugong) and so on (*43*). It is widely used in China for cardio-cerebrovascular diseases and has been extended to diabetes-related vascular disease. A

recent study by Gu *et al.* investigated therapeutic effects of Tongxinluo capsule on diabetic PAD (predominantly LEAD) and its underlying mechanisms (*44*). Using a *db/db* mouse model of diabetic PAD induced by femoral artery ligation, the study demonstrated that Tongxinluo capsule restored hindlimb blood flow and enhanced arteriogenesis. Specifically, Tongxinluo capsule restored endothelial cell functions, including maintaining vasodilation balance and acting as a barrier to reduce inflammation. Mechanistically, Tongxinluo capsule inhibited endothelial cell pyroptosis *via* the ROS/NLRP3/Caspase-1/GSDMD signaling pathway, thereby suppressing persistent inflammation. The study further showed that Tongxinluo capsule reduced ROS, Gasdermin D (GSDMD), cleaved caspase-1, IL-1β, and IL-18 levels in a dose-dependent manner. Additionally, Tongxinluo capsule enhanced endothelial-smooth muscle cell interactions through the Jagged-1/Notch-1/ephrin-B2 signaling pathway. These findings indicate that Tongxinluo capsule exerts anti-inflammatory effects in diabetic LEAD by protecting the endothelial barrier and inhibiting pyroptosis-mediated vascular inflammation, supporting its potential as a therapeutic candidate for diabetic LEAD.

At the level of individual herbal components, several bioactive constituents derived from TCM have demonstrated potent anti-inflammatory activities relevant to diabetic LEAD. APS, a major active constituent of *Astragali Radix*, has been shown to inhibit M1 macrophage polarization and reduce expression of TNF-α, IL-1β, and IL-6 while promoting M2 macrophage polarization and elevating IL-10 levels in diabetic wound tissues, thereby effectively alleviating local inflammatory responses (*45*). Resveratrol, a non-toxic phytoestrogen found in over 70 plants including grapevine and berries, exhibits antioxidant, anti-inflammatory, and antitumor properties (46). Recent studies indicate that it protects against cardiovascular diseases and atherosclerosis and improves glucose homeostasis. Notably, Resveratrol attenuates vascular inflammatory injury in streptozotocin-induced type 2 diabetic rats by inhibiting NF-κB nuclear translocation, reducing circulating IL-1β and IL-6 levels, and down-regulating TNF-α, intercellular adhesion molecule-1 (ICAM-1), and MCP-1 expression in the vascular wall (*47*). Additionally, Pseudostellaria heterophylla polysaccharides, water-soluble polysaccharides extracted from the dried root tubers of *Pseudostellaria heterophylla* (Taizishen), have been shown to inhibit PANoptosis, a process integrating apoptosis, pyroptosis, and necroptosis, and involving NLRP3 inflammasome assembly in high glucose-exposed endothelial cells, thereby improving blood perfusion and microvascular density in diabetic lower limb ischemia (*48*).

In summary, vascular inflammation driven by the NF-κB and NLRP3 inflammasome pathways plays a pivotal role in the initiation and progression of diabetic LEAD, promoting endothelial dysfunction, vascular remodeling, and atherosclerotic lesion formation. TCM bioactive constituents, classical formulas, and patent medicines have demonstrated considerable efficacy in inhibiting these inflammatory cascades through mechanisms involving suppression of pro-inflammatory cytokine production, modulation of macrophage polarization, and inhibition of inflammasome assembly. These multi-target anti-inflammatory properties position TCM as a promising adjunctive therapeutic strategy for prevention and treatment of diabetic LEAD, warranting further investigation through rigorously designed clinical trials and mechanistic studies.

2.4. Improvement of microcirculation and promotion of angiogenesis

Microcirculatory dysfunction and impaired reparative angiogenesis represent hallmark pathological features of diabetic LEAD, and are key modulators of tissue perfusion, ischaemic injury, and long-term limb prognosis (*49*). Under sustained hyperglycaemic stress, angiogenic responses become dysregulated at multiple molecular layers: reduced HIF-1α protein stability, blunted VEGF-A production, and diminished endothelial responsiveness to pro-angiogenic stimuli. In the ischaemic lower limbs, insufficient compensatory neovascularisation fails to establish functional collateral circulation, exacerbating tissue hypoxia and driving progression toward critical limb-threatening ischaemia. Accordingly, restoring microvascular homeostasis and harnessing therapeutic angiogenesis are regarded as promising therapeutic strategies for diabetic LEAD management Frontiers. Mounting evidence indicates that TCM formulas ameliorate lower-limb microcirculatory dysfunction and enhance reparative angiogenesis in diabetic LEAD.

Tangbi capsule, a compound TCM prescription based on the classic formula Huangqi Guizhi Wuwu Decoction, consists of granules made from five herbs: *Astragali Radix* (Huangqi), *Ramulus Cinnamomi* (Guizhi), *Ligusticum Chuanxiong* (Chuanxiong), *Radix Paeoniae Alba* (Baishao), and *Caulis Spatholobi* (Jixueteng). Previous clinical studies have shown that Tangbi capsule improves clinical symptoms such as limb coldness, numbness, pain, burning, and skin dryness in diabetic LEAD patients (*50*). Mechanistically, Tangbi capsule up-regulates HIF-1α, eNOS, and VEGFA while suppressing Calpain-1 and TGF-β expression, thereby reducing endothelial apoptosis and facilitating angiogenesis in diabetic LEAD rats (*51*). Furthermore, integrated metabolomics and transcriptomics analyses indicate that Tangbi capsule modulates arginine biosynthesis, phenylalanine metabolism, and arachidonic acid metabolism, which are biological pathways closely linked to angiogenesis, immune-inflammatory cascades, and cell proliferation.

Danhong injection is a patent injection medicine derived from extracts of *Salvia miltiorrhiza* (Danshen) and *Flos Carthami* (Honghua), which are two Chinese herbal medicines with a property of activating blood circulation and removing blood stasis. It is widely prescribed for treatment of cardiovascular and cerebrovascular disease in clinical practice. Recent studies indicate that Danhong injection improves peripheral arterial repair in type 2 diabetic mice by elevating capillary density and augmenting angiogenesis through coordinated activation of the VEGF-A/VEGFR-2 and PPAR-δ signaling axes, ultimately lowering risk of limb necrosis (*52*). Notably, a defined four-component active fraction purified from DHI has been shown to be both necessary and sufficient to enhance endothelial progenitor cell-driven endothelial repair and local neovascularization.

Danggui Sini Decoction is a classic traditional Chinese medicine formula first documented in "ShangHan Lun". It consists of seven herbs, *Angelicae Sinensis Radix* (Danggui), *Ramulus Cinnamomi* (Guizhi), *Radix Paeoniae Alba* (Baishao), *Asari Radix et Rhizoma* (Xixin), *Glycyrrhizae Radix et Rhizoma* (Gancao), *Tetrapanacis Medulla* (Tongcao), and *Fructus Jujubae* (Dazao). Danggui Sini decoction is traditionally used to warm the meridians, dispel cold, and promote blood circulation, and is widely prescribed in China for diabetic peripheral vascular complications. Recent studies indicate that Danggui Sini Decoction restores hindlimb perfusion and functional recovery in diabetic ischemic mice largely independent of glucose-lowering effects (*53*). Its protective actions are partially attributed to VEGF-PI3K-Akt-driven angiogenesis and rebalancing of the ET-1/eNOS vasomotor axis.

At the level of individual herbal components, several bioactive constituents derived from TCM have demonstrated potent microcirculation-improving and pro-angiogenic activities relevant to diabetic LEAD. Tu *et al.* reported that APS, a major active constituent of *Astragali Radix*, when combined with endothelial progenitor cells, enhanced angiogenesis in diabetic rats with hindlimb ischemia, and the combined treatment produced superior effects at least in part through VEGF/VEGFR and Ang-1/Tie-2 signaling pathways (*54*). Similarly, Pan *et al.* demonstrated that Resveratrol protects vascular endothelial cells from high-glucose-induced ferroptosis by activating the PI3K-AKT-Nrf2 signaling pathway, thereby promoting angiogenesis and accelerating diabetic wound healing (*55*). Heterophyllin B, a cyclopeptide derived from Pseudostellaria heterophylla, markedly improves hindlimb blood flow recovery and promotes angiogenesis in ischaemic muscle, accompanied by elevated CD31, α-SMA, VEGF and endothelial nitric oxide synthase (eNOS) abundance (*56*). Meanwhile, Heterophyllin B dampens local inflammation by lowering TNF-α, IL-6, IL-1β and TGF-β levels. Mechanistically, Heterophyllin B inhibits spermine oxidase (SMOX) to reinforce Nrf2-dependent antioxidant defense and restrain pro-inflammatory signalling, thereby rescuing high-glucose-induced endothelial dysfunction.

In summary, dysregulated angiogenic and vasomotor signaling impairs microcirculation and angiogenesis, which is central to onset and progression of diabetic LEAD, leading to tissue hypoxia, collateral circulation insufficiency, and limb-threatening ischemia. Some TCM bioactive constituents, classical formulas, and patent medicines exert notable effects on improving lower-limb microcirculation and stimulating angiogenesis *via* multi-target modulation of angiogenic and vasomotor signaling cascades, including the HIF-1α/VEGF, VEGF-A/VEGFR-2, PI3K-AKT, Ang-1/Tie-2 axes, as well as Nrf2-mediated antioxidant defense. Such multi-target pro-angiogenic and vasoregulatory features render TCM a promising adjunctive therapy to restore microvascular homeostasis and mitigate limb ischemia in diabetic LEAD. Further well-designed clinical trials and mechanistic studies are therefore warranted.

3. Clinical applications of TCM in diabetic LEAD

In recent years, accumulating clinical studies and evidence-based analyses have continuously validated the unique advantages of traditional Chinese medicine in comprehensive management of diabetic LEAD. Current clinical applications of TCM for diabetic LEAD cover multiple therapeutic dimensions, including perioperative adjuvant medication for endovascular intervention, topical wound rehabilitation for refractory diabetic foot ulcers, and non-pharmacological rehabilitation combined with lifestyle modification (Figure 3). A growing number of systematic reviews and meta-analyses have synthesized empirical clinical data and confirmed that multi-modal TCM interventions can effectively improve lower-limb hemodynamic perfusion, suppress postoperative restenosis, alleviate intractable wound inflammation, delay atherosclerotic progression, and ameliorate long-term clinical prognosis, providing high-level evidence supporting standardized and integrated application of TCM in diabetic LEAD treatment (Table 3).

3.1. TCM-assisted endovascular intervention for diabetic LEAD

Endovascular interventions, including balloon angioplasty and stent implantation, serve as the first-line strategy to rapidly restore lower-limb perfusion in diabetic LEAD patients. However, postoperative restenosis remains the dominant obstacle limiting long-term therapeutic efficacy and contributes to disease recurrence (*57*). Accumulating clinical evidence has demonstrated that postoperative adjuvant treatment with *Qi*-tonifying and blood-activating TCM formulas effectively reduces rates of vascular restenosis and long-term recurrence, while significantly improving

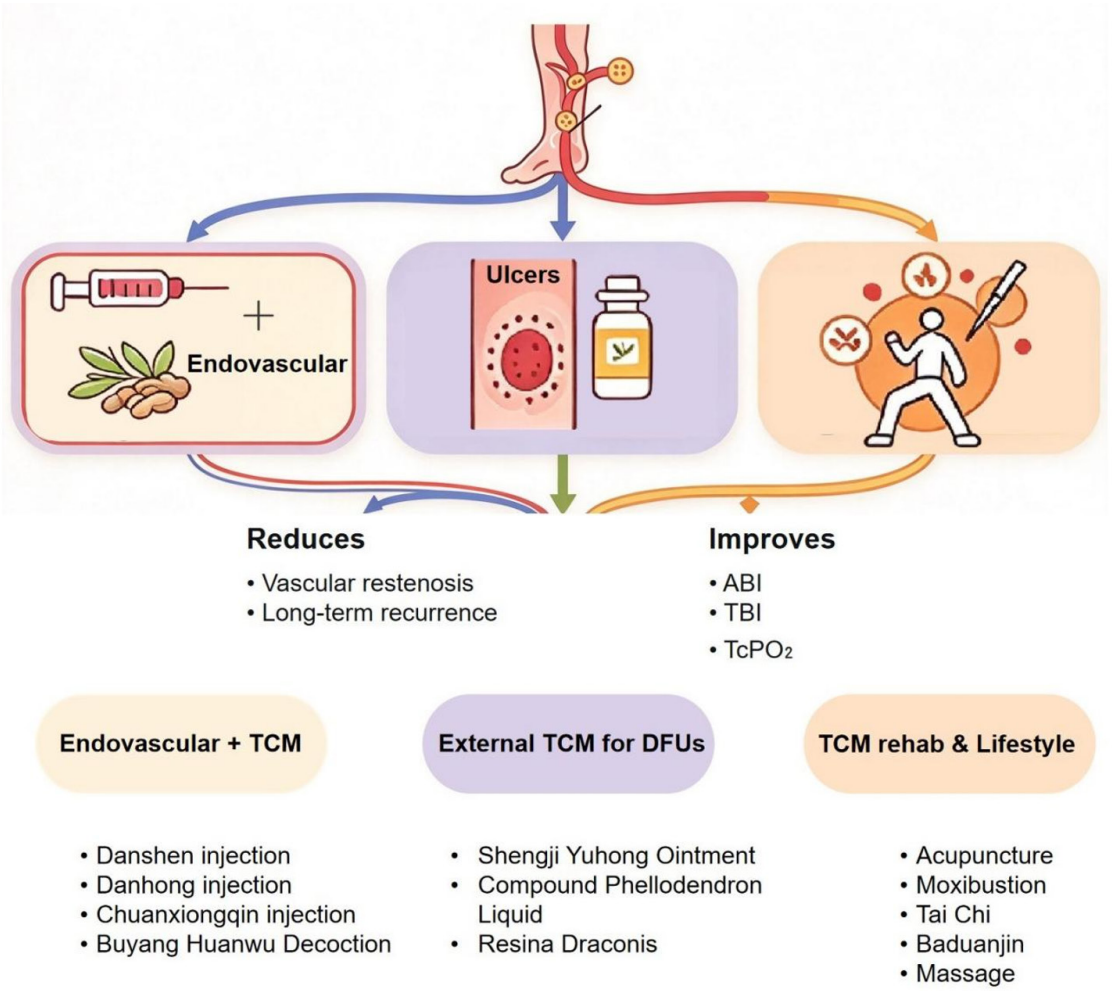


Figure 3. Clinical applications of traditional Chinese medicine (TCM) in diabetic lower-extremity arterial disease (LEAD). Three major TCM-related therapeutic strategies for diabetic LEAD are summarized: perioperative TCM combined with endovascular intervention, topical external TCM therapy for diabetic foot ulcers (DFUs), and nonpharmacological TCM rehabilitation and lifestyle interventions. These multimodal TCM interventions can effectively improve lower-limb hemodynamic perfusion, suppress postoperative restenosis, alleviate intractable wound inflammation, delay atherosclerotic progression, and improve long-term clinical prognosis.

core perfusion indicators including ABI, toe-brachial index (TBI) and transcutaneous oxygen partial pressure ($TcPO_2$).

Buyang Huanwu Decoction, a classic traditional Chinese medicine formula, is composed of seven herbs: *Astragali Radix* (Huangqi), *Angelicae Sinensis Radix* (Danggui), *Paeoniae Radix Rubra* (Chishao), *Chuanxiong Rhizoma* (Chuanxiong), *Persicae Semen* (Taoren), *Carthami Flos* (Honghua), and *Pheretima* (Dilong). The formula is traditionally used for treating blood stasis secondary to *Qi*-deficiency. Clinical studies have reported that adjunctive Buyang Huanwu Decoction treatment after balloon angioplasty significantly improved ABI, TBI, and blood flow velocity of the dorsal pedis and posterior tibial arteries in type 2 diabetic LEAD patients. This combination therapy also reduced the 6-month and 12-month restenosis rates and improved VAS scores and claudication distance (*58*). In addition, a Bayesian network meta-analysis compared efficacy of blood-activating Chinese herbal injections for diabetic LEAD. The results demonstrated that adjuvant use of herbal injections including Danshen injection, Danhong injection, and Chuanxiongqin injection combined with conventional Western therapy significantly improved the ankle-brachial index (ABI), dorsalis pedis artery blood flow velocity, and hemorheological parameters. These findings support that blood-activating Chinese herbal injections represent a valuable adjunctive therapeutic option for patients with diabetic LEAD (*10*).

In summary, TCM adjuvant therapy, represented by *Qi*-tonifying and blood-activating formulas and herbal injections, optimizes lower-limb hemodynamics, improves peripheral perfusion, and reduces postoperative vascular restenosis, thereby enhancing long-term efficacy of endovascular revascularization for diabetic LEAD.

3.2. Application of external TCM therapy in wound management of LEAD-related diabetic foot ulcers

Diabetic foot ulceration and gangrene secondary to

Table 3. Clinical Application of traditional Chinese medicine (TCM) in the treatment of diabetic lower-extremity arterial disease (LEAD)

Therapeutic approach	Specific interventions	Treatment phase	Therapeutic efficacy	Outcome measures	*Ref.*
TCM-assisted endovascular intervention	Adjunctive Buyang Huanwu Decoction after balloon angioplasty	Post-interventional phase	Improving hemodynamics and reducing restenosis	ABI, TBI, blood flow velocity, restenosis rate, VAS score, claudication distance	(*58*)
	Blood-activating herbal injections (Danshen, Danhong, Chuanxiongqin) + conventional Western therapy	Post-interventional phase	Improving hemodynamics and hemorheology	ABI, dorsalis pedis artery blood flow velocity, hemorheological parameters	(*10*)
External TCM therapy for diabetic foot ulcers	Shengji Yuhong Ointment; Compound Phellodendron Liquid	Wound management phase	Eliminating necrotic tissue, controlling infection, and promoting wound healing	Ulcer healing, complete healing rate, healing duration, ulcer size	(*60-63*)
	Resina Draconis	Wound management phase	Improving healing efficiency, diminishing wound area, and downgrading Wagner grades	Healing efficiency, wound area, Wagner grade	(*64*)
TCM-based rehabilitation	Acupuncture	Rehabilitation phase	Improving microvascular perfusion	Microcirculatory parameters, lower-extremity blood flow	(*65*)
	Moxibustion	Rehabilitation phase	Elevating transcutaneous oxygen tension and relieving ischemic manifestations	Transcutaneous oxygen tension, exercise tolerance	(*66*)
	Tai Chi/Baduanjin	Rehabilitation phase	Stabilizing glycemic/lipid profiles and slowing atherosclerotic progression	Glycemic and lipid profiles, vascular function	(*67*)
	Local foot acupoint massage	Rehabilitation phase	Enhancing lower-limb blood perfusion and alleviating numbness/coldness	Lower-limb blood perfusion, clinical symptoms	(*68*)

Note: Abbreviations: ABI, ankle-brachial index; TBI, toe-brachial index; VAS, visual analogue scale.

severe lower-limb ischemia represent the most disabling complications of diabetic LEAD. Conventional debridement and antibiotic treatment often yield unsatisfactory outcomes for refractory ulcers complicated with drug-resistant bacterial infection, especially *Pseudomonas aeruginosa* (*59*). External TCM therapies directly act on local wounds, exerting multiple effects including heat-clearing and detoxifying, necrotic tissue removal, blood circulation activation and granulation promotion. Classic external preparations, such as Shengji Yuhong Ointment and Compound Phellodendron Liquid, are widely applied to eliminate necrotic tissue, control local infection and accelerate wound repair (*60,61*).

A systematic review covering 49 randomized controlled trials (RCTs) demonstrated that adjuvant TCM treatment significantly elevated the complete healing rate of diabetic foot ulcers (RR 1.42-1.76), shortened healing duration by 4-23 days, and reduced ulcer size (*62*). Consistently, another recent meta-analysis of 34 RCTs further validated clinical benefits of topical TCM preparations, confirming that commonly used external TCM patent medicines including Compound Phellodendron Liquid and Shengji Yuhong Ointment substantially improved overall therapeutic efficacy of conventional wound care for diabetic foot ulcers (*63*). Additionally, *Resina Draconis*, a well-known blood-activating TCM resin derived from *Dracaena cochinchinensis*, exerts wound-clearing, detumescent and tissue-regenerating pharmacological properties for cutaneous ulcer management. A systematic review and meta-analysis of RCTs focusing on this agent demonstrated that topical application of *Resina Draconis* extract significantly improved ulcer healing efficiency, diminished wound area, and downgraded Wagner ulcer grades among patients with diabetic foot ulcers (*64*).

In summary, external TCM treatments inhibit local drug-resistant bacterial growth, alleviate intractable wound inflammation and facilitate tissue regeneration, constituting an indispensable component of comprehensive management for LEAD-related diabetic foot ulcers.

3.3. TCM-based rehabilitation and lifestyle modification for diabetic LEAD

Beyond pharmacotherapy and endovascular adjuvant therapy, TCM-based rehabilitation and lifestyle modification constitute critical non-pharmacological components for comprehensive management of diabetic LEAD. These non-pharmacological approaches ameliorate lower-limb microcirculation, stabilize glycolipid homeostasis, and retard atherosclerotic progression.

Acupuncture regulates *Qi*-blood circulation *via* targeted acupoint stimulation and improves peripheral microvascular perfusion. Clinical evidence demonstrates that acupoint-based acupuncture optimizes microcirculatory parameters and augments lower-extremity blood flow in patients with diabetic peripheral arterial disease, which may further facilitate chronic wound repair (*65*). Moxibustion applied at Zusanli (ST36), Sanyinjiao (SP6), and Yongquan (KI1) elevates transcutaneous oxygen tension, improves exercise tolerance, and relieves ischemic manifestations among PAD patients (*66*). Furthermore, traditional Chinese mind-body exercises including Tai Chi and Baduanjin, when combined with treadmill training and dietary regulation, help stabilize glycemic and lipid profiles, preserve vascular function, and slow atherosclerotic progression (*67*). Local foot acupoint massage enhances lower-limb blood perfusion through neuro-reflex modulation and alleviates clinical symptoms such as limb numbness and coldness; notably, massage should be cautiously performed in patients with severe arterial stenosis or skin ulceration to avoid tissue injury (*68*).

Collectively, these multi-modal rehabilitation interventions exert synergistic effects to attenuate vascular lesion progression, reduce ulcer recurrence risk, and improve long-term quality of life.

4. Limitations and future perspectives

Although accumulating preclinical findings have verified the protective efficacy of TCM and its bioactive components against diabetic LEAD, several notable limitations still restrict its clinical translation and standardized application. Current relevant evidence is predominantly derived from rodent hindlimb ischemia models, while high-quality, large-sample clinical randomized controlled trials in diabetic LEAD patients remain insufficient, lacking robust clinical validation for TCM therapeutic effects. Mechanistically, most existing studies merely focus on single bioactive constituents or individual signaling pathways (*e.g.*, Nrf2-mediated antioxidant pathway and HIF-1α/VEGF angiogenesis axis), ignoring the holistic multi-component and multi-target characteristics of TCM formulas, and synergistic interactions among herbal ingredients remain poorly elucidated. In addition, significant heterogeneity in TCM syndrome differentiation, formula composition, dosage and treatment duration across studies impairs reproducibility and comparability of research data. Furthermore, systematic evaluations on long-term safety, adverse reactions and herb-drug interactions between TCM and conventional anti-platelet or lipid-lowering therapies are still lacking.

To address these deficiencies, future research should focus on standardized, rigorously designed clinical trials with unified syndrome stratification and intervention protocols to consolidate clinical efficacy of TCM for diabetic LEAD. Advanced multi-omics technologies are recommended to explore synergistic mechanisms of integral herbal formulas, instead of limited investigation of single monomers. Key signaling axes including SMOX-Nrf2, HIF-1α/VEGF and PI3K-AKT deserve in-depth exploration, as they are critical targets for improving

endothelial dysfunction and restoring lower-limb microcirculation. Moreover, targeted studies are required to clarify the safety profile and herb-drug interactions of TCM adjuvant therapy, to guide its safe and standardized combination with conventional revascularization and symptomatic treatments. Meanwhile, emerging organoid models and large animal ischemic limb models can be applied to bridge the gap between basic research and clinical practice. Integrated basic and clinical studies will further promote innovative development and clinical popularization of TCM-based combinatory strategies for diabetic LEAD.

5. Conclusion

Diabetic LEAD is a severe macrovascular complication of diabetes driven by endothelial dysfunction, glycolipid metabolic disturbance, persistent vascular inflammation, and defective microcirculatory angiogenesis, ultimately leading to limb ischemia, refractory foot ulcers, and increased risks of amputation and mortality. As a complementary and alternative therapeutic approach, TCM offers unique holistic advantages in comprehensive management of diabetic LEAD through multi-component, multi-pathway, and multi-target regulatory mechanisms. This review systematically summarizes modern pharmacological mechanisms of representative TCM bioactive constituents, classical formulas, and patent medicines, encompassing vascular endothelial protection, glycolipid metabolism regulation, vascular inflammatory inhibition, and microcirculation improvement coupled with pro-angiogenic effects. Clinically, various TCM modalities, including perioperative adjuvant herbal medication, topical wound-healing therapies, and traditional rehabilitation combined with lifestyle modification, have been shown to reduce postoperative restenosis, accelerate ulcer repair, improve lower-limb perfusion, and delay atherosclerotic progression. Collectively, TCM-based interventions represent promising adjunctive strategies for diabetic LEAD, and further high-quality evidence is warranted to support their precise, standardized, and personalized clinical application.

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Review

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Cholinergic dysfunction in Alzheimer's disease: Mechanistic perspectives and multi-target effects of traditional Chinese medicine

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SUMMARY: Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder characterized by progressive cognitive decline and complex pathological changes. Cholinergic dysfunction is an early, clinically pivotal feature of AD closely associated with multiple pathological processes, including amyloid-β (Aβ) pathology, neuroinflammatory responses, mitochondrial dysfunction, metal-ion dyshomeostasis, and apolipoprotein E (APOE) ε4-related vulnerability. While disrupted cholinergic signaling impairs memory formation and synaptic function, its bidirectional interaction with AD-related pathological cascades further accelerates disease progression. Traditional Chinese medicine (TCM) has been investigated as a potential multitarget approach with which to modulate cholinergic deficits and related pathological processes. Some TCM-derived compounds, standardized extracts, and formulas have been reported to regulate cholinergic enzymes, acetylcholine levels, cholinergic receptors, oxidative stress, mitochondrial function, neuroinflammatory responses, and Aβ- or tau-related pathology. This review summarizes the role of cholinergic failure in AD pathophysiology and evaluates the evidence surrounding TCM-based interventions, with emphasis on direct cholinergic evidence, indirect collateral mechanisms, and current translational limitations. Indeed, the purpose of these insights is to inform future development of multitarget therapeutic strategies for AD.

Keywords: Alzheimer's disease, cholinergic system, traditional Chinese medicine, multi-target therapy

1. Introduction

As a major neurodegenerative disorder, Alzheimer's disease (AD) predominantly affects older adults worldwide and is characterized by progressive cognitive decline and memory impairment. Patients typically present with initial memory deficits, followed sequentially by language attrition, spatial disorientation, and executive dysfunction. Ultimately, a total loss of independence is observed in the advanced stages (*1*). With the global acceleration of population aging, China faces a particularly acute crisis, harboring the world's largest population of dementia cases, accounting for over 16 million as of 2021 (*2*). Thus, AD imposes a massive burden on patients, families, and healthcare systems worldwide.

The neuropathological landscape of AD is characterized by complex and progressive alterations, including extracellular senile plaques, intracellular neurofibrillary tangles (NFTs), region-specific synaptic loss, and persistent neuroinflammatory activation (*3*). A multi-faceted cascade, including amyloid-β (Aβ) toxicity, pathological tau spreading, cholinergic deficits, oxidative stress, and programmed neuronal death pathways, converges to define selective neuronal vulnerability. Cholinergic deficits have long provided the main rationale for symptomatic treatment in AD (*4*). Cholinergic dysfunction in AD includes diminished choline acetyltransferase (ChAT) activity, impaired high-affinity choline transport, and reduced acetylcholine (ACh) synthesis. These alterations compromise synaptic plasticity and disrupt the integrative function of cognitive networks. The cholinergic system influences Aβ metabolism, neuroinflammatory responses, and mitochondrial bioenergetics in addition to its role in neurotransmission. Loss of cholinergic function can initiate a self-amplifying cascade of complex neurodegenerative processes (*5*).

Rather than representing an isolated neurotransmitter deficit, cholinergic dysfunction interacts with multiple core pathological processes, including Aβ deposition, tau pathology, neuroinflammation, mitochondrial dysfunction, and disturbances in cellular homeostasis. Consequently, cholinergic dysfunction can serve as a

biologically significant entry point for understanding how these mechanisms converge within the broader pathological network of AD. Current pharmacological management includes symptomatic therapies, primarily cholinesterase inhibitors and the NMDA receptor antagonist memantine. These drugs offer mainly symptomatic relief and fail to arrest the underlying neurodegenerative process; long-term administration may be limited by adverse effects, and particularly gastrointestinal disturbances (*5*). Recent advances have led to the clinical approval of anti-amyloid monoclonal antibodies, including lecanemab and donanemab, for select patients with early AD. These disease-modifying therapies have demonstrated the ability to reduce amyloid burden and modestly slow cognitive decline. However, their clinical application remains restricted by stringent patient selection, the need for biomarker confirmation, intravenous administration, intensive imaging monitoring, and safety concerns such as amyloid-related imaging abnormalities (ARIA) (*6*). Their therapeutic benefits are also limited, as they have not yet been shown to fully address the multiple interconnected pathological processes underlying AD.

Against this therapeutic background, complementary strategies capable of acting on multiple pathogenic mechanisms remain of interest. Such a multitarget rationale is particularly relevant for evaluating traditional Chinese medicine (TCM), which has multicomponent and multitarget characteristics that may be suited to modulating interconnected pathological processes. Herbal formulations and TCM-derived compounds have been investigated for their potential effects on cognitive function, activities of daily living, neuropsychiatric symptoms, and AD-related pathological processes (*7*). This review systematically examines cholinergic system dysfunction in AD pathogenesis and evaluates current evidence regarding TCM-mediated modulation of cholinergic function in order to clarify its mechanistic basis, strength of evidence, and translational potential.

2. The cholinergic system in AD

Cholinergic neurotransmission depends on ACh synthesis, vesicular storage, and calcium-triggered release. These cholinergic neurons are widely distributed across the cerebrum and critically regulate learning, memory, and cognition. Pathological degeneration of these projections is a characteristic feature of dementia disorders, and particularly AD. Three features make this system central to AD: early and extensive degeneration, multifunctional disruption, and therapeutic tractability (*8*).

ACh biosynthesis begins with choline acetyltransferase (ChAT), which conjugates choline and acetyl-coenzyme A. The vesicular acetylcholine transporter (VAChT) then loads ACh into synaptic vesicles for release upon calcium-dependent depolarization. Release of ACh activates postsynaptic nicotinic (nAChRs) and muscarinic receptors (mAChRs) until acetylcholine esterase (AChE) hydrolyzes the signal into acetate and choline. Choline returns to the presynaptic terminal *via* the high-affinity choline transporter (CHT1), completing the cycle (*9*) (Figure 1).

Aβ also challenges the integrity of this cycle. In the preclinical stage of AD, physiological $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}16}$ transiently increase the synaptic vesicle pool *via* synaptophysin dephosphorylation and calcineurin activation (*10*). Pathological accumulation of Aβ, however, impairs cholinergic transmission, marking an early transition that precedes neuronal loss and that coincides with initial cognitive decline (*5*).

Cholinergic signaling maintains cerebral homeostasis through two additional mechanisms. These functions extend beyond synaptic transmission. First, ACh serves as an effective modulator and essential prerequisite for the complete expression of sensation-induced neurovascular coupling responses (*11*). Research has established that progressive degeneration of basal forebrain cholinergic neurons in AD leads to chronically reduced cortical ACh levels. These deficits mirror experimental results from models of prolonged ACh deprivation; specifically, both conditions demonstrate substantially impaired neurovascular coupling, constrained dynamic range in sensory processing networks, and distorted interpretation of neural activity through hemodynamic responses (*11*). Second, ACh signaling suppresses neuroinflammation and maintains intracellular homeostasis through multi-level bidirectional regulation *via* protein networks and non-coding microRNAs (*12*). These cortical functions depend on ascending projections from the basal forebrain (*13*). The nucleus basalis of Meynert (NBM) constitutes the principal source of these projections.

The NBM is the largest cholinergic component of the extrathalamic ascending reticular activating system and the primary output nucleus of the central cholinergic system. It integrates dopaminergic, serotonergic (5-HT), and noradrenergic signals from multiple brain regions to govern arousal, attention, and memory encoding (*13*). Its cholinergic Ch4 subdivision, the major cholinergic subdivision of NBM, projects *via* sparse collateral axons to cholinergic neuronal networks in the neocortex, hippocampus, and amygdala, providing the predominant ACh input to both the cerebral cortex and amygdala. Since Ch4 neurons regulate extensive cortical regions through sparse branching, even minor damage disrupts cholinergic signaling across wide territories (*13*). This anatomical vulnerability is associated with molecular susceptibility. Neurofibrillary degeneration within NBM cholinergic neurons emerges during the early stages of AD, strongly associated with the severity of cognitive impairment before substantial neuronal loss occurs. The exact mechanisms linking Ch4 lesions to specific symptoms remain unclear. However, this degeneration is an important factor in early cognitive impairment in AD. Loss of neuroprotective signaling also promotes Aβ and

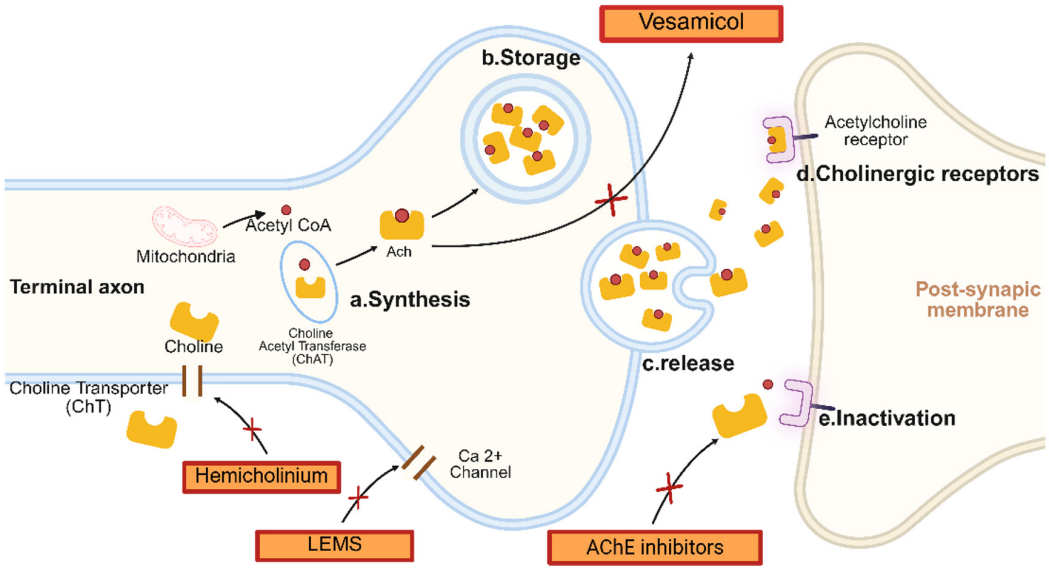


Figure 1. Cholinergic synaptic transmission and pharmacological intervention in Alzheimer's disease (AD). Acetylcholine (ACh) synthesis occurs in the presynaptic terminal, where choline acetyltransferase (ChAT) catalyzes the conjugation of choline and acetyl-CoA. Newly synthesized ACh is sequestered into synaptic vesicles and released into the synaptic cleft following Ca^{2+}-dependent depolarization. Following release, ACh binds to cholinergic receptors on the postsynaptic membrane to initiate signal transmission. Signaling termination proceeds through enzymatic hydrolysis by acetylcholinesterase (AChE), liberating choline for reuptake for subsequent ACh recycling. Pharmacological modulation of cholinergic signaling can target different steps of this process. For example, hemicholinium reduces choline uptake by inhibiting choline transport, vesamicol interferes with vesicular ACh storage through inhibition of the vesicular acetylcholine transporter (VAChT), and AChE inhibitors prolong cholinergic signaling by preventing ACh degradation. Impaired presynaptic release associated with dysfunction of calcium-dependent neurotransmitter release represents another mechanism affecting cholinergic transmission.

tau pathology. These changes further disrupt cholinergic transmission and accelerate cognitive decline (*8*).

The cholinergic crisis in AD encompasses not only presynaptic failure but also postsynaptic deterioration. Concomitant with NBM degeneration, AD reduces the number of nAChRs and mAChRs in basal forebrain cholinergic neurons and their cortical targets.

3. ACh receptors and cholinergic signaling

3.1. Nicotinic ACh receptors

As the first neurotransmitter receptors to be identified, nAChRs serve as prototypical ligand-gated ion channels that are widely distributed in the central and peripheral nervous system. This receptor superfamily also includes γ-aminobutyric acid type A (GABAA) receptors, glycine receptors, and serotonin type 3 (5-HT3) receptors (*14*). The functional diversity of nAChRs arises from various combinations of α and β subunits. At least nine α subunits (α2–α10) and three β subunits (β2–β4) have been identified in the CNS. The prototypical nAChRs typically assemble as a pentamer with an $\alpha_2\beta_3$ stoichiometry, forming a central hydrophilic pore that permits the transmembrane flux of cations such as K^+, Na^+, and Ca^{2+}. Ca^{2+} acts as a critical signaling molecule mediating the sustained regulatory effects of nicotinic receptors. Several neuronal nAChR subtypes, particularly α7 nAChRs, exhibit high Ca^{2+} permeability (*14*). Activation of these receptors increases presynaptic Ca^{2+} levels and promotes the release of multiple neurotransmitters, including glutamate, GABA, dopamine, 5-HT, and ACh.

In recent years, nAChR alterations have been linked to both early diagnosis and disease progression in AD. Positron emission tomography (PET) imaging has consistently demonstrated reduced α4β2 nAChR availability in AD, whereas investigations into α7 nAChRs remain limited but suggest region-specific alterations in receptor binding (*15*). α7 nAChRs are widely distributed and play a critical role in synapse formation, plasticity, learning, memory, and higher cognitive functions. They are reportedly expressed in neurons, microglia, astrocytes, and vascular endothelial cells. In microglia, α7 nAChRs exert neuroprotective effects by mediating anti-inflammatory signaling and modulating oxidative stress; mitochondrial-localized α7β2 nAChRs participate in the regulation of mitochondrial apoptotic pathways (*16*).

Notably, α7 nAChRs exhibit a strong binding affinity for soluble Aβ, forming Aβ-α7 nAChR complexes that undergo clathrin-mediated endocytosis. This process reduces functional α7 nAChRs on neuronal membranes. It also promotes intracellular Aβ accumulation, forming a dual pathological cascade. These observations are consistent with the markedly reduced nAChR expression found in AD brains and suggest a potential link between α7 nAChRs and the progression of AD (*16*). Emerging evidence also suggests that α7nAChRs may modulate

Aβ-induced neurotoxicity and mediate its detrimental effects on synaptic plasticity (*17*). These findings collectively suggest that the interaction between Aβ and α7 nAChRs plays an important role and that its proper balance is required for physiological function.

However, the complexity of α7 nAChR targeting is further compounded by regulatory heterogeneity. At the cellular level, AD patients exhibit upregulated α7 nAChR expression in hippocampal and cortical astrocytes but downregulated neuronal expression (*18*). In AD mouse models, both deletion and stimulation of α7 nAChRs have been reported to improve cognitive function (*19*). Although these findings appear contradictory, they may reflect the context-dependent roles of α7 nAChRs rather than fundamental inconsistencies. Indeed, the functional consequences of α7 nAChR modulation are likely influenced by disease stage, cell type, brain region, and the balance between physiological cholinergic signaling and pathological Aβ–α7 nAChR interactions. Activation of neuronal α7 nAChRs may preserve synaptic plasticity and suppress neuroinflammation during the early stages of disease, whereas under conditions of persistent Aβ accumulation and aberrant receptor signaling, reducing α7 nAChR activity may attenuate downstream neurotoxicity. Moreover, differences in AD models, the timing of the intervention, and experimental paradigms may contribute to the heterogeneity in outcomes across studies. These findings suggest that the function of α7 nAChRs in AD may vary across cell types and brain regions, necessitating precise modulation rather than simple activation or inhibition. Importantly, most evidence supporting α7 nAChR-targeted interventions is derived from preclinical studies, and the lack of consistent efficacy in clinical trials highlights the need for more standardized and disease stage-specific investigations. Although nAChR ligands hold therapeutic promise for some neurodegenerative and psychiatric disorders, no α7 nAChR-targeting ligand has yet succeeded in clinical trials.

3.2. Muscarinic receptors

Current evidence suggests that mAChRs predominantly mediate cholinergic transmission in forebrain regions and their contribution to learning and memory. As G protein-coupled receptors, mAChRs modulate diverse ion channels (*20*). Five subtypes of muscarinic receptors (M1-M5) have been identified and can be functionally grouped into two categories. M1, M3, and M5 couple with Gq/11 proteins. This coupling activates phospholipase C, increases intracellular calcium, and suppresses K^+ channel activity, which enhances neuronal excitability. In contrast, M2 and M4 interact with Gi/o proteins, leading to inhibition of the adenylyl cyclase and modulation of ion channel activity. The M1 subtype significantly influences cognition, attention, and sensory processing. Peripheral M2/M3 activation mediates the main adverse effects of cholinesterase inhibitors and other cholinergic agents, such as bradycardia and gastrointestinal disturbances, reflecting the functional differences between the central and peripheral receptor subtypes (*21*).

Thomson *et al*. examined subtype-specific functions in 2018. Their study showed that M1 and M4 receptors regulate hippocampal circuit formation and influence ACh release, which affects hippocampal function (*22*). Parallel investigations have established that M1 receptor deletion in the mouse prefrontal cortex (PFC) disrupts cholinergic signaling in pyramidal neurons, resulting in behavioral deficits in cue detection tasks (*23*). In contrast, activation of M1 receptors promotes the production of soluble amyloid precursor protein alpha (sAPPα) and reduces Aβ generation (*20*). Collectively, these findings establish M1 mAChR as a promising therapeutic target among the five mAChR subtypes.

Orthosteric agonists activate mAChRs lacking subtype selectivity, often precipitating adverse effects such as salivation, lacrimation, urination, diaphoresis, and gastrointestinal disturbances. To achieve M1 selectivity while avoiding these effects, research efforts have shifted towards allosteric modulation. The positive allosteric modulator VU0467319 (VU319) displays potent M1-mediated efficacy in animal models of AD. It enhances hippocampal synaptic plasticity and increases excitatory activity in the prefrontal cortex. VU319 also improves hippocampus-dependent cognitive function while mitigating the adverse effects of excessive receptor activation, thereby demonstrating an overall favorable safety profile (*24*).

4. Pathological factors associated with the cholinergic system

The hallmark pathological features of AD extend beyond Aβ and NFTs to encompass neuroinflammation, mitochondrial dysfunction, metal ion dyshomeostasis, and genetic abnormalities. These pathological characteristics interact with cholinergic pathology in a complex manner.

4.1. Aβ pathology

Aβ accumulation is a hallmark pathological feature in both familial AD and sporadic AD, and mounting evidence indicates that it is also one of the earliest pathological events contributing to central cholinergic dysfunction. Aβ is generated through sequential cleavage of amyloid precursor protein (APP), a transmembrane glycoprotein, by β-secretase and γ-secretase. This processing occurs predominantly within the endolysosomal system. Under physiological conditions, immune-mediated mechanisms efficiently clear Aβ peptides, whereas pathogenic mutations or impaired clearance promote progressive accumulation of insoluble

Aβ species and ultimately drive senile plaque (SP) formation (*25*) (Figure 2).

Clinical and experimental evidence indicates that abnormal Aβ deposition is closely associated with early impairment of the central cholinergic system. Postmortem evidence indicates that cognitively normal individuals with existing Aβ plaques already exhibit markedly reduced ChAT activity: 70.9% in the superior frontal gyrus and 79.5% in the inferior temporal gyrus. In addition, ChAT activity correlates negatively with Aβ concentration in the inferior temporal gyrus (*26*).

Aβ preferentially damages cholinergic projection regions involved in learning and memory, and particularly the basal forebrain and hippocampus. In Tg2576 transgenic mice, aged animals typically exhibit increased AChE staining around neocortical and hippocampal Aβ plaques. Moreover, cholinergic fibers in these areas exhibit marked structural distortion (*27*). Intracerebral injection of $A\beta_{42}$ in male rats impairs cholinergic function. Observed changes include lower ACh levels, reduced ChAT activity, and increased AChE activity, along with a compromised mitochondrial structure (*28*).

Aβ oligomers (AβOs) have been found to cause multi-target neurotoxicity. Direct interaction with α7 nAChRs represents one of the major mechanisms underlying cholinergic dysfunction. In addition, AβOs disrupt glutamatergic signaling by interfering with synaptic glutamate recycling. As a result, this induces abnormal extracellular glutamate accumulation, excessive NMDAR activation, and subsequent suppression of long-term potentiation (LTP) and exacerbation of long-term depression (LTD), ultimately resulting in a synaptic plasticity imbalance (*29*).

Interestingly, Aβ plaques and phosphorylated tau protein exhibit synergistic effects in the progression of AD. This interaction involves Aβ-facilitated tau aggregation and propagation within neuritic plaques. Tau is a microtubule-associated protein responsible for maintaining cytoskeletal stability (*30*). In AD, aberrant Aβ activates intracellular kinases that phosphorylate tau and promote its detachment from microtubules, which produces neurofibrillary tangles, destabilizes microtubules, impairs normal neuronal function, and may ultimately trigger neurodegeneration or apoptosis (*30*). The Aβ–tau cascade contributes not only to progressive neurodegeneration but also to sustained deterioration of cholinergic function. Postmortem studies have confirmed that Aβ pathology typically precedes and accelerates neocortical tau propagation, synergistically driving cognitive decline, which is closely associated with cholinergic dysfunction (*31*). Cholinergic depletion exacerbates cognitive impairment by promoting tau pathology and Aβ deposition, as shown in animal models. In rodent models of AD, selective basal forebrain cholinergic damage correlates significantly with increased hippocampal and cortical Aβ deposition and tau hyperphosphorylation (*32*). Beyond direct cytotoxicity, aberrant tau oligomers propagate intercellularly (*33*). These species activate neuroinflammatory responses

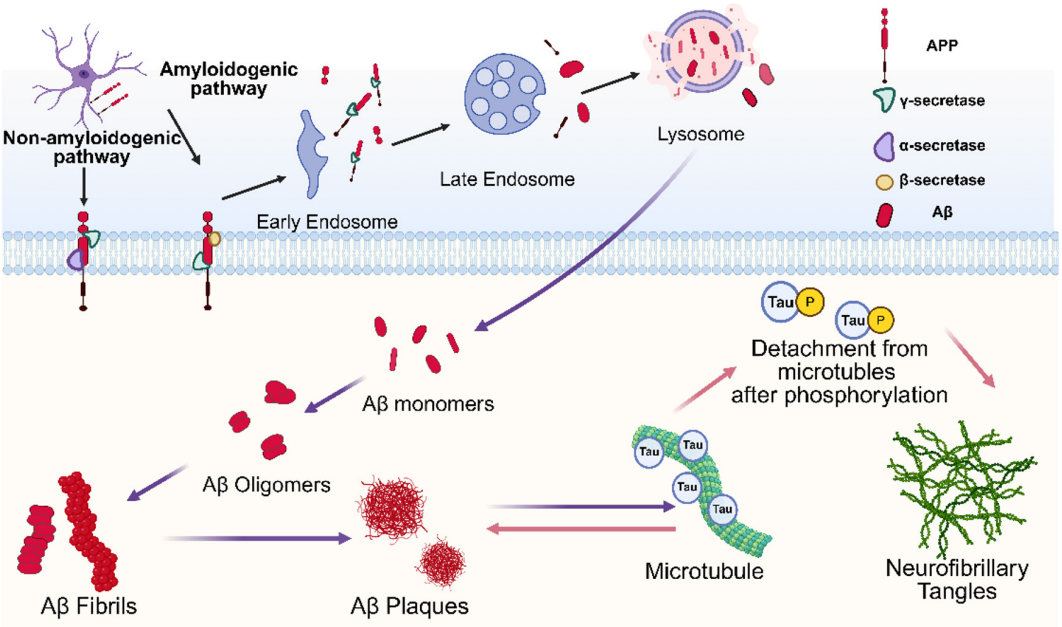


Figure 2. Mechanisms of amyloid-β processing and tau pathology in AD. Aβ accumulation constitutes a hallmark pathological feature of both familial and sporadic AD. Aβ is generated through sequential cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase within the endosomal–lysosomal system. Following internalization from the plasma membrane, APP is trafficked through early and late endosomes and lysosomes, where amyloidogenic processing occurs. The resulting Aβ peptides are released as monomers that progressively assemble into oligomers, fibrils, and ultimately extracellular plaques. In parallel, Aβ-associated pathology promotes tau phosphorylation, triggering its detachment from microtubules and subsequent aggregation into neurofibrillary tangles. These interconnected processes drive the progressive neuropathological alterations characteristic of AD.

and, through astrocyte and microglia interactions, induce programmed cell death (*33*). The cholinergic system possesses intrinsic anti-inflammatory properties, and compromised cholinergic function may amplify these pathological processes. These findings collectively suggest that Aβ-mediated cholinergic impairment is not merely a consequence of amyloid pathology. Loss of cholinergic function may further encourage Aβ accumulation and tau-associated neurodegeneration, thereby accelerating disease progression.

4.2. Neuroinflammation

Neuroinflammation is closely linked to cholinergic dysfunction in AD, driven in part by the disruption of the cholinergic anti-inflammatory pathway (CAP). The CAP was first characterized in 2000 by Borovikova and colleagues, who showed that the vagus nerve and its neurotransmitter ACh suppress excessive inflammatory responses by modulating immune cell function. This interaction inhibits key signaling pathways (including NF-κB), downregulates pro-inflammatory cytokine production, and thereby limits excessive inflammation while protecting tissue homeostasis (*34*).

Impaired CAP signaling weakens cholinergic regulation of neuroinflammation and contributes to persistent glial activation during the progression of AD. Microglia and astrocytes are the principal drivers of central nervous system inflammation. In AD, cerebral Aβ clearance depends largely on microglial phagocytosis. Given that cholinergic signaling can restrain glial activation through CAP, impairment of this pathway may render microglia and astrocytes more susceptible to persistent inflammatory activation. However, chronic microglial activation induced by Aβ accumulation triggers sustained release of inflammatory factors, which paradoxically impairs phagocytic and degradative capacity. This inflammatory loop increases Aβ deposition and promotes tau aggregation, culminating in neuronal injury and cell death and accelerating the progression of AD (*35*). *In vitro* studies have demonstrated that Aβ exposure induces microglia and astrocytes to generate neurotoxic reactive oxygen species (ROS) and reactive nitrogen species, activate the NLRP3 inflammasome, and release pro-inflammatory cytokines (*36*). Simultaneously, activated microglia can indirectly induce astrocytes to adopt an A1-like reactive phenotype through the release of IL-1α, TNF, and the complement component C1q. This transformation causes astrocytes to lose their normal neuroprotective functions, including support of neuronal survival, synapse formation, and synaptic pruning, while reducing neurotrophic factor expression, thereby further exacerbating neuronal injury (*37*). Pathological cholinergic neuronal loss, coupled with declining ACh release, attenuates CAP-mediated control, effectively removing a vital regulatory checkpoint that restricts neuroinflammatory cascades. Persistent neuroinflammation, in turn, further compromises cholinergic neuronal survival and neurotransmission, forming a feed-forward cycle that amplifies cholinergic dysfunction throughout the progression of AD (*34*).

4.3. Mitochondrial dysfunction

Cholinergic neurons are particularly vulnerable to mitochondrial dysfunction given their exceptionally high metabolic demands. Mitochondrial dysfunction is an early feature of AD. It involves reduced energy metabolism, disrupted Ca^{2+} homeostasis, and increased oxidative stress. Mitochondria facilitate neuronal survival not only by generating ATP through oxidative phosphorylation but also by regulating neurotransmitter synthesis and Ca^{2+} buffering, both of which are essential to maintaining cholinergic neurotransmission (*38*).

Mitochondrial metabolism is directly coupled to cholinergic function. Under physiological conditions, mitochondria generate ATP and critical metabolites including acetyl-CoA, the acetyl donor required for ACh synthesis, through the tricarboxylic acid (TCA) cycle. Given that ACh synthesis relies on both acetyl-CoA availability and an adequate ATP supply, mitochondrial dysfunction can directly compromise ACh production and cholinergic synaptic activity (*39*) (Figure 3). Reduced ATP production also impairs the maintenance and function of cholinergic neurons, thereby exacerbating cholinergic dysfunction during the progression of AD.

In addition to facilitating ACh synthesis, mitochondrial metabolism influences neuronal survival through multiple protective mechanisms. For example, the TCA cycle intermediate oxaloacetate (OAA) plays several protective roles in neurons. It enhances energy metabolism, promotes mitochondrial biogenesis, reduces neuroinflammation, and facilitates hippocampal neurogenesis. These effects may help preserve cholinergic neuronal function, although they are attenuated under the hypometabolic conditions characteristic of AD (*40*). Enhancing mitophagy facilitates the clearance of Aβ plaques and hyperphosphorylated tau, restores mitochondrial homeostasis, and may interrupt the propagation of pathological cascades and even reverse memory impairment (*38*). These observations highlight the close metabolic dependence of cholinergic neurotransmission on mitochondrial integrity throughout the progression of AD.

4.4. Dysregulation of metal ion homeostasis

Disruption of metal ion homeostasis is closely associated with cholinergic dysfunction in AD. Iron, copper, and calcium affect cholinergic neurons through distinct but convergent mechanisms, including oxidative stress, mitochondrial dysfunction, abnormal cholinesterase regulation, and calcium homeostasis disruption, particularly within the vulnerable basal forebrain

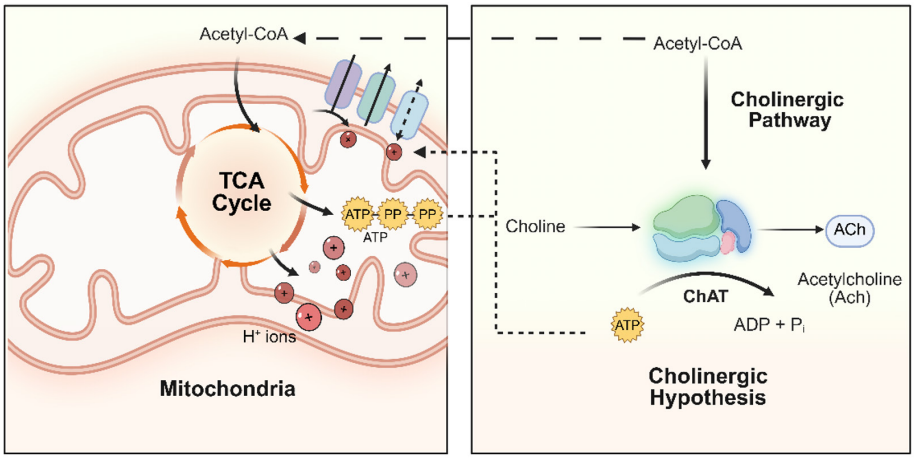


Figure 3. Mitochondrial metabolic regulation of the cholinergic pathway in AD. The tricarboxylic acid (TCA) cycle generates ATP and metabolic intermediates, including acetyl-CoA, which supply essential substrates and energy for ACh synthesis. Acetyl-CoA condenses with choline *via* ChAT to produce ACh. In AD, mitochondrial hypometabolism and impaired mitophagy promote the accumulation of dysfunctional mitochondria, disrupting energy homeostasis and amplifying the mitochondrial cascade. Reduced metabolic output restricts acetyl-CoA availability and ATP production, thereby impairing cholinergic function. Restoration of mitochondrial integrity may attenuate pathological protein accumulation and indirectly facilitate cholinergic neuronal survival and signaling.

cholinergic system (*41*). Iron has been extensively implicated in cholinergic degeneration. Excess iron and copper may also indirectly damage cholinergic neurons by promoting APP expression, accelerating Aβ production, and enhancing tau hyperphosphorylation.

Cholinergic neurons exhibit heightened susceptibility to iron dysregulation (*42*). Abnormal iron accumulation promotes oxidative stress and ferroptosis, thereby increasing the vulnerability of cholinergic neurons. Anatomical evidence indicates that the ventral extension area of the iron-rich globus pallidus overlaps highly with the distribution of cholinergic neurons (AChE-positive neurons). This anatomical overlap may, to some extent, account for the selective susceptibility of cholinergic neurons to iron-mediated oxidative injury (*43*). Iron overload further enhances ROS generation through the Fenton reaction, leading to oxidative damage, lipid peroxidation, and progressive neuronal degeneration (*38*). Iron dysregulation may also impair cholinergic neurotransmission by altering butyrylcholinesterase (BChE) activity. In addition, current evidence suggests that abnormally elevated BChE activity in the hippocampus and cortex accelerates ACh hydrolysis, thereby further exacerbating cholinergic dysfunction (*42*).

Disruption of copper homeostasis represents another mechanism linking a metal ion imbalance to cholinergic dysfunction in AD. Excessive intracellular copper can trigger cuproptosis, a form of regulated cell death associated with mitochondrial metabolism disruption. Several cuproptosis-related genes, including *FDX1*, *DLAT*, *LIAS*, and *PDHA1*, have been reported to be dysregulated in AD and may contribute to mitochondrial dysfunction and oxidative stress. Reduced PDHA1 activity limits acetyl-CoA production, thereby not only impairing ATP generation but also restricting ACh synthesis. Dysregulated copper homeostasis may therefore impair cholinergic neurotransmission by disrupting mitochondrial metabolism and promoting cuproptosis-associated neuronal injury (*44*).

Iron dysregulation and calcium homeostasis disruption are closely interconnected, together intensifying neuronal injury in AD. Iron-mediated calcium signaling activates downstream kinase cascades involved in synaptic plasticity. Under physiological conditions, iron-dependent ROS generation facilitates normal calcium-dependent signaling, whereas excessive ROS disrupts calcium signaling and mitochondrial function, ultimately impairing neurons, including cholinergic neurons (*45*). Given the dependence of cholinergic neurotransmission on tightly regulated intracellular Ca^{2+} dynamics, persistent disruption of calcium homeostasis is likely to further exacerbate cholinergic dysfunction during the progression of AD (*46*). Taken together, these findings suggest that metal ion dyshomeostasis may diminish cholinergic neurotransmission and reduce neuronal survival, thereby contributing to a self-reinforcing degenerative process during the progression of AD.

4.5. Apolipoprotein E

The apolipoprotein E (APOE) ε4 allele, the strongest genetic risk factor for sporadic AD, is closely associated with cholinergic vulnerability in AD. In addition, ApoE is essential for cholesterol transport, synaptic maintenance, and neuronal repair in the central nervous system. (*47*).

Compared to non-carriers, individuals carrying the APOE ε4 allele have been reported to exhibit poorer therapeutic responses to cholinesterase inhibitors such as tacrine, possibly owing to impaired ACh biosynthesis (*48*). ApoE4 transgenic mice were found to display

a reduced capacity to regenerate cholinergic nerve fibers following entorhinal cortex injury (*49*), while postmortem studies have demonstrated significantly lower ChAT activity in APOE ε4 carriers than in non-carriers. Although a direct causal relationship between APOE ε4 and reduced ChAT activity has not been established, available evidence suggests that this genotype may exacerbate cholinergic degeneration by impairing neuronal repair and limiting the functional reserve of the cholinergic system (*50*).

APOE ε4 also contributes to cholinergic dysfunction through its effects on Aβ pathology. In this respect, ApoE4 promotes the early deposition and spread of Aβ while reducing its clearance, thereby increasing pathological stress on vulnerable cholinergic neurons (*51*). In addition, Aβ can interact with BChE and ApoE to form a soluble complex with enhanced ACh-hydrolyzing activity, thereby increasing ACh degradation and further disrupting cholinergic neurotransmission (*52*).

These interrelated pathological events do not occur in isolation but converge on and interact with the cholinergic system, forming a key point of intersection among multiple neurodegenerative cascades. A cholinergic-centered perspective helps integrate these mechanisms and highlights the complex interplay underlying disease progression, as shown in Figure 4.

5. Progress in TCM for the treatment of AD

At present, the main medications for AD in clinical use include centrally acting AChE inhibitors, such as rivastigmine, galantamine, and donepezil, together with the NMDA receptor antagonist memantine. Although these agents can alleviate cognitive symptoms to some extent, they have limited ability to prevent the progressive loss of cholinergic neurons or modify the complex pathological processes underlying AD, and their long-term use may be constrained by adverse effects, including gastrointestinal disturbances and sleep disorders (*5*). Consequently, multitarget therapeutic strategies that simultaneously affect cholinergic dysfunction and its associated pathological networks have attracted increasing attention. In this context, TCM and its bioactive constituents have, given their multicomponent composition and multitarget characteristics, been investigated as potential therapeutic approaches for AD. Current evidence indicates that some TCM interventions directly modulate cholinergic-related markers, including ACh, AChE, BChE, ChAT, and cholinergic receptors. Other studies suggest that their reported effects may involve the regulation of AD-related pathological processes closely associated with cholinergic degeneration, including Aβ deposition, tau pathology, oxidative stress, mitochondrial dysfunction, and neuroinflammation (*7*).

Accordingly, the available evidence was categorized into three groups: direct cholinergic evidence, indirect cholinergic-related evidence, and supportive or weak evidence. Direct cholinergic evidence is defined as studies demonstrating the direct modulation of established cholinergic markers, including ACh, AChE, BChE, ChAT, or cholinergic receptors. Indirect cholinergic-related evidence includes studies in which the main findings involve AD-associated

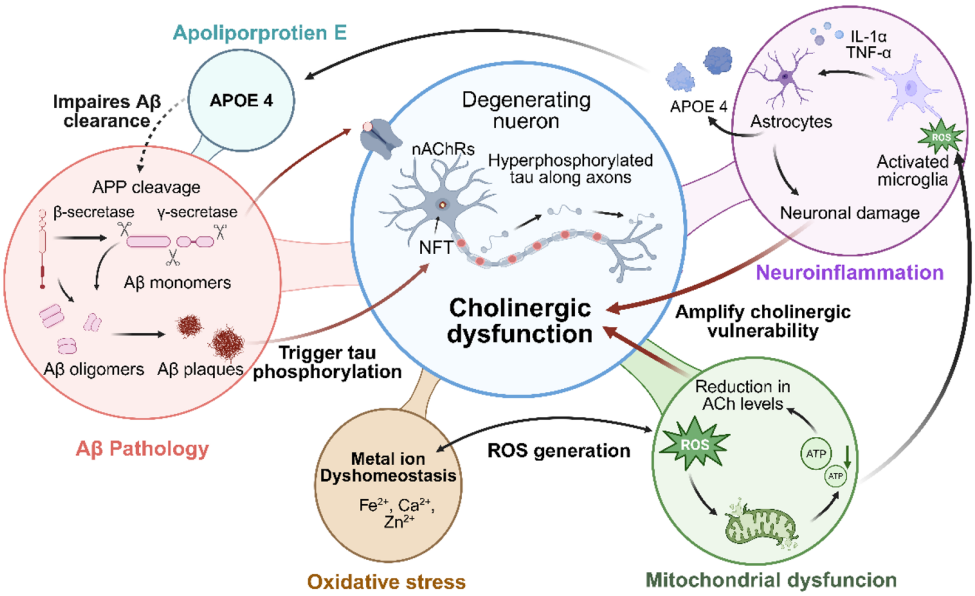


Figure 4. Interconnected mechanisms underlying cholinergic dysfunction in AD. APP cleavage by β- and γ-secretases generates Aβ monomers, oligomers, and plaques, along with impaired Aβ clearance, and particularly in the presence of APOE4. These changes are linked to tau hyperphosphorylation, neurofibrillary tangle (NFT) formation, and neuronal degeneration. Oxidative stress is reflected by reactive oxygen species (ROS) production and metal ion dyshomeostasis (Fe^{2+}, Cu^{2+}, Ca^{2+}). Mitochondrial dysfunction is characterized by reduced ATP production and decreased ACh levels. Astrocytes and activated microglia release pro-inflammatory mediators, contributing to neuronal damage. These alterations converge, resulting in cholinergic dysfunction. Red arrows indicate detrimental effects on the cholinergic system.

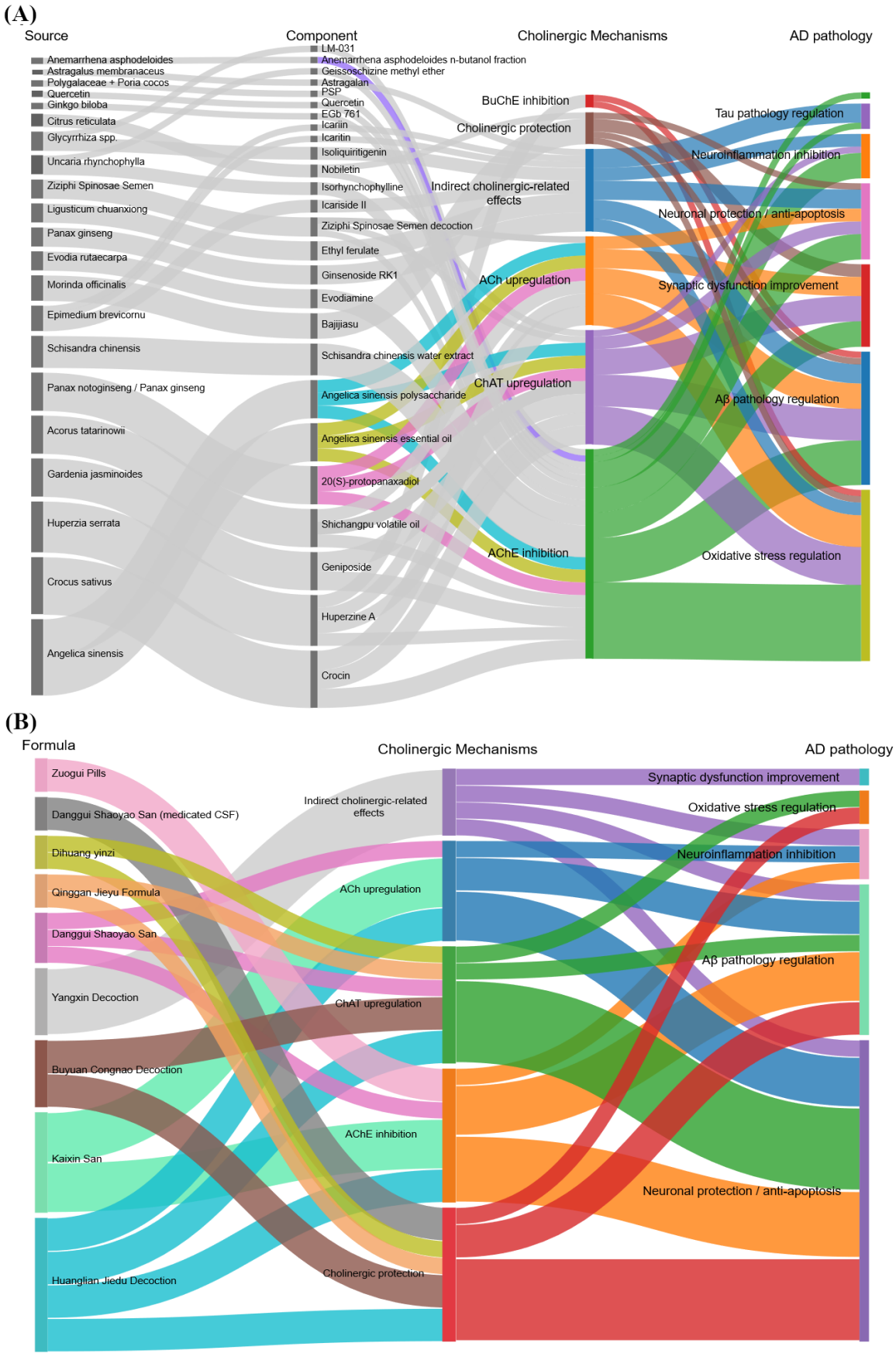


Figure 5. Sankey diagrams of active components from single herbs and herbal formulations in traditional Chinese medicine (TCM). **(A)** Sankey diagram of TCM compounds regulating the cholinergic system in AD. **(B)** Sankey diagram of TCM herbal formulations regulating cholinergic mechanisms in AD. The Sankey diagrams were generated using the online visualization tool RAWGraphs 2.0 (https://app.rawgraphs.io/)

pathological processes, such as Aβ deposition, tau pathology, oxidative stress, mitochondrial dysfunction, neuroinflammation, or synaptic impairment, without direct assessment of cholinergic markers. Supportive or weak evidence refers to studies reporting only general neuroprotective effects, showing limited relevance to disease models, or lacking clear experimental validation. Based on this classification scheme, the experimental evidence evaluating single herbs, standardized extracts, and pure TCM-derived constituents is shown in Table 1, with multi-herb formulas summarized in Table 2. Table 3 presents a comprehensive summary of the available clinical evidence and translationally relevant information.

5.1. Single herbs, standardized extracts, and TCM-derived compounds

Among the currently investigated TCM-based therapeutic strategies, bioactive constituents that directly modulate cholinergic neurotransmission have yielded relatively substantial experimental evidence. Among these, Huperzine A, an active constituent isolated from *Huperzia serrata* (serrate clubmoss), represents the most extensively investigated example. Huperzine A is a potent AChE inhibitor that enhances cholinergic neurotransmission by reducing the degradation of ACh. In APP/PS1 mice, intranasal administration of Huperzine A reduced AChE activity, increased ChAT expression, and alleviated Aβ pathology through the modulation of APP processing and activation of the Wnt/β-catenin signaling pathway (*53*). These findings collectively suggest that the effects of directly enhancing cholinergic function are not limited to the regulation of neurotransmitter signaling. Direct cholinergic modulation may also influence multiple interconnected pathological pathways in AD, thereby producing broader modulation of AD-related pathological processes.

In addition to Huperzine A, several compounds derived from TCM have been reported to have direct cholinergic activity by targeting different components of the cholinergic system. For example, nobiletin, a polymethoxylated flavonoid isolated from *Citrus reticulata* (tangerine peel), restored ChAT expression and cholinergic fiber density in the hippocampus of olfactory bulbectomized mice (*54*). Likewise, crocin, a major bioactive constituent of *Crocus sativus* (saffron), increased ACh and ChAT levels while reducing AChE activity in animal models of AD (*55*). Similar cholinergic regulatory activities have been reported for geniposide, *Schisandra chinensis* (Chinese magnolia vine) extract, isorhynchophylline, *Angelica sinensis* (Chinese Angelica) polysaccharides, 20(S)-protopanaxadiol, β-asarone, and other natural products summarized in Table 1 (*56-61*). Collectively, these findings indicate that TCM-derived compounds may directly regulate cholinergic signaling at multiple levels, including neurotransmitter synthesis, cholinergic neuron integrity, and other key components of the cholinergic system.

Notably, many compounds with direct cholinergic activity modulate pathological processes that are closely associated with cholinergic neurodegeneration. In addition to restoring cholinergic neurotransmission, crocin attenuates oxidative stress, mitochondrial dysfunction, and Aβ deposition. Geniposide not only normalizes abnormalities in ChAT and AChE but also reduces the amyloid burden by suppressing the Aβ-RAGE/MAPK signaling pathway (*56*). Likewise, *Schisandra chinensis* extract decreases AChE activity while ameliorating neuroinflammation and metabolic disturbances (*57*). Taken together, these findings suggest that cholinergic dysfunction in AD is not an isolated neurotransmitter deficiency but an integral component of the broader pathological network underlying disease progression.

Another group of compounds has not been evaluated for their direct effects on cholinergic markers, such as ACh, AChE, ChAT, or cholinergic receptors. At present, these compounds are more appropriately regarded as indirect cholinergic-related evidence. Representative examples include ginsenoside RK1, icariin, icariside II, icaritin, and ethyl ferulate. Their reported effects mainly involve the regulation of Aβ production and clearance, tau phosphorylation, oxidative stress responses, mitochondrial homeostasis, and neuronal survival (*62-65*). In addition, some compounds have been assessed in models with limited relevance to AD-related cholinergic dysfunction. In this regard, quercetin has been reported to improve abnormal AChE activity only in a cadmium-induced neurotoxicity model and is therefore classified as supportive or weak evidence in Table 1. Collectively, evidence from isolated compounds and standardized extracts indicates that TCM-derived interventions may act through both direct cholinergic regulation and indirect modulation of AD-related pathological processes. Nevertheless, the assessment of cholinergic markers remains inconsistent across studies, and the translational value of these findings needs to be validated further.

5.2. TCM formulas

Compared to individual compounds, TCM formulas are characterized by multicomponent composition, multitarget actions, and network-based regulation. These features theoretically align well with the complex pathological network in which cholinergic dysfunction develops during the progression of AD. In contrast to several TCM-derived natural compounds with mechanisms that have been investigated in considerable detail, only a limited number of TCM formulas have been supported by direct evidence of cholinergic regulation. Most studies have instead demonstrated their ability to modulate pathological processes that are closely associated with cholinergic degeneration. Accordingly, the collective evidence for TCM formulations remains

Table 1. Preclinical evidence of TCM-derived compounds and standardized extracts targeting cholinergic dysfunction in AD

Intervention	Main constituent(s)	Model	Dose	Evidence category	Cholinergic evidence	Main findings	Reference
Intranasal administration with Huperzine A	Huperzine A	APP/PS1 mice	167 or 500 μg/kg/day, intranasal, 4 months	Direct	AChE↓, ChAT↑	Improved cognition; reduced Aβ plaques; activated Wnt/β-catenin signaling.	(*53*)
Nobiletin (*Citrus reticulata*)	Nobiletin	OBX mice	50 mg/kg/day, i.p., 11 days	Direct	ChAT↑; restored cholinergic fiber density	Improved memory through ERK/CREB signaling.	(*54*)
Crocin (*Crocus sativus*)	Crocin	HT22 cells; D-galactose/AlCl3 mice	5 or 20 mg/kg/day	Direct	ACh↑, ChAT↑, AChE↓	Reduced Aβ deposition; attenuated oxidative stress and mitochondrial dysfunction.	(*55*)
Geniposide	Geniposide	APP/PS1 mice; primary hippocampal neurons	12.5–50 mg/kg/day for 3 months	Direct	ChAT↑, AChE↓	Reduced amyloid pathology *via* the Aβ–RAGE/MAPK pathway.	(*56*)
Schisandra chinensis extract	Gomisin A, Schisandrin, Longikaurin A	$A\beta_{25–35}$ rats	0.5% SCF for 7 weeks	Direct	AChE↓	Reduced neuroinflammation and Aβ deposition; improved cognition.	(*57*)
Isorhynchophylline	Isorhynchophylline	AlCl3-induced AD in mice	20 or 40 mg/kg/day for 8 weeks	Direct	AChE↓	Improved cognition; suppressed oxidative stress and NF-κB activation.	(*58*)
Angelica sinensis polysaccharide	Angelica sinensis polysaccharide	$A\beta_{25–35}$ rats	2 mL/100 g/day for 28 days	Direct	ACh↑, ChAT↑, AChE↓	Reduced APP/Aβ levels and oxidative stress.	(*59*)
20(S)-Protopanaxadiol	20(S)-PPD	Scopolamine-induced memory impairment in mice	20 or 40 μmol/kg/day for 14 days	Direct	AChE↓, ChAT↑, ACh↑	Improved cognition; reduced oxidative stress.	(*60*)
β-Asarone (*Acorus tatarinowii*)	β-Asarone	$A\beta_{1–42}$ rats	25 mg/kg/day for 28 days	Direct	ACh↑, ChAT↑, AChE↓	Increased NGF and reduced neuronal apoptosis.	(*61*)
Ginsenoside RK1	Ginsenoside RK1	PC12 cells; mice with AD	Not reported	Indirect	Not directly evaluated	Improved cognition; mitochondrial protection; activated AMPK/Nrf2.	(*62*)
Icariin/Icaritin	Icariin; Icaritin	OA-treated SH-SY5Y cells	ICA 2.5 μM; ICT 1 μM	Weak	Not directly evaluated	Reduced tau phosphorylation through GSK-3β inhibition.	(*63*)
Icariside II	Icariside II	APP/PS1 mice	10 or 30 mg/kg/day for 3 months	Indirect	Not directly evaluated	Reduced Aβ production; improved cognition.	(*64*)
Ethyl ferulate	Ethyl ferulate	$A\beta_{1–42}$ rats	100 mg/kg/day for 6 weeks	Indirect	Not directly evaluated	Reduced Aβ, oxidative stress and neuroinflammation.	(*65*)

Table 1. Preclinical evidence of TCM-derived compounds and standardized extracts targeting cholinergic dysfunction in AD (continued)

Intervention	Main constituent(s)	Model	Dose	Evidence category	Cholinergic evidence	Main findings	Reference
Quercetin	Quercetin	Cadmium-exposed rats	5–50 mg/kg/day	Direct	Prevented abnormal AChE↑	Neuroprotection in cadmium toxicity rather than AD.	(*78*)
LM-031 (chalcone–coumarin hybrid)	LM-031	Tet-On Aβ-GFP293/SH-SY5Y cells	Not reported	Indirect	AChE inhibition	Reduced Aβ aggregation and oxidative stress.	(*79*)
Anemarrhena asphodeloides n-butanol fraction	Timosaponins	Scopolamine-induced memory impairment in mice	20 mL/kg/day for 21 days	Direct	AChE↓	Improved cognition and antioxidant capacity.	(*80*)
Geissoschizine methyl ether	Geissoschizine methyl ether	AChE assay	IC50 18.3 μM	Direct	Reversible AChE inhibition	Potential AChE inhibitor.	(*81*)
Ziziphi Spinosae Semen	Jujuboside A/B	PCPA-induced insomnia in mice	Not reported	Direct	AChE mRNA↓, ACh↑, ChAT↑	Improved cholinergic neurotransmission.	(*82*)
Angelica sinensis essential oil	Angelica sinensis Essential oil	$A\beta_{25-35}$ rats	Not reported	Direct	ACh↑, ChAT↑, AChE↓	Improved cognition and reduced APP/Aβ.	(*83*)
PSP (*Polygala tenuifolia* saponins + *Poria cocos* polysaccharides)	PSP	Memory-impaired mice	Not reported	Direct	AChE↓	Improved learning and antioxidant capacity.	(*84*)
EGb 761 + donepezil	Standardized Ginkgo biloba extract	Scopolamine-induced memory impairment in rats	EGb761 8.27 mg/kg + donepezil	Direct	AChE↓	Enhanced cognitive improvement with donepezil.	(*85*)
Bajijiasu	Bajijiasu	$A\beta_{25-35}$ rats	See original study	Direct	Attenuated cholinergic dysfunction	Improved cognition and reduced neuronal apoptosis.	(*80*)
Evodiamine	Evodiamine	STZ-induced AD in mice	50 or 100 mg/kg/day for 21 days	Direct	AChE↓	Improved cognition; suppressed oxidative stress and neuroinflammation.	(*81*)

Table 2. Preclinical evidence of TCM formulas targeting cholinergic dysfunction in AD

Formula	Main herbal components	Model	Dose	Evidence category	Cholinergic evidence	Other major mechanisms	Main findings	Major limitations	Reference
Huanglian Jiedu Decoction	*Coptis chinensis, Scutellaria baicalensis, Phellodendron chinense, Gardenia jasminoides*	$A\beta_{1-42}$-induced AD rats	1.5, 3, or 6 g/kg/day	Direct	ACh↑; ChAT↑; AChE↓; α7nAChR↑	Anti-Aβ activity; neuroprotection	Increased ACh, ChAT and α7nAChR expression while reducing AChE activity, accompanied by improved cognition and alleviated neuronal injury.	Downstream cholinergic signaling was not investigated; receptor antagonist validation lacking.	(*66*)
Danggui Shaoyao San	*Angelica sinensis, Paeonia lactiflora, Atractylodes macrocephala, Alisma orientale, Poria cocos, Ligusticum chuanxiong*	Ibotenic acid (IBO)-induced AD rats	0.139, 0.372, or 1 g/kg/day	Direct	ACh↑; ChAT↑; AChE↓	Neuroprotection; behavioral improvement	Restored cholinergic function by increasing ACh and ChAT and decreasing AChE, while improving neuronal survival and cognitive performance.	Limited mechanistic investigation beyond cholinergic biomarkers.	(*67*)
Kaixin San	*Panax ginseng, Polygala tenuifolia, Acorus tatarinowii, Poria cocos*	SCOP-induced cognitive impairment in C57BL/6 mice	6.5 g/kg/day	Direct	ACh↑; AChE↓	NF-κB/STAT3 inhibition; anti-neuroinflammation; neuroprotection	Increased ACh, reduced AChE, improved cognitive function, and inhibited NF-κB/STAT3 signaling *in vivo*.	Only selected predicted targets were experimentally validated.	(*68*)
Yang Xin Decoction	*Poria cocos, Polygala tenuifolia, Panax ginseng, Astragalus membranaceus, Angelica sinensis, etc.*	Primary mouse cortical neurons; ReNcell VM; SH-SY5Y; PC12 cells	100 μg/mL (extract); Polygala tenuifolia extract 10 μg/mL	Indirect	None (no direct cholinergic evidence)	Anti-Aβ aggregation; anti-inflammatory activity; CREB/ERK activation; neuronal differentiation; neuroprotection	Reduced Aβ aggregation, suppressed inflammation, activated CREB/ERK signaling, and promoted neuronal differentiation.	No *in vivo* validation; cholinergic biomarkers were not assessed; synergistic interactions remain unclear.	(*69*)
Zuogui Pills	*Rehmannia glutinosa, Dioscorea opposita, Cornus officinalis, Lycium barbarum, etc.*	D-galactose + $A\beta_{1-40}$-induced AD in rats	2.7 or 5.4 g/kg/day	Direct	AChE↓	HSP70 upregulation; anti-apoptosis; neuroprotection	Suppressed brain AChE activity, enhanced HSP70 expression, and protected hippocampal neurons.	Active constituents and downstream pathways remain unclear.	(*88*)
Buyuan Congnao Decoction	*Astragalus membranaceus, processed Polygonum multiflorum, etc.*	$A\beta_{1-42}$ + IBO-induced AD in rats	~2.39 g/kg/day	Direct	ChAT↑; AChE modulation	Antioxidant activity; neuroprotection	Improved learning and memory while increasing serum ChAT activity and antioxidant capacity.	Mechanistic pathway remains unclear.	(*89*)
Qinggan Jieyu Formula	*Angelicae Sinensis Radix, Paeoniae Radix Alba, Poria, Atractylodis Macrocephalae Rhizoma, etc.*	Naturally occurring dementia in aged mice	Not reported	Direct	ChAT↑; AChE-positive fiber density↑	Neuroprotection	Enhanced central cholinergic activity and improved learning and memory in aged mice.	Formula composition and dosage were incompletely reported.	(*90*)

Table 2. Preclinical evidence of TCM formulas targeting cholinergic dysfunction in AD (continued)

Formula	Main herbal components	Model	Dose	Evidence category	Cholinergic evidence	Other major mechanisms	Main findings	Major limitations	Reference
Dihuang Yinzi	*Rehmannia glutinosa, Morinda officinalis, Cornus officinalis, Dendrobium officinale, etc.*	2VO-induced chronic cerebral hypoperfusion in rats	10 or 20 g/kg/day	Indirect	ChAT↑; AChE↑	Neuroprotection	Improved cognitive performance and partially restored hippocampal cholinergic activity in chronic cerebral hypoperfusion rats.	Disease model differs from classical AD pathology. The increase in AChE complicates interpretation of its cholinergic effect.	(*91*)

heterogeneous, with the extent of mechanistic validation varying substantially across studies (Table 2).

Huanglian Jiedu Decoction, Danggui Shaoyao San, and Kaixin San are among the few representative TCM formulas for which direct evidence of cholinergic regulation has been reported. In animal models of Aβ-induced synaptic dysfunction, Huanglian Jiedu Decoction increased the levels of ACh and the expression of ChAT and hippocampal α7nAChR while reducing AChE activity. It also alleviated neuronal injury, suggesting that the formula regulates both cholinergic neurotransmission and related pathological processes (*66*). Similarly, Danggui Shaoyao San restored cholinergic function by increasing ChAT and ACh levels while decreasing AChE activity. It also improved neuronal survival and behavioral performance (*67*). Kaixin San was consistently found to increase ACh levels and reduce AChE activity *in vivo*. Further experimental studies showed that its cholinergic effects were accompanied by inhibition of the NF-κB/STAT3 signaling pathway (*68*). These studies collectively indicate that the effects of TCM formulas on cholinergic signaling are unlikely to occur in isolation. Instead, their cholinergic effects usually extend to multiple interconnected pathological pathways.

At present, most TCM formulas have yielded only evidence indirectly related to cholinergic dysfunction. The reported mechanisms primarily involve the regulation of amyloid pathology, neuroinflammation, oxidative stress, mitochondrial homeostasis, synaptic function, or neuronal survival. In many of these studies, however, classical cholinergic biomarkers were not evaluated. For example, Yangxin Decoction has demonstrated broad neuroprotective activities, including inhibition of Aβ aggregation and inflammatory signaling pathways, activation of the CREB/ERK-dependent pathway, and promotion of neuronal differentiation. However, direct regulation of AChE, ChAT, or other cholinergic biomarkers by this formula has not been demonstrated (*69*). For studies of TCM formulas that rely solely on network pharmacology predictions or preliminary computational target analyses without experimental validation, the findings can only suggest potential regulatory mechanisms rather than provide mechanistic confirmation. Accordingly, these studies were classified as supportive or weak evidence in Table 2.

Overall, the available evidence suggests that TCM formulas may influence cholinergic dysfunction in AD through the coordinated regulation of interconnected pathological processes (Table 2). Compared to studies of individual compounds, the principal challenge in investigating TCM formulas is not the identification of a single molecular target. Rather, it lies in the incomplete characterization of their chemical basis, key bioactive constituents, and the synergistic interactions among different components. At present, only a limited number of studies have directly evaluated ACh levels, AChE or

Table 3. Clinical evidence of TCM interventions targeting cholinergic dysfunction in AD

Intervention	Disease type	Treatment duration	Dose	Clinical evidence	Main clinical outcomes	Safety	Reference
Huperzine A	AD	8 weeks–6 months	0.2–0.45 mg/day, oral	Systematic review, meta-analysis, and TSA (25 studies; *n* = 1,813); RCT (*n* = 90)	Improved MMSE, HDS-R, memory performance, ADL, and overall clinical response.	Mild cholinergic adverse events (mainly gastrointestinal discomfort and dizziness); serious adverse events uncommon.	(*70*)
EGb 761 (*Ginkgo biloba* extract)	Mild dementia (including probable AD, VD, and mixed dementia)	22–24 weeks	240 mg/day, oral	Meta-analysis (*n* = 782)	Improved cognition, ADL, global assessment, and quality of life, and alleviated neuropsychiatric symptoms.	Generally well tolerated; no significant increase in serious adverse events.	(*71*)
Buyuan Congnao Decoction + piracetam	AD	12 weeks	Buyuan Congnao Decoction + piracetam (0.8 g, three times daily)	RCT (*n* = 196)	Improved MMSE, ADAS-Cog, overall clinical response; increased IGF-1 and reduced Aβ.	No obvious treatment-related adverse events reported.	(*72*)
Dihuang Yinzi + donepezil	Mild-to-moderate AD	3 months	Dihuang Yinzi (1 dose/day) + donepezil (5 mg/day)	RCT (*n* = 114)	Improved MMSE, ADAS-Cog, ADL; regulated Notch1, ADAM10, BACE1, and acrolein.	Similar incidence of adverse events between groups; no serious adverse events.	(*73*)
Jianpi Yangxin Decoction + donepezil	Mild-to-moderate AD	3 months	Jianpi Yangxin Decoction (1 dose/day) + donepezil (5–10 mg/day)	RCT (*n* = 150)	Improved MMSE, ADL, and TCM syndrome scores.	Lower incidence of adverse events than donepezil alone.	(*74*)
Hengqing II + donepezil	AD	3 months	Hengqing II Formula (1 dose/day) + donepezil (5–10 mg/day)	RCT (*n* = 107)	Improved MMSE, MoCA, ADAS-Cog, ADL, and NPI; reduced APP, Aβ, inflammatory markers, Hcy, and CysC.	Well tolerated; no significant difference in adverse events between groups.	(*75*)
Bushen Capsules	aMCI	24 months	4 capsules, three times daily	Randomized, double-blind, placebo-controlled trial (*n* = 60)	Improved episodic memory and altered brain activation patterns on fMRI.	Similar adverse-event profile to a placebo.	(*76*)
Di-Tan Decoction	AD	24 weeks (+6-week follow-up)	13.5 g twice daily, oral	Randomized, double-blind, placebo-controlled study protocol (planned *n* = 40)	Primary outcomes: ADAS-Cog, C-MMSE, C-DAD (trial ongoing).	Safety monitoring predefined; no efficacy or safety results available.	(*92*)
Buyuan Congnao Decoction + acupuncture	AD	12 weeks	Buyuan Congnao Decoction (1 dose/day) + acupuncture	RCT (*n* = 98)	Improved MMSE, overall clinical response; reduced serum Hcy and Aβ.	No serious safety concerns reported.	(*93*)

ChAT activity, or the expression of cholinergic receptors. Most investigations remain focused on the modulation of pathological pathways or on predictions derived from network pharmacology. Future studies should establish standardized quality control procedures and provide comprehensive chemical characterization of TCM formulas. Further investigations need to be conducted to verify their direct effects on the cholinergic system and to clarify how these effects are related to the regulation of multiple pathological processes.

5.3. Clinical evidence and translational considerations

Clinical studies of TCM and related interventions for AD have primarily focused on a limited number of standardized extracts, isolated compounds, TCM formulas, Chinese patent medicines, and combination therapies. The most assessed outcomes include cognitive function, activities of daily living, overall clinical improvement, and select AD-related pathological biomarkers (Table 3).

Among the currently available interventions, Huperzine A has yielded one of the relatively strongest bodies of clinical evidence supporting a cholinergic therapeutic approach. As a well-characterized AChE inhibitor, its clinical efficacy has been evaluated in multiple randomized controlled trials and is further supported by systematic reviews and meta-analyses. Collectively, these studies suggest that Huperzine A is associated with improvements in cognitive function, as assessed with the Mini-Mental State Examination (MMSE), the Revised Hasegawa Dementia Scale (HDS-R), and memory-related evaluations. Improvements in activities of daily living have also been reported, indicating potential functional benefits. Although mild cholinergic adverse events, such as gastrointestinal discomfort and dizziness, were reported more frequently in the treatment groups than in the control groups, serious adverse events were uncommon (*70*).

The standardized extract EGb 761 from *Ginkgo biloba* L. is another intervention supported by a relatively substantial body of clinical evidence. A recent meta-analysis involving patients with mild dementia reported improvements in cognition, activities of daily living, global clinical assessment, and quality of life and alleviation of neuropsychiatric symptoms after 22–24 weeks of treatment. However, the study populations likely included patients with AD, vascular dementia (VD), and mixed dementia. In addition, treatment efficacy was evaluated solely based on clinical outcome measures, without assessing cholinergic function (*71*).

Current clinical evidence for TCM formulas is derived primarily from studies in which they were used as adjunctive therapies in combination with conventional treatments, such as donepezil, piracetam, or acupuncture. In those randomized controlled trials, formulas such as Buyuan Congnao Decoction, Dihuang Yinzi, Jianpi Yangxin Decoction, and Hengqing II were reported to improve cognitive outcomes, including scores on the MMSE, the Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-Cog), and the Montreal Cognitive Assessment (MoCA), and activities of daily living. Changes in AD-related biomarkers, including Aβ, amyloid precursor protein (APP), inflammatory mediators, insulin-like growth factor-1 (IGF-1), and Notch-associated signaling molecules, were also reported (Table 3) (*72-75*). In addition to studies involving patients with clinically diagnosed AD, the current clinical evidence includes investigations conducted in different stages of cognitive impairment and using diverse study designs. For example, Bushen Capsules have been evaluated in patients with amnestic mild cognitive impairment (aMCI). The study findings highlight the potential value of TCM-based interventions during the prodromal stage of cognitive decline (*76*). Overall, the current clinical evidence suggests that some TCM-derived interventions may provide cognitive or functional benefits, particularly when standardized compounds, extracts, and TCM formulas are used as adjunctive therapies. Nevertheless, considerable heterogeneity remains across studies with respect to patient populations, intervention strategies, outcome measures, and study design. These factors should be taken into account when interpreting the evidence, and particularly in the context of the translational limitations discussed below.

5.4. Translational limitations, standardization, and quality control

The translational reliability of the clinical evidence described thus far remains subject to several important limitations. From the perspective of the cholinergic system, which is the focus of this review, the most important limitation is the lack of mechanistic endpoints in clinical studies. Most studies have relied predominantly on neuropsychological assessments, activities of daily living, or global clinical improvement as their primary endpoints. Mechanistic indicators, including ACh, AChE, ChAT, VAChT, cholinergic receptors, and cholinergic neuroimaging, have rarely been incorporated into clinical evaluations. Taken together, the available clinical studies provide preliminary support for selected TCM-based interventions, and particularly as adjunctive therapies. However, the current evidence remains insufficient to establish their cholinergic-specific clinical effects in AD.

The methodological quality of the available clinical evidence is also inconsistent. Although most studies were described as randomized controlled trials, details regarding allocation concealment, blinding procedures, and follow-up were often incomplete. Moreover, the investigations were predominantly limited by small sample sizes and single-center designs, with diagnostic criteria varying substantially across the literature, including the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), the

National Institute on Aging-Alzheimer's Association (NIA-AA) criteria, the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria, and Chinese diagnostic guidelines. Some studies also enrolled patients with vascular dementia, mixed dementia, or aMCI. This heterogeneity limits the extent to which their findings can be directly interpreted as evidence in patients with clinically diagnosed AD. Evidence regarding long-term safety also remains limited. Most studies reported only mild and transient adverse events, and a consistent increase in serious adverse events was not observed. Nevertheless, treatment durations were typically limited to three to six months, precluding a comprehensive assessment of long-term safety. Combination therapy is another important factor affecting the interpretation of the available clinical evidence. In many studies, TCM formulas were administered as adjuncts to treatments such as donepezil, piracetam, or acupuncture, hampering attribution of the observed cognitive or functional improvements solely to the TCM intervention. Moreover, few studies have systematically evaluated potential pharmacokinetic or pharmacodynamic interactions associated with combination therapy. Whether co-administration with AChE inhibitors alters drug exposure or increases the risk of cholinergic adverse effects remains unclear.

Standardization and quality control remain major challenges for TCM formula-based preparations. Most clinical studies provide limited information on botanical authentication, chemical characterization, batch-to-batch consistency, and quality assurance. This limits the reproducibility and comparability of findings across studies. Future studies should include well-designed multicenter clinical trials using standardized TCM preparations and rigorous methodological standards. Longer follow-up periods and systematic safety evaluations are also needed. In addition, validated cholinergic biomarkers should be incorporated to bolster the translational evidence linking TCM-based interventions to cholinergic dysfunction in AD (*77*).

6. Summary and prospects

AD is a multifactorial disorder with multiple pathological changes. One early feature is the selective vulnerability of the basal forebrain cholinergic system. The cholinergic hypothesis emphasizes the importance of this system in the pathology of AD and links cholinergic deficits to Aβ deposition, neuroinflammation, and mitochondrial bioenergetics. These interactions suggest that the cholinergic system may act as an important contributor to disease progression.

Current cholinesterase inhibitors offer limited symptomatic benefit without demonstrable disease-modifying effects, highlighting the urgent need for novel therapeutic strategies. TCM may, given its multicomponent and multitarget characteristics, provide a complementary perspective. Some TCM-derived compounds, standardized extracts, and formulas have been reported to modulate cholinergic neurotransmission, influence Aβ metabolism, reduce neuroinflammation, and regulate other AD-related pathological processes. Emerging clinical evidence also suggests potential cognitive and functional benefits of selected TCM-derived interventions, and particularly when used as standardized preparations or adjunctive therapies. In addition, combining TCM with conventional drugs may be a promising complementary therapeutic strategy, although this possibility requires further confirmation in well-designed clinical studies.

TCM research faces several challenges, including the complexity of herbal components, unclear mechanisms, lack of standardized methods of preparation, and insufficient clinical evidence. Greater emphasis should also be placed on improving study quality, standardization, quality control, and translational validation, including the incorporation of mechanistic cholinergic biomarkers in future clinical studies. Future efforts should integrate traditional TCM theories with modern biomedical approaches, elucidate mechanisms at the molecular level, and establish standardized evaluation systems.

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Review

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The spatiotemporal ecosystem of gallbladder cancer: From inflamed areas to invasive margins — Integrating biomechanical injury, the bile microenvironment, precursor lineages, and spatial niches

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SUMMARY: Gallbladder cancer (GBC) is usually described as the end point of chronic cholelithiasis-associated inflammation, and yet this linear formulation does not explain why only a small fraction of people with gallstones develop cancer, why histologically similar precursor lesions follow different evolutionary routes, or why invasion preferentially advances through anatomically and immunologically distinct interfaces. We propose a spatiotemporal ecosystem model that links five domains: gallstones and reflux as precipitating conditions; bile as a chemically active habitat; resident or transient microorganisms as context-dependent modifiers; metaplastic, biliary intraepithelial and intracholecystic precursor lineages as alternative epithelial trajectories; and stromal-immune niches that select for invasive phenotypes. Evidence from epidemiology, pathology, genomics, organoid and animal models, single-cell sequencing, and spatial profiling is integrated while preserving distinctions between association, mechanism, and clinical prediction. The strongest causal chain currently links prolonged mechanical or reflux injury with cycles of epithelial loss and repair, metaplastic or dysplastic changes, and acquisition of driver alterations. Microbial findings are biologically plausible and increasingly supported by functional Salmonella studies, but most human biliary microbiome datasets remain small and vulnerable to low-biomass contamination and treatment-related confounding. Precursor studies substantiate the existence of both BilIN progression in affected areas and alternative polypoid or BilIN-independent routes, arguing against a single obligatory sequence. Recent single-cell and spatial studies have identified organized, region-specific interactions among malignant epithelial states, macrophages, exhausted CD8 T cells, neutrophils, endothelial cells, fibroblasts, hepatocytes, and nerves at invasive margins. Liver-facing, serosal, perineural, lymphovascular, and nodal interfaces should not be assumed to share one ecology. These data shift the central question from which mutation is present to where a clone resides, which neighboring cells sustain it, and when the niche becomes permissive. The model suggests testable priorities: longitudinal sampling of high-risk gallbladders, paired bile-mucosa profiling with rigorous controls, lineage-resolved multi-section spatial analysis, and boundary-aware biomarkers validated in prospective cohorts.

Keywords: gallbladder cancer, gallstones and bile, spatiotemporal ecosystem model, stromal-immune niches, spatial transcriptomics, invasive margin

1. Introduction: Why gallbladder cancer requires a spatiotemporal model

Gallbladder cancer (GBC) is uncommon globally but highly lethal, with a pronounced geographic and ethnic concentration and a persistent tendency to present after invasion of the liver or regional structures. Contemporary syntheses have established its epidemiology, pathology, molecular alterations, and clinical management, but most frameworks still arrange these elements as a list of risk factors followed by a linear histological sequence (*1-4*). That arrangement is useful for describing the cancer but not for indicating the mechanism of its onset. Gallstones are common, whereas GBC is rare; chronic cholecystitis is widespread, whereas dysplasia is focal; and invasive behavior varies distinctly in regions of the same tumor. A

model that generalizes the entire organ or tumor therefore loses information that is central to causation.

The term "ecosystem" is used here in a restricted, testable sense. It refers to interactions among epithelial clones, bile constituents, microorganisms, immune cells, stromal cells, and the local anatomy of the gallbladder over time. An affected "area" denotes a mucosal region altered by repeated injury and repair, not proof that every cell in that area is premalignant. A invasive "margin" denotes the interface through which tumor cells enter adjacent liver or other tissues. This vocabulary helps separate three questions that are often conflated: what creates a susceptible mucosal region; which epithelial lineage acquires and retains malignant competence; and which local niche permits invasion.

This narrative review focuses specifically on GBC. PubMed-indexed and major peer-reviewed studies available through August 2026 were prioritized, with an emphasis on original human cohorts, systematic reviews, mechanistic infection models, precursor lesion studies, multi-region genomics, single-cell sequencing, and spatial profiling. Studies of cholangiocarcinoma or pan-biliary cancers are cited only when they provide an indispensable methodological or therapeutic context. Because the evidence base is heterogeneous, statements are explicitly calibrated as an epidemiological association, histological inference, experimental mechanism, prognostic association, or clinical-trial evidence (Figure 1).

2. Creation of an inflamed area

2.1. Gallstones are the predominant precipitator of an inflamed area's creation but not a sufficient cause

The most reproducible environmental factor associated with GBC is cholelithiasis. A recent field-wide review identified 215 studies and performed 350 meta-analyses, and its results confirmed that a biliary infection is closely associated with gallbladder cancer while adiposity and related metabolic factors were somewhat associated; however, heterogeneity, reverse causation, and residual confounding remained important limitations (*5*). A separate global analysis of 115 studies encompassing more than 32 million participants estimated a gallstone prevalence of 6.1%, with substantial geographic and sex variation, underscoring the mismatch between a common factor and a rare cancer outcome (*6*). Classic clinical reviews and modern pathology agree that gallstones should be understood as a prolonged source of mechanical abrasion, obstruction, bile stasis, and recurrent epithelial injury rather than as a direct cause of cancer in every carrier (*7,8*).

Evidence from cholecystectomy specimens substantiates the concept of an affected area. In a study of 350 gallstone-bearing gallbladders, hyperplasia, metaplasia, dysplasia, and carcinoma in situ were identified at different frequencies, and molecular loss-of-heterozygosity accumulated across the pathological continuum (*9*). A meta-analysis has also linked gallstones and their characteristics to the risk of biliary tract cancer, but estimates vary with stone ascertainment, latency, anatomical site, and the choice of stone-free or stone-bearing controls (*10*). These observations establish population-level associations and mucosal plausibility, but they do not show that an individual gallstone will initiate a particular tumor clone.

Obesity, diabetes, and smoking may make an affected area more susceptible through bile supersaturation, systemic inflammation, altered motility, oxidative stress, or shared lifestyle determinants. Meta-analyses reported an increased risk of GBC with obesity, with higher figures in women, and less of an association

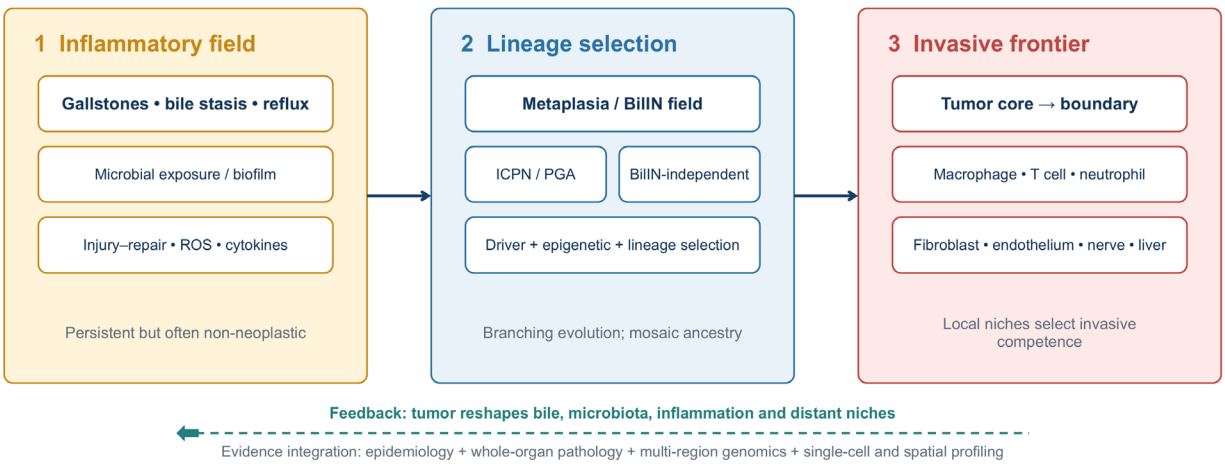


Figure 1. A spatiotemporal ecosystem model of gallbladder carcinogenesis. Prolonged injury creates an inflamed area; epithelial lineages then branch through BilIN-associated, polypoid precursor-associated, or apparently independent routes; and local stromal-immune niches select for invasive competence. The dotted arrow indicates feedback from established cancer to bile, microbiota, inflammation, and distant niches. *Abbreviations:* BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; PGA, pyloric gland adenoma; ROS, reactive oxygen species.

with overweight (*11,12*). Type 2 diabetes was associated with an approximately 1.5-fold pooled risk in observational studies, although confounding by adiposity and gallstones could not be fully eliminated (*13*). Smoking was associated with a pooled relative risk of approximately 1.45 in an analysis dominated by case-control studies (*14*). These effect sizes are materially smaller than those reported for chronic biliary infection or selected anatomical abnormalities and should not be presented as equivalent causal drivers.

Host susceptibility is also non-random. A genome-wide association study in India implicated variants near ABCB1 and ABCB4, genes related to hepatobiliary transport, while population studies in Shanghai showed that a family history of gallstones clustered with gallstone and biliary cancer risk (*15,16*). The most defensible interpretation is interaction: inherited or acquired differences in bile handling and inflammation may determine which stone-bearing mucosa sustains prolonged injury, but no current germline marker alone provides support for population screening for GBC.

2.2. Pancreaticobiliary maljunction (PBM) as a natural experiment in reflux-driven carcinogenesis

PBM provides unusually coherent evidence that anatomy can create an area where cancer is more likely to develop. In a Japanese nationwide survey of 2,529 patients, adults with congenital biliary dilatation had a bile duct cancer frequency of 6.9% and a GBC frequency of 13.4%; among adults with PBM without dilatation, the corresponding frequency of bile duct cancer was 3.1% and that of GBC was 37.4% (*17*). Because the pancreatic and bile ducts join outside sphincter control, pancreatic juice can reflux into the gallbladder, where activated enzymes, secondary bile acids, and prolonged stasis repeatedly injure the mucosa (*18*).

Histopathological studies of "reflux-associated cholecystopathy" describe diffuse mucosal hyperplasia and proliferative changes that precede carcinoma, providing a morphological bridge between abnormal anatomy and neoplasia (*19*). Importantly, PBM-associated GBC is not restricted to East Asian practice: a Western pathological series identified comparable lesions and distinctive clinicopathological features, corroborating PBM as a general model of anatomy-induced physicochemical carcinogenesis rather than a regional curiosity (*20*). PBM nevertheless represents a specific high-risk pathway and should not be used to infer that pancreatic reflux drives most gallstone-associated cancers.

2.3. Chronic infection: Strongest for Salmonella, uncertain for a universal biliary microbiome

Chronic Salmonella carriage has the most substantiated evidence chain for infection leading to GBC. Using anti-Vi serology and culture-based definitions, a Chilean case-control study combined with a meta-analysis found an increased GBC risk, although confidence intervals in the primary study were wide (*21*). An earlier systematic review of 17 studies estimated an overall odds ratio of 4.28 for chronic Salmonella typhi carriage, with stronger associations in South and Southeast Asian datasets (*22*). Detection of non-typhoidal Salmonella DNA in tumor specimens widened the question beyond S. typhi (*23*).

Mechanistic work has shifted the association beyond serology. In epithelial and organoid models, Salmonella manipulation of MAPK and AKT signaling promoted transformation-related phenotypes, and chronic infection accelerated tumor formation in susceptible experimental settings (*24*). More recent triangulation across human microbiomes, cultured S. typhimurium isolates, gallstone-bearing mice, GBC cells, and xenografts implicated KDM6B-dependent host epigenetic remodeling; KDM6B inhibition reduced tumor growth in the experimental model (*25*). This does not prove that Salmonella is required for human GBC, but it establishes a biologically coherent route by which persistent bacteria can cooperate with gallstones and inflammation.

Evidence for Helicobacter species is less consistent. A multicountry analysis detected Helicobacter DNA in some gallbladder and extrahepatic biliary cancers, but differences in sampling, assays, geography, and contamination control preclude a universal conclusion (*26*). The literature on infection therefore supports a hierarchy: Salmonella has convergent epidemiological and functional evidence; other taxa are candidates whose effects may be restricted to particular ecological and geographic contexts (Table 1).

3. Bile and microbiota: A coupled but technically fragile habitat

3.1. Human sequencing studies show dysbiosis but have yet to demonstrate a reproducible cancer-specific consortium

Bile is not merely a carrier fluid. It is a concentrated mixture of bile acids, phospholipids, cholesterol, proteins, microbial products, and host-derived inflammatory molecules. Gallstone obstruction, infection, fasting, antibiotics, endoscopic instrumentation, and tumor-related cholestasis can each alter that mixture. Consequently, a cross-sectional difference between cancer bile and control bile may be a cause, a consequence, or both.

Small human studies nevertheless provide useful signals. Metagenomic analysis of gallbladder bile from seven GBC and 30 cholelithiasis patients identified distinct microbial communities and candidate organisms, but the small cancer group limited inference (*27*). Shotgun metagenomics of mucosal biopsies subsequently described microbial shifts along the chronic cholecystitis-to-GBC progression, shifting the sampled compartment from free bile towards the tissue-associated habitat

Table 1. Evidence linking major factors or conditions field-forming exposures to the development of gallbladder cancer

Factor or condition	Human evidence	Quantitative signal	Mechanistic interpretation	Principal limitation
Gallstones/chronic cholecystitis	Meta-analyses, case-control studies, cholecystectomy pathology	Strong association, but estimates vary by stone phenotype and comparator (*5,9,10*)	Repeated abrasion, stasis, necrosis-repair cycles, oxidative injury	Common exposure; low absolute progression rate; reverse causation in short-latency studies
Obesity/metabolic disease	Cohort and case-control meta-analyses	Obesity RR about 1.6; diabetes RR about 1.5 (*11-13*)	Cholesterol supersaturation, motility, systemic inflammation	Residual confounding by gallstones and lifestyle
PBM/pancreatic reflux	Nationwide survey and pathology series	Gallbladder cancer reported in 37.4% of adults with non-dilated PBM in the Japanese survey (*17*)	Enzyme-rich reflux, secondary bile acids, diffuse hyperplasia	Rare anatomy; referral and registry effects
Chronic Salmonella carriage	Case-control studies, meta-analysis, cellular and animal models	Pooled odds ratios around 4 in historical meta-analyses (*21,22*)	Persistent colonization, inflammatory signaling, epigenetic remodeling	Geographic concentration; exposure misclassification
Other biliary bacteria	Small sequencing cohorts	Taxa differ across studies (*26-30*)	Biofilm, metabolite production, innate immune activation	Low biomass, antibiotics, obstruction, instrumentation, contamination

(*28*). Bile microbiome profiling across normal, chronic cholecystitis, and GBC groups also found disease-associated dysbiosis (*29*), while a study spanning pancreaticobiliary and gallbladder cancers associated gallbladder microbial composition with prognosis (*30*). A 2024 mechanistic review summarized the emerging bacteria-metabolite-inflammatory pathways but also noted that study designs and taxonomic signals remain heterogeneous (*31*).

The central limitation is low microbial biomass. Contaminating DNA from extraction kits, reagents, endoscopes, the room environment, or batch effects may constitute a substantial fraction of the apparent signal. Salter and colleagues experimentally demonstrated that reagent contamination can critically distort sequence-based microbiome studies, and later guidance for low-biomass research emphasized negative controls, randomized extraction batches, absolute microbial quantification, and transparent contaminant removal (*32,33*). These are not optional refinements for GBC: bile is often collected after antibiotics or instrumentation, and cancer cases frequently differ from stone controls in obstruction, jaundice, hospitalization, and drainage.

3.2. Bile acids, lipids, and microbial products may link injury to selection

Bile acids regulate epithelial physiology through membrane effects and receptor-mediated signaling, while microbial transformation modifies the primary-secondary bile acid pool. General hepatobiliary physiology supports links among bile acids, barrier function, inflammation, FXR/TGR5 signaling, and epithelial stress (*34*). In the gallbladder, however, direct longitudinal evidence that a defined bile acid species initiates a human precursor clone is sparse.

Multi-omics has increased resolution. A 2022 study used a training cohort of 87 bile samples, a metaproteomic subset of 42, and a validation cohort of 38 to derive lipid and microbial-peptide signatures that distinguished GBC from benign gallbladder disease; the reported eight-lipid panel achieved very high diagnostic performance in the study setting (*35*). The result is encouraging but should be treated as a biomarker-development signal and not as a population-ready test because spectrum effects, sample handling, biliary obstruction, and independent external validation determine transportability.

Older biochemical studies also reported increased lipid peroxidation products in GBC bile, consistent with oxidative injury but not specific for carcinogenesis (*36*). Together, these findings justify paired chemical and microbial profiling, but they do not establish that any single bile constituent leads to cancer in humans.

The most plausible synthesis is a feedback loop. Stones or reflux alter flow and damage epithelium; altered bile chemistry and inflammation change microbial fitness; selected organisms or biofilms modify metabolites and innate immune signaling; and the resulting habitat imposes selection on clones repairing the epithelium. The direction and magnitude of each arrow are patient-specific. This formulation avoids the unsupported claim that there is one "GBC microbiome," while preserving the experimentally substantiated possibility that particular microbes, under appropriate chemical and anatomical conditions, can become causal cofactors (Figure 2).

4. Precursor lineages: One area, several evolutionary routes

4.1. The flat metaplasia-BilIN-carcinoma route

The fifth edition of the WHO Classification of Digestive System Tumours recognizes pyloric gland adenoma, biliary intraepithelial neoplasia (BilIN), and intracholecystic papillary neoplasm (ICPN) as distinct gallbladder epithelial precursor or preinvasive entities (*37*). BilIN is a microscopic flat or micropapillary lesion graded in a two-tier system: low-grade BilIN incorporates the former BilIN-1 and BilIN-2 categories, whereas high-grade BilIN corresponds broadly to the former BilIN-3. High-grade BilIN requires extensive sampling to exclude invasion and is difficult to detect by imaging or gross examination (*38*). ICPN, in contrast, is a grossly visible intraluminal mass-forming neoplasm; any associated invasive carcinoma should be documented separately. Contemporary reviews emphasize that BilIN, ICPN, and pyloric gland adenoma are related but not interchangeable, with different morphology, lineage markers, and associated invasive cancers (*39,40*).

The classic route associated with an inflamed area begins with repeated epithelial loss and repair in a stone-bearing gallbladder. Pseudopyloric, foveolar, or intestinal metaplasia may occur, followed by low-grade and high-grade BilIN and then invasive carcinoma. A mapping study showed that intestinal metaplasia and dysplasia cluster in gallbladders with carcinoma and that routine limited sampling under-detected focal lesions (*41*). Cross-sectional age differences led to an estimate of roughly 15 years from dysplasia to advanced carcinoma, but this is an ecological inference from different patients rather than a direct longitudinal observation (*42*). A histological review similarly corroborated a sequence while cautioning that not every metaplasia progresses and that pseudopyloric change is common in benign inflammation (*43*).

Whole-organ mapping reinforces the spatial nature of an affected area. Dysplasia may be patchy, multifocal, and more extensive than the gross lesion, which is why complete or additional sampling is required when high-grade dysplasia is found (*44*). The area therefore contains multiple epithelial states at the same time. Histology alone cannot determine which patch is ancestral to the invasive tumor.

4.2. Polypoid precursors and alternative lineages

ICPN is a macroscopically visible, mass-forming intraepithelial neoplasm. In a clinicopathological study of 123 cases, ICPNs displayed biliary, gastric, intestinal, or oncocytic differentiation and were frequently associated with invasive carcinoma, but the invasive component and outcome differed from conventional flat-pathway GBC (*45*). Gallbladder adenomas also display molecular heterogeneity. The observation that CTNNB1 exon 3 mutations were more frequent in adenomas than carcinomas supports the view that many conventional adenomas are not obligatory intermediates of ordinary GBC (*46*).

Rare invasive phenotypes further caution against collapsing all endpoints into one lineage. A 2024 population-based analysis found that gallbladder signet-ring cell carcinoma presented with more aggressive clinicopathological features and poorer survival than conventional adenocarcinoma (*47*). That study established prognostic heterogeneity but not a distinct precursor sequence: registry morphology cannot

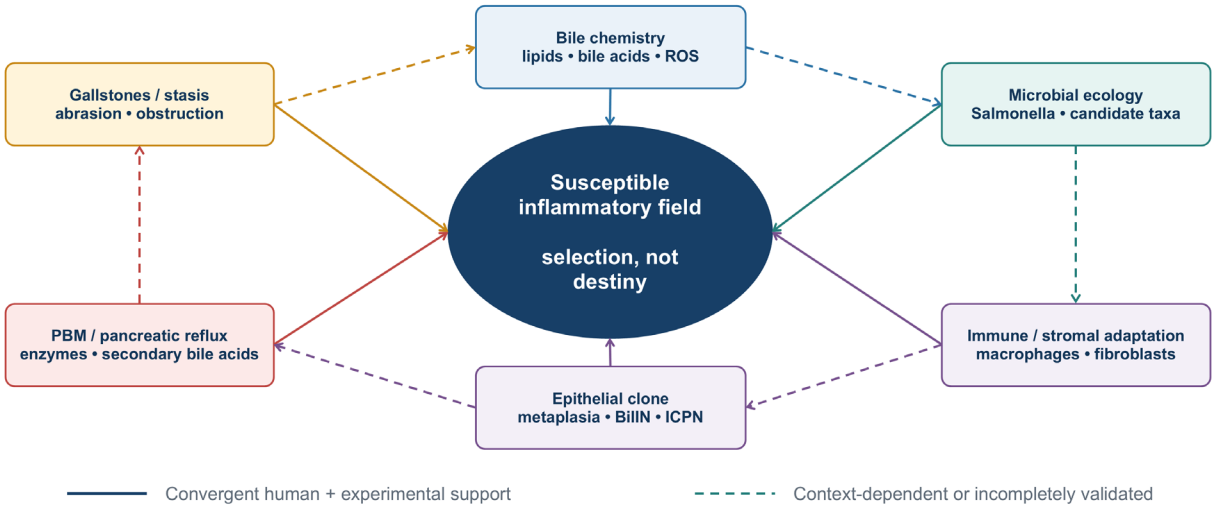


Figure 2. The gallstone-bile-microbiota-mucosa feedback system. Gallstones and pancreaticobiliary reflux have convergent human and mechanistic factors that create affected areas. Microbial, metabolic, immune, stromal, and epithelial components interact bidirectionally, but many individual taxa and metabolites remain context-dependent candidates rather than validated universal causes. *Abbreviations:* PBM, pancreaticobiliary maljunction; BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; ROS, reactive oxygen species.

determine whether a signet-ring phenotype has arisen through BilIN, ICPN, or an unsampled route.

Early molecular pathology further indicated pathway divergence. KRAS mutations were detectable in subsets of dysplasia and carcinoma (*48*), microsatellite alterations were found in chronic cholecystitis and preneoplastic lesions (*49*), and loss of E-cadherin expression or gene alterations increased across precursor-to-carcinoma progression (*50*). Those studies were limited by small cohorts and older assays, but they anticipated the contemporary conclusion that histologically similar lesions may have different clonal histories.

4.3. Multi-region genomics and spatial transcriptomics reveal branching evolution

Multi-region sequencing provides more direct evidence of alternative routes. Whole-exome analysis of 44 microdissected regions from 11 T1 GBCs with coexisting lesions found both BilIN-dependent and apparently BilIN-independent cancers; six tumors were classified as BilIN-independent and five as BilIN-dependent, although the rarity and early stage of the cohort limited generalization (*51*). A two-patient spatial transcriptomic study sampled 24 and 32 regions of interest spanning normal epithelium, low-grade BilIN, high-grade BilIN, and adenocarcinoma. The sequence of pathway activation differed between patients, and SEMA4A repression emerged as a shared potential event, with functional support in organoids and cell-line-derived tumors (*52*). That study is mechanistically informative precisely because it does not claim population prevalence based on two cases.

A 2025 study of papillary neoplasms of the bile duct and gallbladder added genomic and spatial-transcriptomic evidence for sequential evolution from intraductal or intracholecystic papillary neoplasm to invasive cancer, while also showing that molecular trajectories differed by anatomical site (*53*). Because the cohort combined biliary locations, its gallbladder findings support the plausibility of an ICPN-associated route but should not be generalized to flat BilIN-associated GBC.

Mouse models supply controlled tests of potential drivers. Concurrent Kras and canonical Wnt activation induced premalignant gallbladder or extrahepatic biliary lesions resembling human papillary-tubular precursors and allowed progression under defined genetic conditions (*54*). More recent human pathology studies are attempting to refine high-risk metaplastic states: CLDN18 expression was absent in normal epithelium and TFF2-positive pseudopyloric metaplasia but was detected in intestinal metaplasia, BilIN, ICPN, and a subset of GBC; however, most lesions were cancer-adjacent and the 37-cancer cohort was unable to establish prospective risk (*55*). Single-cell analysis of adenomyomatosis and GBC likewise identified epithelial and macrophage states of interest, and yet adenomyomatosis should not be reclassified as a premalignant condition without longitudinal data on its incidence (*56*) (Table 2).

5. Genomic evolution: Drivers are necessary information but not the whole explanation

Exome sequencing established recurrent disruption of the ErbB signaling pathway in GBC, including ERBB2 and ERBB3 alterations, alongside TP53, KRAS, PIK3CA, and other context-dependent events (*57*). Functional work later linked ERBB2/ERBB3 mutations to PD-

Table 2. Precursor routes and the limits of a single linear sequence

Precursor route	Typical spatial form	Representative molecular features	Evidence substantiating progression	Boundary of inference
Metaplasia → BilIN → invasive adenocarcinoma	Flat, patchy, often within thickened stone-bearing mucosa	TP53/cell-cycle disruption, variable KRAS, adhesion and chromosomal changes (*38,41,48-50*)	Whole-organ mapping, age-stratified pathology, coexisting lesions, multi-region genomics	Cross-sectional sequence is not a patient-level clock; many metaplasias never progress
ICPN-associated carcinoma	Grossly visible polypoid/ papillary-tubular lesion	Lineage-dependent alterations; heterogeneous gastric, intestinal, biliary, oncocytic phenotypes (*39,40,45*)	Large clinicopathological series and associated invasive components	ICPN biology should not be merged with flat BilIN
Conventional adenoma/pyloric gland adenoma	Localized polypoid lesion	CTNNB1 enriched in some adenomas; lineage-specific programs (*39,46*)	Morphology and molecular divergence	Many adenomas may be evolutionary side branches
PBM-associated hyperplasia-dysplasia	Diffuse reflux-associated mucosal hyperplasia	Proliferative and injury-response programs, followed by driver acquisition (*17-20*)	Strong anatomical exposure and characteristic pathology	Specific to reflux anatomy; not a model for all gallbladder cancer
BilIN-independent/de novo route	Invasive focus without clonally related sampled BilIN	Distinct regional mutation profiles (*51*)	Multi-region sequencing of early gallbladder cancer	"Independent" may reflect unsampled or extinct precursors

L1-mediated immune escape, directly connecting an epithelial driver to microenvironmental selection (*58*). An integrative study of 190 patients, including 92 whole exomes and a multi-omic subset, reported a low median mutation burden, TP53 as the most frequently mutated gene, and three transcriptomic survival groups distinguished more strongly by hypoxia, desmoplasia, myeloid suppression, and T-cell dysfunction than by a single driver (*59*).

Large targeted sequencing has reinforced both diversity and actionability. In 244 samples from 233 patients, TP53, CDKN2A, ERBB2, and KRAS were among the common alterations; potentially actionable changes were identified in 35% of those patients, and 18% of patients with metastatic disease received genomically guided off-label or trial therapy (*60*). Smaller targeted studies in high-incidence populations identified overlapping somatic alterations but also highlighted limited cohort size and geographical variation (*61*). Older allelotyping and morphological synthesis had already suggested stepwise chromosomal loss and divergent histogenetic pathways (*62*).

Epigenetic and RNA-regulatory layers provide another possible link between an affected area and phenotype. Epigenome-wide profiling across cholelithiasis, dysplasia, and GBC identified recurring methylation changes along the pathological sequence, but bulk cross-sectional specimens cannot establish when or in which epithelial clone those changes arose (*63*). Functional studies suggested lncRNA-HGBC, through a HuR/miR-502-3p/SET/AKT axis, and circAATF, through AKT/miR-142-5p/PD-L1 signaling, as regulators of proliferation, invasion, or immune signaling (*64,65*). The circAATF study combined paired tissue analyses with humanized mouse models rather than a prospective immunotherapy cohort. Those data justify integrating methylome and non-poly(A) RNA assays with lineage and spatial methods; they do not yet show that noncoding RNA creates an inflamed area or predicts clinical response.

Metabolic selection can also alter both tumor fitness and treatment sensitivity. In paired human specimens, cell lines, and xenografts, fatty-acid synthase promoted GBC progression and reduced gemcitabine sensitivity through PI3K/AKT signaling (*66*). A contemporaneous GBC-focused review catalogued additional resistance mechanisms but emphasized that most remain preclinical (*67*). These findings justify incorporating lipid metabolism into ecosystem models, while avoiding the stronger claim that FASN defines a clinically validated immune-metabolic subtype.

These data argue against using the mutation list as the complete model of carcinogenesis. The same driver can occur in different epithelial lineages, and the same histological lesion can be embedded in different inflamed areas and stroma. Genomics is most informative when paired with location and state: whether an alteration is clonal or subclonal, whether it is present in adjacent BilIN, whether the clone resides near reflux-injured mucosa or an invasive margin, and which immune or stromal cells are present.

6. Single-cell reconstruction of ecosystem remodeling

6.1. From an inflamed gallbladder to an immunosuppressive tumor

Single-cell studies have changed the level of analysis from average gene expression to interacting cell states. A 2022 atlas of 24 fresh gallbladder samples profiled 140,870 cells across chronic cholecystitis, primary GBC, and metastasis. Chronic cholecystitis contained MDSC-like macrophages, tissue-resident CD8 T cells, and CCL2-positive regulatory fibroblasts, whereas GBC contained an abundance of tumor-associated macrophages, regulatory and exhausted T-cells, and STMN1-positive tumor-promoting fibroblasts (*68*). This indicates continuity between an adapted inflamed area and a more suppressive tumor ecosystem but does not prove that each inflammatory cell state is a direct ancestor of its cancer counterpart.

Earlier immunological synthesis had already implicated macrophages, neutrophils, cytotoxic T cells, regulatory T cells, and checkpoint pathways in GBC (*69*). The newer atlases refine that picture by separating cell states and neighborhoods: immune cells are not simply recruited in increasing numbers but are progressively reorganized from injury-associated repair toward myeloid dominance, T-cell dysfunction, and stromal exclusion.

In ErbB-pathway-mutated GBC, single-cell analysis identified an MDK-dependent communication program linking malignant cells to immunosuppressive myeloid populations; the study provided a concrete example of genotype-to-niche coupling (*70*). An early five-patient atlas profiled 24,887 cells from primary tumors, lymph-node metastases, and adjacent normal tissue, identifying altered T-cell, macrophage, and ACKR1-positive endothelial interactions across primary and nodal compartments (*71*). Its mixed histologies and small patient number preclude a general metastatic trajectory, but the study showed why a draining lymph node should not be treated as a duplicate of the primary tumor. Separate work indicated reactivation of human endogenous retroviral elements in selected malignant and immune states, suggesting an additional layer of transcriptional plasticity (*72*).

A larger 2024 study profiled 230,737 cells from 43 specimens spanning 15 GBCs, cholecystitis, polyps, adenomas, and adjacent tissues. It identified distinct stromal ecosystems, including an iCAF-tip endothelial interaction associated with poor outcome, and implicated epithelial OLFM4 in immune evasion through MAPK-AP1-dependent PD-L1 regulation (*73*). Focused T-cell profiling further resolved 12 T-cell states and described

tumors dominated by exhausted CD8 cells, Tregs, or macrophage-rich suppressive patterns (*74*). A 2025 study of 98,113 cells spanning normal tissue, high-grade intraepithelial neoplasia, and GBC extended the temporal axis and emphasized changing intercellular communication during malignant transformation (*75*).

Pan-biliary comparisons highlight the need for anatomical specificity. Single-cell RNA/T-cell receptor sequencing of 36 samples from 16 patients, integrated with 355 pathological slides, showed that T-cell states, tertiary lymphoid structures, and desert phenotypes differed among intrahepatic, extrahepatic, and gallbladder cancers (*76*). These data are a valuable context for GBC but cannot substitute for GBC-only validation. Likewise, integrated immunogenomic analysis of 31 resected biliary tract cancers associated a KRAS mutation with fewer CD8 T cells and antigen-presenting cells and greater tumor-APC-T-cell separation (*77*); whether the same spatial relationship predicts adjuvant outcome specifically in GBC still needs to be verified.

The largest current ecosystem analysis combined single-cell, spatial, and other multi-omic data from 102 patients, encompassing 1,117,245 cells, and described five tumor microenvironmental ecosystems associated with progression. An AREG-EGFR-pERK-EGR1-CXCL5 circuit linked malignant epithelial signaling to myeloid recruitment and adverse ecology (*78*). Its scale strengthens the taxonomy, but the ecosystems still require prospective testing as predictors of treatment response.

6.2. Immune suppression is spatially heterogeneous

Before single-cell profiling, immunohistochemistry had already shown that immune context matters. A spatiotemporal analysis of 139 biliary precursor lesions documented changing immune infiltration across carcinogenesis, although the cohort was not limited to GBC (*79*). In 211 GBCs, lower CD8-cell density and higher BTLA/CD8, Cbl-b/CD8, and FOXP3/CD4 ratios were associated with worse survival (*80*). Classification by CD8 infiltration and PD-L1 expression provided an additional prognostic immune framework (*81*), and a separate series found that high stromal CD3 or CD8 infiltration favored survival mainly in smaller tumors, suggesting stage- and geometry-dependent effects (*82*).

Multiplex immunohistochemistry later demonstrated co-expression of PD-1 and TIM-3 and differences among the tumor core, hepatic invasive margin, and liver metastases (*83*). These observations caution against treating a single core biopsy as a complete immune phenotype. Mechanistic studies also identified stromal-metabolic regulators: nicotinamide N-methyltransferase promoted M2 macrophage polarization through IL-6 and myeloid-derived suppressor-cell conversion through GM-CSF (*84*). The recurrent pattern is not simply "more" or "less" immunity; it is the presence of specific suppressive cell states in specific locations.

Methodological limits must be treated as part of the biological interpretation. Current GBC includes small precursor-mapping and multimodal liver-front cohorts (*51,52,85-87*). Standard 10x Visium capture spots are approximately 55 μm in diameter and usually contain transcripts from multiple cells, so assignment of a 'niche' or ligand-receptor interaction depends on deconvolution, the reference single-cell atlas, histological annotation, and section orientation (*88*). Dissociation can under-represent fragile cell states in scRNA-seq, while a tissue section remains a thin two-dimensional sample of a three-dimensional boundary. RNA quality, archival fixation, treatment exposure, and batch effects further constrain sensitivity and comparability. For retrospective GBC cohorts, probe-based FFPE-compatible single-nucleus RNA sequencing can recover cell-state information from archived blocks, while imaging-based platforms such as Xenium and CosMx can localize targeted transcripts at a single-cell or subcellular resolution (*89,90*). These methods expand access to historical specimens but remain sensitive to block age, RNA fragmentation, panel design, segmentation error, and platform-specific detection bias; they complement rather than replace fresh-tissue whole-transcriptome discovery. Spatial candidates therefore require orthogonal immunohistochemistry or in situ validation, multi-section sampling, multicenter replication, and prospective treatment cohorts (Table 3).

6.3. Immune editing is dynamic across progression and treatment

The transition from a premalignant inflamed area to an early tumor microenvironment is bidirectional, gradual, and temporally overlapping: chronic inflammation can select for epithelial clones, while emerging dysplastic or malignant clones progressively remodel the same area. The available evidence supports a staged, but not strictly linear, model of immune editing. During chronic cholecystitis, innate recruitment and tissue repair may initially contain epithelial injury while simultaneously exposing regenerating cells to cytokines, reactive species, and growth signals. With dysplasia and clonal expansion, epithelial programs such as ERBB-MDK, OLFM4-PD-L1, AREG-CXCL5, and NNMT-dependent myeloid polarization can convert this inflammatory setting into selective immune pressure (*58,70,73,78,84*). During invasion, suppressive macrophages, metabolically adapted neutrophils, dysfunctional CD8 T cells, Tregs, fibroblasts, and abnormal endothelium assemble in different ways at each anatomical boundary. This is better described as immune-state replacement and spatial redistribution than as a uniform transition from "hot" to "cold."

Clinical observations are consistent with this model but do not yet validate its mechanisms. In a single-center retrospective cohort of 80 ICI-treated GBC patients, the objective response rate was 13.75%; high

Table 3. Representative single-cell and spatial studies defining gallbladder cancer ecosystems

Study	Material and scale	Spatial/temporal coverage	Principal ecosystem finding	Key limitation
Wang *et al.*, 2022 (*68*)	24 fresh tissues; 140,870 cells	Cholecystitis, primary gallbladder cancer, metastasis	Transition from adapted inflammatory macrophage/fibroblast states to TAM-, Treg-, exhausted-T-cell-rich tumors	Cross-sectional patients; state continuity is inferred
Zhang *et al.*, 2021 (*70*)	Gallbladder cancer single-cell atlas with ErbB stratification	Tumor and adjacent compartments	MDK-dependent immunosuppressive communication in ErbB-mutated tumors	Genotype-selected context; external validation needed
Chen *et al.*, 2021 (*71*)	Primary tumors, lymph-node metastases, and adjacent normal tissue from 5 patients; 24,887 cells	Primary-to-nodal metastatic compartments	Immunosuppressive T-cell/ macrophage remodeling and ACKR1-positive endothelial interactions	Five patients, mixed histologies, no spatial boundary map
He *et al.*, 2024 (*73*)	43 samples; 230,737 cells plus spatial/histological validation	Normal, cholecystitis, polyps, adenomas, gallbladder cancer	OLFM4-MAPK-AP1-PD-L1 axis; heterogeneous stromal ecosystems	Observational ecosystem-outcome links
He *et al.*, 2025 (*75*)	98,113 cells	Normal, high-grade intraepithelial neoplasia, gallbladder cancer	Transformation-associated cell-state and communication trajectories	Limited number of patients per pathological stage
Zhou *et al.*, 2025 (*78*)	102 patients; 1,117,245 cells with multi-omics	Multiple disease stages and spatial contexts	Five ecosystems; AREG-EGFR-pERK-EGR1-CXCL5 myeloid-recruiting circuit	Predictive utility not prospectively established
Rao *et al.*, 2024 (*85*)	scRNA-seq, spatial transcriptomics, proteomics, mIHC	Tumor core and liver-invasive front	KRT17-positive tumor-fibroblast niche supports OLR1-positive oxLDL-absorbing neutrophils	Functional model requires clinical intervention testing
Hou *et al.*, 2025 (*86*)	Single-cell and experimental validation	Primary tumors and metastatic phenotypes	CD34-positive/CD90-positive endothelial cells acquire mesenchymal features through TGF-β signaling	Preclinical target evidence; clinical prevalence is uncertain
Li *et al.*, 2026 (*87*)	27 scRNA-seq samples from 8 patients (140,213 cells); 40 spatial slides from 4 patients; validation cohort	Tumor-to-liver interface	CXCL9-positive macrophage niches recruit or exhaust CD8 T cells according to position and LGALS4	Small discovery cohort; retrospective response analysis
He *et al.*, 2026 (*91*)	86 treatment-naive Gallbladder cancers profiled with 16-marker multiplex IHC; transcriptomics in 9 cases	Tumor core, margin, peritumor, and nerve-centered regions	Inflamed, excluded, and desert phenotypes; PNI-associated PD-L1-positive myeloid/exhausted-CD8 niche with IL-6/IGF1 signaling	Retrospective treatment associations; transcriptomic subset was small

CD8 infiltration and an inflamed phenotype were associated with disease control and longer survival, but only 31 archival tumors underwent molecular and immunohistochemical analysis, and treatment regimens were not randomized (*91*). Conversely, a 2026 single-cell study of successfully downstaged advanced GBC reported treatment-associated expansion of T follicular helper cells, tertiary lymphoid structures in half of treated tumors, cytotoxic/exhausted CD8 states, and NK cells, with fewer tumor-promoting macrophages and angiogenic fibroblasts (*92*). That analysis selected successful responders and lacked a randomized pretreatment-to-posttreatment design, so it revealed that therapy can remodel the ecosystem but did not indicate which baseline niche caused that response.

7. The invasive margin: Local anatomy becomes an ecological selector

The gallbladder has a thin wall, lacks a conventional submucosa, and lies directly against the liver. Once epithelial clones cross the muscular layer, they encounter vascular, lymphatic, neural, fibrous, and hepatic compartments over very short distances. The invasive margin is therefore not just the outermost part of the tumor; it is a new habitat that selects for motility, matrix remodeling, immune evasion, and metabolic adaptation.

7.1. The surface facing the liver

Multi-modal profiling of liver-invasive GBC identified

enrichment of neutrophils at the front. KRT17-positive tumor cells and surrounding fibroblasts formed a niche that promoted OLR1-dependent uptake of oxidized LDL by neutrophils, reducing cytotoxicity and enhancing invasion-related behavior (*85*). Single-cell analysis also identified a CD34-positive/CD90-positive endothelial population with endothelial-to-mesenchymal features, TGF-β activation, and association with metastasis; inhibition of TGF-β-related signaling attenuated the pro-migratory effect in experimental systems (*86*). The two studies linked local lipid availability and vascular plasticity to invasion, but both are based on preclinical hypotheses.

Pan-biliary experimental work provides an adjacent mechanism linking the vascular state to immune resistance: adrenomedullin disrupted endothelial function and reduced the efficacy of combined immunotherapy and targeted therapy in biliary tract cancer models (*93*). This finding is conceptually compatible with the endothelial states observed in GBC, but it should not be offered as GBC-specific clinical evidence.

The most direct boundary map comes from paired single-cell and spatial transcriptomics of the tumor-liver interface. Twenty-seven single-cell samples from eight patients and 40 spatial slides from four patients were used to identify CXCL9-positive macrophage-rich niches. A liver-adjacent CXCL9-positive/TRAC-positive niche recruited CD8 T cells through CXCL9-CXCR3, whereas a more tumor-proximal CXCL9-positive/C1QB-positive niche was associated with LGALS4-mediated T-cell exhaustion. In a validation cohort, high CXCL9 and low LGALS4 at the boundary correlated with a better outcome and anti-PD-1 response (*87*). The initial and validation cohorts were small, so the marker pair cannot be used for routine selection yet, but that study established a crucial principle: the same chemokine-rich interface can contain spatially adjacent immune recruitment and immune failure.

7.2. Parallel surfaces are non-equivalent anatomically

The serosal/peritoneal surface, lymphovascular channels, cystic-duct mucosa, draining lymph nodes, and perineural spaces impose different physical and cellular constraints. A liver-facing front includes hepatocytes, sinusoidal endothelium, liver-resident immune cells, and a lipid-rich parenchyma; a serosal breach entails entry into a mesothelial and peritoneal compartment. Direct GBC spatial comparisons of these two interfaces in GBC are limited, so mechanisms defined at the edge of the liver should not be generalized to the serosal edge without matched sampling.

Lymphovascular and cystic-duct routes add continuity with mucosa, endothelium, and regional immune tissue. The aforementioned five-patient single-cell atlas that included lymph-node metastases demonstrated compartmental immune and endothelial remodeling but did not indicate the anatomical route from a primary tumor to a node (*71*). A matched primary tumor, invasive lymphatic channel, cystic-duct margin, sentinel or regional node, and distant metastasis therefore need to distinguish between dissemination-associated selection and organ-specific adaptation.

Perineural invasion (PNI) represents another specialized aspect. Spatial profiling of 86 treatment-naive GBCs identified inflamed, immune-excluded, and immune-desert phenotypes and a nerve-centered niche rich in PD-L1-positive myeloid cells, exhausted CD8 T cells, and IL-6/IGF1-related signaling with lipid-metabolic rewiring (*94*). Retrospective identification of an association with survival and treatment suggested clinical relevance, but causal therapeutic benefit cannot be inferred from non-randomized comparisons. Together, the liver, serosal, lymphovascular/nodal, and neural interfaces show that invasion should be sampled by boundary type, not collapsed into a generic "tumor margin."

Mechanistic evidence published in 2026 further showed that GBC-derived extracellular vesicles can transfer O-GlcNAcase to neurons, induce neuronal necroptosis, and facilitate PNI in experimental systems (*95*). This tumor-neuron mechanism complements, rather than proves, the spatially observed immune-evasion niche: neuronal injury and immune exclusion were measured in different studies and require joint validation in the same specimens (Figure 3).

8. An integrated three-stage model

The evidence supports a three-stage model with branching rather than obligatory progression.

First, a susceptible inflamed area is created by prolonged injury. Gallstones, PBM-related reflux, chronic Salmonella carriage, metabolic bile changes, and host susceptibility contribute to this, with different effect sizes and in different populations. The area is characterized by repeated epithelial death and repair, altered bile chemistry, stromal activation, and immune adaptation. This stage can persist for decades without cancer developing.

Second, epithelial lineages diversify. Metaplastic cells, BilIN, ICPN, pyloric gland adenoma, and apparently de novo clones acquire different combinations of driver, chromosomal, epigenetic, and lineage alterations. Selection occurs within a mosaic mucosa, so the most atypical lesion sampled histologically is not necessarily the ancestor of the invasive tumor. Multi-region sequencing and spatial transcriptomics are required to distinguish shared ancestry from coexistence.

Third, invasive competence is selected for at boundaries. Malignant epithelial states interact with fibroblasts, macrophages, neutrophils, endothelial cells, T cells, hepatocytes, and nerves. The local ecology may simultaneously recruit immune cells and disable them. This stage explains why molecular profiling of the tumor

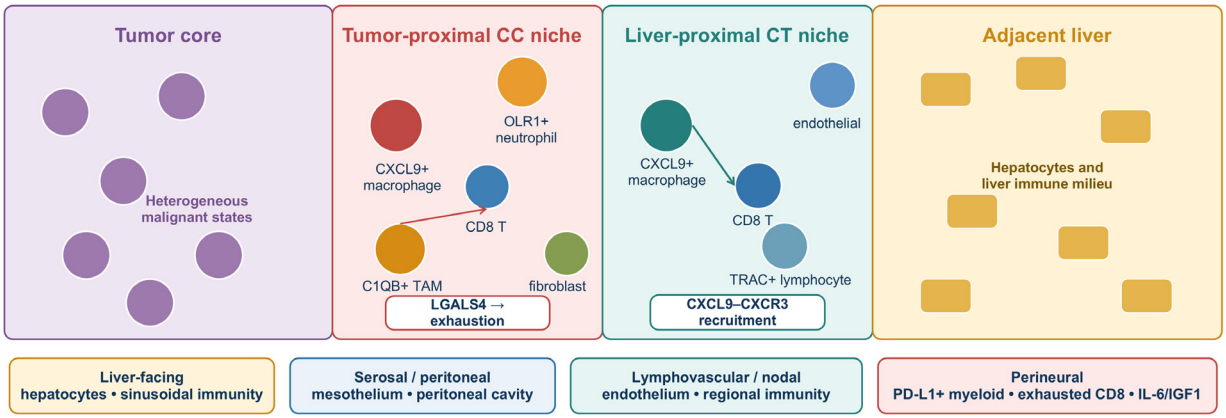


Figure 3. Anatomically distinct invasive margins in gallbladder cancer. Cancer cell (CC) niche denotes the CXCL9-positive/C1QB-positive macrophage-rich compartment, and connective tissue (CT) niche denotes the CXCL9-positive/TRAC-positive T-cell-recruitment compartment. At the best-mapped liver interface, a tumor-proximal CXCL9-positive/C1QB-positive macrophage niche is associated with LGALS4-mediated T-cell exhaustion, whereas a more liver-proximal CXCL9-positive/TRAC-positive niche encourages CXCL9-CXCR3-dependent CD8 T-cell recruitment. OLR1-positive neutrophils, fibroblasts, and specialized endothelial cells provide additional invasion-associated states. Serosal/peritoneal, lymphovascular/nodal, and perineural routes impose different anatomical constraints and require matched sampling; they should not be inferred from the edge of the liver. This diagram is a synthesis, not a diagnostic assay. *Abbreviations:* CC, cancer cell; CT, connective tissue; TAM, tumor-associated macrophage; CD8, cluster of differentiation 8; C1QB, complement C1q B chain; CXCL9, C-X-C motif chemokine ligand 9; CXCR3, C-X-C motif chemokine receptor 3; OLR1, oxidized LDL receptor 1; TRAC, T-cell receptor alpha constant; LGALS4, galectin-4; PD-L1, programmed death ligand-1; IL-6, interleukin-6; IGF1, insulin-like growth factor 1.

core may fail to predict behavior at the liver-facing edge and why spatially resolved biomarkers may outperform whole-tumor averages.

The stages overlap. A tumor can continue to reshape bile and microbiota; an invasive niche can feed back on core epithelial states; and treatment can remodel every compartment. The model is therefore a set of testable relationships, not a fixed chronology for every patient.

9. Clinical and research implications

9.1. Prevention and early detection should be risk-structured

The model does not justify prophylactic cholecystectomy for all asymptomatic gallstones. Absolute cancer risk remains low for most carriers, and surgery has its own harms. It does support more precise risk stratification regarding the duration and phenotype of stones, PBM, suspicious wall thickening or polyps, geography, infection history, and host factors. When dysplasia is found, extensive histological sampling is essential because the area is patchy and occult invasion may be focal.

Bile multi-omics and microbial assays are attractive because they sample the organ habitat, but current signatures require prospective validation with standardized pre-analytic variables, antibiotic and drainage metadata, negative controls, and benign controls matched for obstruction and inflammation. A clinically useful assay must distinguish cancer-associated signals from the consequences of cholestasis and instrumentation.

Imaging can identify where the area has become morphologically abnormal, although it cannot depict an immune niche. In a 2025 retrospective study of 151 patients with gallbladder lesions, contrast-enhanced ultrasound and contrast-enhanced computed tomography had broadly comparable diagnostic performance, with important limitations due to unpaired testing and small subgroups (*96*). Such data support the multimodal assessment of suspicious wall or polypoid lesions but do not support population screening or assignment of a histological lineage.

9.2. Use of biomarkers must bear compartments in mind

Routine molecular profiling identifies actionable alterations in a subset of advanced GBC, but tissue location should be recorded. A core biopsy, primary resection, invasive margin of the liver, and metastasis may contain different immune and stromal states even when driver alterations overlap. Future trials should predefine where tissue is obtained and should not validate a spatial marker in whole-tumor homogenates alone.

Incidental GBC creates a distinct sampling problem. Approximately half of GBCs may be detected during or after cholecystectomy in some contemporary series, although the proportion varies by geography and referral pattern (*97*). Once the index operation has removed the intact organ, the untreated invasive margin is no longer prospectively accessible. Spatial analysis should therefore remap the archived cholecystectomy specimen—hepatic-side wall, cystic-duct margin, serosal breach, lymphovascular and perineural foci—and, when completion radical re-resection is performed, pair these findings with the gallbladder bed, regional nodes, and any residual disease. Such maps should complement,

not replace, pathological stage, margin status, cross-sectional imaging, and the assessment of resectability. In unresectable GBC, image-guided biopsy protocols should record whether tissue represents the tumor core, liver-facing edge, nodal disease, or distant metastasis and, when safe, obtain multiple cores for orthogonal histological and molecular assays.

Trials of systemic treatment in biliary tract cancer provide proof that immune modulation can improve outcomes, but they do not validate the proposed GBC niches. Gemcitabine-cisplatin established a systemic-therapy backbone in advanced biliary tract cancer (*98*). Adding durvalumab improved overall survival in TOPAZ-1, with a hazard ratio of 0.80 and 24-month survival of 24.9% versus 10.4% (*99*). KEYNOTE-966 similarly indicated a median overall survival of 12.7 versus 10.9 months with pembrolizumab plus gemcitabine-cisplatin and a hazard ratio of 0.83 (*100*). Those trials enrolled mixed biliary tract cancers; GBC-specific, spatially stratified predictive analyses need to be performed before CXCL9, LGALS4, OLFM4, MDK, OLR1, or neural-interface signatures guide therapy.

Updated TOPAZ-1 follow-up confirmed a durable overall-survival benefit without a new safety signal (*101*), and a 2024 network meta-analysis of seven phase III first-line trials (4,033 patients) indicated that durvalumab plus gemcitabine-cisplatin ranked high in progression-free and overall survival (*102*). These analyses strengthen the pan-biliary standard but do not deal with the smaller and statistically uncertain GBC subgroup effects. In a separate phase II pembrolizumab study of 32 metastatic biliary tract cancers, integrated tissue and blood profiling sought to identify response biomarkers in microsatellite-stable disease (*103*); sample size and mixed anatomy again limited any GBC-specific inferences. The orphan disease perspective remains relevant because a low incidence constrains dedicated trials and drug development (*104*), but rarity should encourage the assembly of coordinated GBC cohorts rather than justifying uncritical extrapolation from cholangiocarcinoma.

Spatial findings offer a plausible mechanistic explanation for heterogeneous checkpoint-inhibitor benefit but not a validated resistance test. At the edge of the liver, a CXCL9-rich liver-proximal niche can recruit CD8 T cells, whereas the adjacent tumor-proximal CXCL9-positive/C1QB-positive macrophage niche and LGALS4-positive malignant cells are associated with T-cell exhaustion; high CXCL9 with low LGALS4 correlated with anti-PD-1 response only in a small retrospective cohort (*87*). PNI-associated excluded or desert regions likewise place exhausted T cells beside PD-L1-positive myeloid cells (*94*). These observations suggest that the tumor core and an invasive edge may contain opposing immune programs that are averaged out in bulk assays. However, TOPAZ-1 and KEYNOTE-966 neither selected patients by these niches nor prospectively sampled boundary-specific tissue, so the spatial states cannot yet be called mechanisms of clinical resistance.

9.3. A practical research agenda

Four study designs would materially strengthen this field. First, longitudinal cohorts should enroll patients with PBM, selected high-risk gallstones, or suspicious mucosal lesions before cancer, banking serial plasma, bile when clinically indicated, imaging, and eventual whole-organ pathology. Second, bile and mucosa should be sampled as paired but non-equivalent compartments, with absolute microbial load and rigorous contamination controls. Third, precursor studies should combine laser microdissection, multi-region DNA sequencing, single-cell or single-nucleus profiling, and spatial transcriptomics to reconstruct lineage rather than infer it from morphology alone. Fourth, invasive margin studies should standardize orientation and collect a matched tumor core, gallbladder-facing margin, liver-facing margin, serosal/peritoneal breach, lymphovascular channel, cystic-duct margin, PNI, draining lymph node, distant liver, and metastasis sample. For retrospective cohorts, pathology-guided serial sections should pair FFPE-compatible single-nucleus profiling with targeted imaging-based spatial transcriptomics, while fresh tissue should be reserved for broader examination whenever feasible. Clinical endpoints and treatment exposure must be prospectively defined.

The decisive validation step is intervention. A niche marker becomes clinically meaningful only if it reproducibly predicts an outcome or treatment response in an independent cohort, and a proposed driver becomes actionable only if perturbing it changes disease behavior without unacceptable toxicity. Until then, spatial and microbial signals should be presented as mechanistic or prognostic candidates, not as established clinical tests.

10. Conclusions

Gallbladder carcinogenesis is not adequately explained by a single mutation, a single organism, or a single histological staircase. The strongest evidence supports a layered process in which stones or reflux create an area of chronic injury; bile chemistry, selected microorganisms, host susceptibility, and immune-stromal adaptation modify that area; epithelial precursors follow branching lineages; and invasive clones are selected for within anatomically distinct regions. Single-cell and spatial studies have made this framework measurable by depicting cell states and their neighborhoods, but the cohorts in which they were found remain small relative to the biological and geographical heterogeneity of GBC.

The immediate opportunity is methodological rather than speculative: sample the correct compartments, preserve orientation, distinguish association from

causation, and validate proposed biomarkers prospectively. If those standards are met, the transition from an inflamed area to an invasive margin may become not only a conceptual account of GBC but also a practical map for prevention, early detection, and context-specific therapy.

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Original Article

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Multidimensional social capital and latent anxiety severity profiles among Chinese medical graduate students: A multicenter cross-sectional study

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SUMMARY: Mental health issues, such as anxiety, are highly prevalent among medical graduate students. However, the potential relationship between social capital and anxiety symptoms in medical graduate students has not yet been investigated. Therefore, data were collected in 2024 from a multicenter sample of 2,587 medical graduate students obtained through multistage stratified sampling across northern, central, and southern Anhui Province. Anxiety symptoms were assessed using the 20-item Self-Rating Anxiety Scale (SAS-20). Social capital was evaluated across six dimensions: social participation, social support, social connection, trust, sense of belonging, and reciprocity. Latent profile analysis (LPA) was used to identify latent anxiety severity profiles. Multinomial logistic regression was used to examine associations between social capital dimensions and anxiety severity-profile membership. The prevalence of anxiety symptoms among participants was 30.5%. Three latent anxiety severity profiles were identified: low (50.6%), moderate (18.6%), and high (30.8%). Lower levels of social support, social connection, and trust were associated with greater odds of membership in the moderate- and high-anxiety severity profiles. A higher sense of belonging was associated with lower odds of membership in the high-anxiety profile, whereas higher social participation was associated with greater odds of membership in the high-anxiety profile. Compared with the moderate-anxiety profile, membership in the high-anxiety profile was also associated with higher social participation and lower trust. Cognitive social capital, particularly trust and supportive connections, was consistently associated with lower anxiety severity, whereas structural participation showed a context-dependent association. These findings suggest that the quality and context of social relationships may warrant as much attention as the quantity of participation.

Keywords: medical graduate students, anxiety symptoms, social capital, latent profile analysis

1. Introduction

The global expansion of higher education has positioned medical students as an increasingly vital component of the healthcare workforce. Statistics show that the number of medical graduates in OECD countries rose from 93,000 in 2000 to 114,000 in 2010, reaching 160,000 by 2021 (*1*). China's postgraduate medical education system comprises master's and doctoral tiers, featuring diversified training modalities such as the "5+3" integrated clinical medicine professional degree program, research-focused master's programs, and eight-year combined bachelor-master-doctoral pathways (*2*). Based on degree type, medical postgraduates are categorized into academic (PAD) and professional (PPD) tracks, with the former research-oriented and the latter focused on clinical practice skills (*2,3*). Interestingly, while the need for skilled clinical practitioners in China's healthcare system is increasing, fewer medical postgraduates have been pursuing clinical careers recently (*4,5*). Several investigations have highlighted that medical graduate students often encounter difficulties like extended academic courses, research stress (*3,6,7*) and significant financial and interpersonal burdens (*8*). Collectively,

these pressures may compromise career commitment to clinical medicine and precipitate adverse mental health outcomes.

Anxiety, a prevalent global mental disorder, notably affects medical students in this scenario. The World Health Organization states that around 4% of people worldwide experience anxiety symptoms. Research shows that medical students experience significantly greater mental health challenges than their non-medical counterparts, with anxiety disorders being the most notable difference (*9-11*). A study revealed that medical students scored 7.70 on anxiety symptoms, compared to 5.51 for workers of the same age (*11*). A meta-analysis covering 40,348 medical students showed that the prevalence of anxiety symptoms among medical students worldwide ranged from 29.2% to 38.7% (*12*).

Cross-cultural studies consistently report elevated anxiety symptom prevalence among medical students, with significant international variations. For instance, the anxiety symptom scores of medical students in Pakistani institutions were 19.2 ± 11.2, with 74% meeting moderate-to-severe anxiety criteria (*13*). A US cohort study revealed 20.3% of medical trainees met diagnostic thresholds for generalized anxiety disorder, with nearly one-third reporting clinically significant academic impairment attributable to anxiety symptoms (*14*). Research in China indicates that 33.8% of medical graduate students experience depression symptoms, while 22.2% suffer from anxiety symptoms. At the same time, more than half of the respondents experienced burnout, and 58.4% of the respondents considered leaving school (*4*).

There has been a growing interest in studies examining the influence of social capital on mental health. Social capital encompasses the resources and assistance people gain from their social connections, and it has multiple dimensions. There are two elements to social capital: structural and cognitive (*15,16*). The former comprises objective measures: social networks and participation. The latter encompasses trust in other community members, reciprocal norms, and perceived social support (*17,18*). Earlier research has investigated how social capital relates to anxiety symptoms across various groups, employing diverse methods to assess social capital and yielding varied outcomes. Research conducted on rural women in Kenya revealed that enhancing social capital (trust, cohesion) helped reduce anxiety levels in adult women (*19*). In Australia, a randomized controlled trial demonstrated that enhanced social capital—particularly interactions with friends—significantly alleviated anxiety symptoms and loneliness symptoms among elderly nursing home residents (*20*). A Japanese study examined associations between multidimensional social capital components—including fairness, trust, reciprocity, social participation, and support – and anxiety symptoms among disaster survivors. The results showed that fairness, trust, and reciprocity can serve as protective factors against anxiety symptoms, while social participation may exacerbate anxiety symptoms, and social support's impact is minimal (*21*). Interestingly, research conducted on female domestic workers in China showed that anxiety levels were not significantly related to either cognitive social capital (trust, social harmony, perceived fairness, and sense of belonging) or structural social capital (membership, support, and activity) (*22*). Another study carried out in China discovered that medical students with higher levels of social capital, including social participation, support, connections, trust, cohesion, and reciprocity, experienced significantly fewer symptoms of anxiety and depression, and this relationship was stronger in medical students compared to non-medical fields (*23*).

Although existing research has extensively examined the prevalence of anxiety symptoms among student populations, there remain significant limitations in the exploration of the underlying mechanisms. As one of the core social determinants of mental health, the role of social capital in anxiety symptoms has been preliminarily validated; however, most studies examine social capital as a single, aggregate indicator, overlooking the fundamental differences in the mechanisms of action between structural social capital (*e.g.*, social participation, social ties) and cognitive social capital (*e.g.*, trust, sense of belonging, reciprocity). The practice of simply summing up total scores tends to obscure the heterogeneous effects across different dimensions and may even underestimate the true impact due to opposing effects that cancel each other out. This is particularly true for medical graduate students, a unique population who hold dual identities as both research scholars and future clinical practitioners. They are constantly under multiple pressures—including research evaluations, clinical rotations, and professional development—and their social interactions are highly dependent on academic and professional contexts. Social participation often blends voluntary socializing, academic tasks, and obligatory work, which means that the impact of social participation on mental health may present a "double-edged sword"—it may alleviate anxiety through social support, but it may also exacerbate emotional burdens due to additional role demands and performance pressures. This unique aspect has not yet received sufficient empirical attention. According to the stress buffering model, social support, trust, and social connections can buffer academic, clinical, and occupational stress. At the same time, resource conservation theory suggests that social capital can provide emotional, informational, and instrumental resources, but participation itself may also consume time, energy, and identity resources. Based on the above, this study proposes the following research hypotheses: H1 Higher levels of cognitive social capital, particularly trust, perceived support, social connection, and belonging, are associated with lower anxiety severity. H2

Structural social capital, particularly social participation, may show associations with anxiety that differ in direction from those of cognitive social capital. We further explored whether the associations of individual social-capital dimensions with anxiety severity differed in direction and magnitude.

Furthermore, previous studies have largely used continuous scores or binary thresholds based on general norms to characterize anxiety levels, making it difficult to accurately identify the naturally occurring stratification of anxiety severity within the target population. LPA provided a data-driven complementary approach to characterize severity strata without imposing prespecified clinical thresholds. Therefore, LPA was used in this study to stratify the severity of anxiety symptoms among medical graduate students.

Overall, previous studies have largely treated social capital as a single, holistic beneficial resource, generally focusing on its protective associations with mental health while paying less attention to its potential adverse or context-dependent associations. Second, existing studies mostly use the total social capital score as the core indicator, failing to sufficiently distinguish between structural and cognitive dimensions and thereby potentially obscuring heterogeneous associations across different dimensions. Third, few studies have stratified participants by anxiety severity to examine whether different dimensions of social capital are associated with anxiety in different directions across severity levels. This study therefore aimed to identify latent anxiety severity profiles among medical graduate students and to examine how different dimensions of social capital are associated with membership in these profiles. The findings may inform the development of more targeted psychological support strategies in medical educational institutions to address anxiety symptoms.

2. Materials and Methods

2.1. Research design and data collection

This study was conducted in Anhui Province from October to December 2024, using a multistage stratified random sampling method to select study participants. Anhui Province was divided into three regions—southern, central, and northern—based on geographic location, and the sampling was carried out in three stages:

Stage 1 involved city sampling: simple random sampling was used in each of the three regions—southern, central, and northern—to select one city representative of medical education in that region. Ultimately, Wuhu (south), Hefei (central), and Bengbu (north) were selected as the survey cities, ensuring both randomness and geographic coverage in city selection.

Stage Two involved sampling survey institutions: Within each selected city, the sampling frame consisted of medical universities and their directly affiliated hospitals offering postgraduate medical programs. Representative institutions were randomly selected as survey sites based on the scale of postgraduate training, hospital grade, and disciplinary composition. All selected institutions agreed to participate in this study, and a total of five university-affiliated hospitals were ultimately included in the survey.

The third stage involved sampling the study participants: Using the Probability Proportional to Size (PPS) sampling principle, questionnaire distribution quotas were allocated proportionally to each institution based on the total number of enrolled medical graduate students at each survey site. A total of 2,599 questionnaires were distributed. Due to differences in the scale of graduate student training across institutions, there were some variations in sample size distribution among the institutions. These variations corresponded to the actual training scales of each institution, were consistent with the logic of the sampling design, and did not compromise the overall representativeness of the sample.

The Ethics Committee of the First Affiliated Hospital of Anhui Medical University approved the ethics application for this study (PJ 2025-03-68). Study participants were required to possess normal language and cognitive abilities, be at least 18 years of age, and voluntarily consent to participate. The survey was conducted *via* an online questionnaire platform, ensuring the anonymity and confidentiality of data collection throughout the process. Prior to the formal survey, all participants signed a written informed consent form. A total of 2,599 questionnaires were collected for this survey. After excluding invalid questionnaires with incomplete responses or logical inconsistencies, 2,587 valid questionnaires were ultimately obtained, resulting in an individual-level response rate of 99.5%. The sample size is adequate based on the prevalence rates among graduate students reported in previous studies (Figure 1).

2.2. Measurement

2.2.1. Measurement of Anxiety symptoms

The Zung Self-Rating Anxiety Scale (SAS) (*24*) was used to evaluate anxiety symptoms in medical graduate students. This scale is widely used for screening anxiety symptoms rather than for clinical diagnosis. Every item is evaluated on a 4-point Likert scale, with scores from 1 (never or rarely) to 4 (most of the time). Anxiety levels are categorized as none (25–49 points), mild (50–59 points), moderate (60–69 points), and severe (70–100 points). In the study sample, the SAS scale showed good internal consistency, indicated by a Cronbach's α of 0.935.

2.2.2. Measurement of social capital

Referring to previous studies on social capital

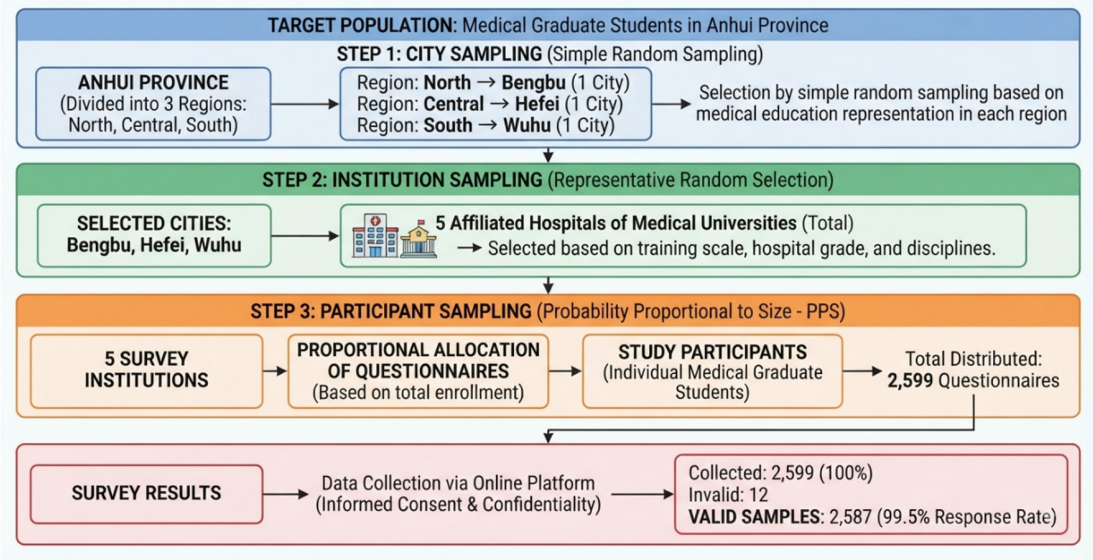


Figure 1. Data acquisition flowchart.

measurement methods (*25-27*), this study selected and adapted some items based on the characteristics of medical graduate students to form a set of social capital measurement tools suitable for medical graduate students. The scale includes six aspects: social participation, social support, social connection, trust, sense of belonging, and reciprocity, with a total of 24 items. Each item is evaluated using a five-point Likert scale, with choices ranging from 1 for "never" to 5 for "very often". Elevated scores reflect increased levels of social capital in the dimension or overall. We used the median method to dichotomize scores for each dimension: social participation (high ≥ 8), social support (high ≥ 12), social connection (high ≥ 12), trust (high ≥ 21), sense of belonging (high ≥ 19), and reciprocity (high ≥ 12). The statistical analysis revealed that the scale's overall Cronbach's α was 0.936, suggesting high reliability of the tool. Discriminant validity among the six dimensions was evaluated using the heterotrait–monotrait ratio (HTMT); all values ranged from 0.492 to 0.728 and were below the prespecified threshold of 0.85. The multicollinearity analysis did not reveal any multicollinearity (Supplementary Table S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*).

2.2.3. Measurement of Other Variables

The variables also encompass age (21–25, 26–30, and ≥ 31), residence (urban, rural), gender (male, female), degree (master's students, doctoral students), grade (1, 2, 3), income level (< 15,000, 15,000-18,000, > 18,000), frequency of communication with family and tutors (once every six months, once a month, once a week and more), exercise (never, 1-2 times per week, 3 or more times per week), smoking and drinking (never, former, current), chronic diseases (yes, no), *etc*.

2.3. Statistical analysis

The sample's general characteristics were analyzed using descriptive statistics. Mean ± standard deviation (x ± s) was used to express continuous variables, whereas categorical variables were shown as frequency and percentage. Chi-square tests (χ^2) were employed to examine variations in social capital scores and demographic variables across different anxiety levels, and ANOVA was used to determine if social capital varied across different dimensions among various anxiety levels. SAS was utilized as observed variables for latent profile analysis using Mplus version 8.3. The selection of the optimal model was guided by several indicators. The Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and sample size-adjusted BIC (aBIC) were observed to decrease progressively with an increasing number of categories, indicating that lower values correspond to better model fit. The Lo-Mendell-Rubin likelihood ratio test (LMR) and the Bootstrapped Likelihood Ratio Test (BLRT) yielded p-values of less than 0.05, suggesting that a model with k categories is more appropriate than one with k-1 categories. Entropy was used as an accuracy indicator for evaluating category classification, with values of ≥ 0.8 indicating classification accuracy exceeding 90%. Beyond these statistical indicators, practical significance and interpretability were also taken into consideration. Covariates were related to latent class membership using the three-step procedure, which accounts for uncertainty in class assignment. A large number of random starting values were used to reduce the likelihood of local maxima, and the best log-likelihood value was replicated. Based on the above, we constructed a multinomial

logistic regression model to investigate the link between different elements of social capital and varying degrees of anxiety symptoms.

To test the robustness of the results, a two-level random-intercept model was used as an exploratory sensitivity analysis to account for clustering of participants within institutions. The analysis employed a multilevel data structure comprising 2,587 individuals (Level 1) nested within 5 schools (Level 2). Multilevel linear regression analysis was performed using continuous SAS scores as the dependent variable. The model was estimated *via* restricted maximum likelihood (REML). The intraclass correlation coefficient (ICC) was calculated as 0.04, meaning that only a minor proportion of the total variance in anxiety severity was explained by between-institution differences. $P < 0.05$ is regarded as statistically significant. We utilized IBM SPSS Statistics 27.0 and Mplus 8.3 for our data analysis.

3. Results

3.1. Demographic characteristics

The demographic details of the study, sorted by gender, are outlined in Table 1 ($N = 2,587$), comprising 1,138 males (44%) and 1,449 females (56%). Most of the respondents were aged between 21 and 30 (92%). The proportion of female students from rural areas (59.6%) was slightly higher than that of male students (56.6%). In terms of degree and grade, the proportion of female master's students was slightly higher (88.2%) than that of male students (87.0%), and about half of the population was concentrated in the first grade, accounting for 52.5% and 46.0% of males and females, respectively. The majority, 59.5%, of individuals have an income that is under 15,000 yuan. Females are more likely to communicate with their families at least once a week (87.5% *vs*. 80.8%), while males are more likely to communicate with their tutors at least once a week (69.3% *vs*. 67.6%). In terms of exercise, about a quarter (23.4%) of male students exercise 3 or more times per week, while only 10.7% of female students do so. The proportion of female students who never smoke (99.3%) and never drink (93.7%) is significantly higher than that of male students, at 89.3% and 64.9% respectively. Only 5.5% of participants suffer from chronic diseases. Anxiety symptoms are present in 30.5% of medical graduate students, affecting 32.0% of males and 29.3% of females.

3.2. Latent profile analyses

This study used LPA to identify latent anxiety severity classes among medical graduate students. Based on statistical fit indices, including AIC, BIC, aBIC, entropy, LMR, and BLRT, models with 1 to 6 features were evaluated, as shown in Supplementary Table S2 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*). The latent profile model of anxiety symptoms is depicted in Figure 2, illustrating the structure and class probabilities of the three identified subgroups. The results showed that the scores for items across the three latent categories exhibited an overall parallel upward trend; the ranking of scores among items was consistent with no obvious overlap.

Notably, the six-profile model's AIC, BIC, and aBIC values gradually declined, with the four-profile model attaining the peak Entropy value of 0.983. However, the LMR p-values for the models with four and five profiles exceeded 0.05, and the six-profile model had too few participants in certain profile proportions, so these three were excluded. Despite the 3-profile model having a slightly lower Entropy than the 2-profile model, it still maintained a high value of 0.954, suggesting that the three-category division was distinct. In addition, the values for AIC, BIC, and aBIC were significantly reduced relative to the 2-profile model, indicating better model fit. Additionally, there are no extremely small categories, and the 51%, 18%, and 31% proportions are very acceptable structures in this study. Therefore, the 3-profile model was ultimately selected. Supplementary Table S3 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*) shows that each respondent attributed high probabilities to their most likely trait (0.986 for Profile 1, 0.940 for Profile 2, and 0.991 for Profile 3), which demonstrates the reliability of the categorization.

3.3. Inter-profile characteristic differences

The research revealed that 50.6% of medical graduate students had low anxiety symptoms, 18.6% had moderate symptoms, and 30.8% had high symptoms. As shown in the chi-square test in Supplementary Table S4 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*), age, residence, gender, Degree, grade, income level, frequency of communication with family and tutors, exercise, smoking and drinking, chronic diseases, and various aspects of social capital were all significantly correlated with different levels of anxiety symptoms, $P < 0.05$.

The box plots in Figure 3 illustrate the score distributions across the various dimensions of social capital for different anxiety symptoms level groups, visually showing that the low anxiety symptoms group generally scored higher across all dimensions of social capital compared to the moderate or high anxiety symptoms groups. Analysis of variance with social capital as the dependent variable revealed statistically significant differences between low, moderate, and high anxiety levels across all dimensions of social capital, with $P < 0.001$. This shows a strong link between latent anxiety severity classes and every aspect of social capital, see Supplementary Table S5 (*https://www.*

Table 1. Basic characteristics of the study population

Variables	Male [*N* (%)]	Female [*N* (%)]	Total [*N* (%)]
Age(years)			
21-25	475 (41.7)	645 (44.5)	1120 (43.3)
26-30	554 (48.7)	707 (48.8)	1261 (48.7)
≥ 31	109 (9.6)	97 (6.7)	206 (8.0)
Residence			
Urban	494 (43.4)	585 (40.4)	1079 (41.7)
Rural	644 (56.6)	864 (59.6)	1508 (58.3)
Degree			
Master's students	990 (87.0)	1278 (88.2)	2268 (87.7)
Doctoral students	148 (13.0)	171 (11.8)	319 (12.3)
Grade			
1	598 (52.5)	667 (46.0)	1265 (48.9)
2	271 (23.8)	423 (29.2)	694 (26.8)
3	269 (23.6)	359 (24.8)	628 (24.3)
Income level (RMB)			
<15,000	681 (59.8)	858 (59.2)	1539 (59.5)
15,000-18,000	245 (21.5)	323 (22.3)	568 (22.0)
>18,000	212 (18.6)	268 (18.5)	480 (18.6)
Frequency of communication with family			
Once every six months	43 (3.8)	18 (1.2)	61 (2.4)
Once a month	176 (15.5)	163 (11.2)	339 (13.1)
Once a week and more	919 (80.8)	1268 (87.5)	2187 (84.5)
Frequency of communication with tutors			
Once every six months	37 (3.3)	36 (2.5)	73 (2.8)
Once a month	312 (27.4)	433 (29.9)	745 (28.8)
Once a week and more	789 (69.3)	980 (67.6)	1769 (68.4)
Exercise			
Never	228 (20.0)	440 (30.4)	668 (25.8)
1-2 times per week	644 (56.6)	854 (58.9)	1498 (57.9)
3 or more times per week	266 (23.4)	155 (10.7)	421 (16.3)
Smoking			
Never	1016 (89.3)	1439 (99.3)	2455 (94.9)
Former	39 (3.4)	2 (0.1)	41 (1.6)
Current	83 (7.3)	8 (0.6)	91 (3.5)
Drinking			
Never	738 (64.9)	1358 (93.7)	2096 (81.0)
Former	108 (9.5)	23 (1.6)	131 (5.1)
Current	292 (25.7)	68 (4.7)	360 (13.9)
Chronic disease			
No	1068 (93.8)	1376 (95.0)	2444 (94.5)
Yes	70 (6.2)	73 (5.0)	143 (5.5)
Social participation			
Low	460 (40.4)	553 (38.2)	1013 (39.2)
High	678 (59.6)	896 (61.8)	1574 (60.8)
Social support			
Low	332 (29.2)	375 (25.9)	707 (27.3)
High	806 (70.8)	1074 (74.1)	1880 (72.7)
Social connection			
Low	435 (38.2)	540 (37.3)	975 (37.7)
High	703 (61.8)	909 (62.7)	1612 (62.3)
Trust			
Low	485 (42.6)	651 (44.9)	1136 (43.9)
High	653 (57.4)	798 (55.1)	1451 (56.1)
Sense of belonging			
Low	485 (42.6)	651 (44.9)	1136 (43.9)
High	653 (57.4)	798 (55.1)	1451 (56.1)
Reciprocity			
Low	484 (42.5)	621 (42.9)	1105 (42.7)
High	654 (57.5)	828 (57.1)	1482 (57.3)
Anxiety symptoms			
No	774 (68.0)	1025 (70.7)	1799 (69.5)
Yes	364 (32.0)	424 (29.3)	788 (30.5)

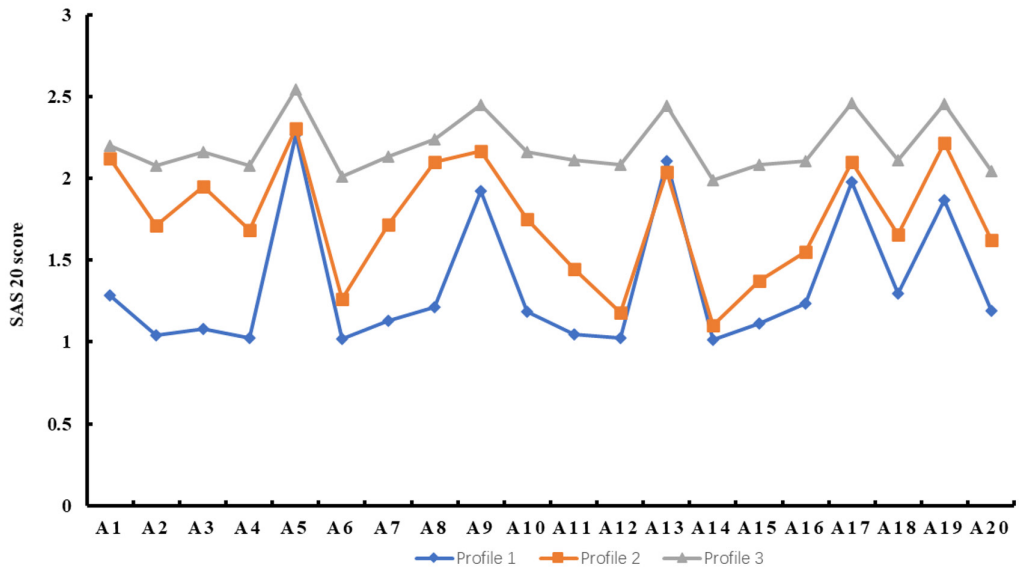


Figure 2. Latent profile model of anxiety symptoms.

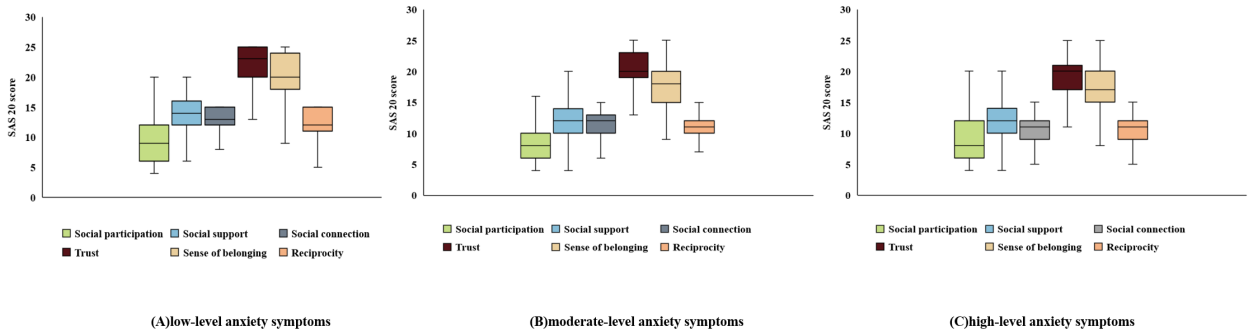


Figure 3. The average social capital score distribution comparison of different categories of anxiety symptoms.

biosciencetrends.com/action/getSupplementalData.php?ID=318).

3.4. Analysis of latent anxiety severity profiles using multinomial logistic regression

Table 2 shows the factors associated with latent anxiety severity-profile membership after adjustment for relevant covariates. Using the low-anxiety profile as the reference, high social support (OR = 0.720; 95% CI: 0.545-0.951), high social connection (OR = 0.643; 95% CI: 0.487-0.848), and high trust (OR = 0.561; 95% CI: 0.432-0.729) were associated with lower odds of membership in the moderate-anxiety profile. For the high-anxiety profile versus the low-anxiety profile, high social participation was associated with greater odds of membership (OR = 1.302; 95% CI: 1.040-1.629), whereas high social support (OR = 0.715; 95% CI: 0.557-0.919), high social connection (OR = 0.506; 95% CI: 0.394-0.650), and high trust (OR = 0.324; 95% CI: 0.257-0.409) were associated with lower odds. When the moderate-anxiety profile was used as the reference, high social participation was associated with greater odds of membership in the high-anxiety profile (OR = 1.340; 95% CI: 1.042-1.725), whereas high trust was associated with lower odds (OR = 0.627; 95% CI: 0.476-0.825). Reciprocity was not significantly associated with profile membership.

In the sensitivity analysis (Supplementary Table S6, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*), this study conducted a multiple linear regression using the SAS total score as a continuous outcome. After adjusting for relevant factors, the results showed the same direction of effect as the main analysis. Specifically, it was once again confirmed that higher levels of social participation were associated with higher SAS scores (B = 0.044, 95% CI: 0.008, 0.080). Compared with the reference group, medical graduate students' social support (B = −0.070, 95% CI: −0.108, −0.033), social connection (B = −0.124, 95% CI: −0.167, −0.082), trust (B = −0.246, 95% CI: −0.290, −0.209), and sense of belonging (B = −0.091, 95% CI: −0.135, −0.047) were associated with lower SAS scores. No statistically significant correlation was found between reciprocity and anxiety level. In the multiple linear regression model incorporating the six dimensions of social capital as independent variables,

Table 2. Multinomial logistic regression of anxiety symptoms profiles

Variables	Low (*ref*) *vs.* Moderate		Low (*ref*) *vs.* High		Moderate (*ref*) *vs.* High	
	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
Age(years)						
21-25 (reference)						
26-30	1.040 (0.789-1.371)	0.780	0.756 (0.588-0.972)	0.029	0.725 (0.535-0.982)	0.038
≥ 31	1.149 (0.717-1.840)	0.564	0.695 (0.444-1.089)	0.112	0.685 (0.402-1.167)	0.164
Residence						
Urban (reference)						
Rural	0.827 (0.657-1.042)	0.106	1.149 (0.933-1.415)	0.190	1.340 (1.051-1.708)	0.018
Gender						
Male (reference)						
Female	1.397 (1.075-1.816)	0.013	0.895 (0.717-1.118)	0.330	0.662 (0.507-0.864)	0.002
Degree						
Master's students (reference)						
Doctoral students	0.845 (0.578-1.237)	0.387	0.620 (0.426-0.904)	0.013	0.808 (0.515-1.268)	0.354
Grade						
1 (reference)						
2	0.877 (0.663-1.161)	0.360	0.864 (0.670-1.115)	0.260	1.030 (0.757-1.402)	0.850
3	1.020 (0.747-1.395)	0.899	1.375 (1.031-1.834)	0.030	1.205 (0.859-1.690)	0.280
Income level(RMB)						
<15,000 (reference)						
15,000-18,000	0.843 (0.636-1.117)	0.234	0.598 (0.463-0.771)	<0.001	0.761 (0.563-1.030)	0.077
>18,000	0.888 (0.655-1.205)	0.447	0.586 (0.438-0.784)	<0.001	0.693 (0.495-0.970)	0.032
Frequency of communication with family						
Once every six months (reference)						
Once a month	1.414 (0.565-3.539)	0.459	0.734 (0.357-1.509)	0.400	0.499 (0.220-1.132)	0.096
Once a week and more	1.120 (0.462-2.717)	0.802	0.658 (0.331-1.309)	0.233	0.523 (0.237-1.154)	0.109
Frequency of communication with tutors						
Once every six months (reference)						
Once a month	1.167 (0.593-2.294)	0.655	1.266 (0.657-2.437)	0.481	1.239 (0.649-2.368)	0.516
Once a week and more	0.968 (0.496-1.887)	0.923	1.307 (0.686-2.489)	0.415	1.539 (0.813-2.914)	0.185
Exercise						
Never (reference)						
1-2 times per week	0.772 (0.588-1.012)	0.061	1.003 (0.779-1.293)	0.980	1.262 (0.956-1.665)	0.100
3 or more times per week	0.573 (0.389-0.844)	0.005	0.701 (0.496-0.991)	0.044	1.251 (0.823-1.900)	0.295
Smoking						
Never (reference)						
Former	1.348 (0.538-3.380)	0.524	1.684 (0.744-3.811)	0.211	1.081 (0.421-2.773)	0.871
Current	1.688 (0.902-3.159)	0.102	1.127 (0.622-2.040)	0.693	0.984 (0.532-1.822)	0.960
Drinking						
Never (reference)						
Former	1.516 (0.884-2.598)	0.130	1.368 (0.831-2.251)	0.218	0.801 (0.453-1.414)	0.444
Current	1.447 (1.011-2.069)	0.043	1.304 (0.943-1.803)	0.108	0.852 (0.591-1.230)	0.393
Chronic disease						
No (reference)						
Yes	1.595 (0.978-2.601)	0.061	1.420 (0.895-2.255)	0.137	0.969 (0.611-1.535)	0.893
Social participation						
Low (reference)						
High	1.001 (0.782-1.281)	0.996	1.302 (1.040-1.629)	0.021	1.340 (1.042-1.725)	0.023
Social support						
Low (reference)						
High	0.720 (0.545-0.951)	0.021	0.715 (0.557-0.919)	0.009	1.115 (0.847-1.466)	0.438
Social connection						
Low (reference)						
High	0.643 (0.487-0.848)	0.002	0.506 (0.394-0.650)	< 0.001	0.798 (0.598-1.066)	0.127
Trust						
Low (reference)						
High	0.561(0.432-0.729)	< 0.001	0.324 (0.257-0.409)	< 0.001	0.627 (0.476-0.825)	< 0.001
Sense of belonging						
Low (reference)						
High	0.821(0.620-1.088)	0.170	0.760 (0.582-0.992)	0.043	0.914 (0.673-1.241)	0.563
Reciprocity						
Low (reference)						
High	0.766(0.585-1.002)	0.052	0.885 (0.685-1.144)	0.353	0.972 (0.731-1.294)	0.848

represented by their original continuous scores, and SAS scores as the dependent variable, the findings aligned with those obtained from the main analysis (Supplementary Table S7, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=318*).

4. Discussion

This study examined latent anxiety severity profiles among medical graduate students and their associations with multiple dimensions of social capital. Medical graduate students in Anhui Province, China, showed a substantial burden of anxiety symptoms, with three empirically derived severity profiles: low, moderate, and high. Trust, social connection, social support, and sense of belonging were generally inversely associated with anxiety severity, whereas higher social participation was associated with greater anxiety severity. Reciprocity showed no independent association in the adjusted analyses.

Our findings indicate that 30.5% of medical graduate students experienced anxiety symptoms, which is higher than the 18.0% average prevalence reported among Chinese medical students (*28*) and higher than the rates of 14.0% (*4*) and 22.2% (*29*) reported in two previous studies of Chinese medical postgraduates. However, the observed prevalence was slightly lower than the global estimate of 33.8% for medical students (*12*). These differences may reflect variation in academic, research, and career-development pressures, as well as differences in cultural context, geographic sampling, assessment instruments, and study design.

Social support was inversely associated with anxiety severity, consistent with prior evidence (*29-31*). Previous studies suggest that social support may help medical graduate students manage academic and daily-life challenges (*29*). Among nursing graduate students, social support has also been reported to mediate the association between work stress and anxiety symptoms during intensive clinical internships (*30*). Emotional reassurance, practical assistance, and access to coping resources may therefore be plausible pathways linking stronger perceived support with better mental health (*32*).

Lower trust was associated with greater anxiety severity in our sample. Consistent with this finding, a Swedish cohort study reported inverse associations of trust with anxiety and depressive symptoms (*33*). One possible explanation is that trusting relationships may facilitate expression of psychological needs and help-seeking (*34*). In medical graduate training, trust in family members, peers, and supervisors may also reduce social uncertainty and improve the quality of communication in research and clinical settings. These processes could lessen perceived threats related to academic evaluation and peer competition. This pattern is consistent with the stress-buffering framework, in which trust may function as an important cognitive social-capital resource.

Social connection also showed a consistent inverse association with anxiety severity. Medical graduate students who maintain close ties with family members, supervisors, and peers may have greater access to empathy, practical support, and opportunities to discuss concerns. Previous studies similarly suggest that stronger social connection and group identity are associated with lower anxiety and stress levels among young people (*35*). In high-pressure medical training environments, supportive interpersonal connections may therefore represent an important psychological resource (*36,37*).

Sense of belonging was inversely associated with anxiety severity, particularly when the high-anxiety and low-anxiety profiles were compared. Prior research has linked stronger university belonging with lower academic anxiety (*38*). Feeling accepted, recognized, and supported within a social setting is an important component of social connectedness (*39,40*), whereas weak belonging may accompany difficulties in adaptation and greater psychological stress.

A notable finding was that higher social participation was associated with greater anxiety severity, in contrast to studies in which social participation has generally been linked to better mental health outcomes (*41-43*). Other evidence, however, suggests that the effects of participation can be context-dependent (*44*), and medical-student volunteering in post-disaster settings has also been associated with additional psychological strain (*45*). Several explanations are plausible. First, frequent participation may consume time and energy that would otherwise be available for research, clinical work, rest, and sleep. Second, some forms of participation may be obligatory or performance-oriented, being tied to academic evaluation, organizational responsibilities, or supervisor expectations rather than voluntary social engagement. Third, reverse selection is possible: students with higher anxiety may seek more social interaction or may be assigned more group-related tasks. Accordingly, the positive association observed here should not be interpreted as evidence that social participation causes anxiety. It may reflect excessive or obligatory participation, unmeasured role strain, reverse selection, or other residual confounding.

Beyond social capital, several demographic and lifestyle variables were associated with anxiety severity, including sex, income level, physical activity, and alcohol consumption. Female students and students with lower income levels showed greater anxiety severity in some comparisons, broadly consistent with previous reports (*46-48*). Such differences may reflect a combination of biological susceptibility, gendered social expectations, differential exposure to academic or caregiving burdens, and differences in symptom recognition and reporting (*49-51*). Lower income may also increase financial strain and concerns about debt (*52*). Regular physical activity was associated with lower anxiety severity, particularly among students exercising three or more times per

week, in line with previous studies (*53*). Potential pathways include effects on sleep, stress regulation, and neurobiological systems involved in mood and anxiety (*54*). Alcohol use, by contrast, was associated with greater anxiety vulnerability in some analyses, consistent with the possibility that alcohol-based coping may fail to address underlying stressors and may worsen longer-term coping capacity (*29,55,56*).

Several limitations should be considered. First, the cross-sectional design does not establish temporal or causal relationships between social capital and anxiety symptoms; anxiety may itself influence perceptions of social capital, and reverse causality is possible. Second, the study relied primarily on self-reported questionnaires and may therefore be affected by recall or social-desirability bias. Third, participants were medical postgraduates from Anhui Province, and only a small number of institutions were included, which limits generalizability and prevents comprehensive assessment of institution-level differences. Fourth, potentially relevant psychological factors, such as personality traits and coping strategies, were not measured. Fifth, the latent profiles primarily represented severity strata rather than clinically distinct symptom patterns. Finally, the social-participation measure did not distinguish voluntary and supportive participation from mandatory or performance-oriented participation. Future longitudinal and multicenter studies should address these limitations and further clarify the contextual mechanisms linking different dimensions of social capital with anxiety severity.

5. Conclusions

Social capital was associated with anxiety severity among medical graduate students, but the direction of association differed across dimensions. Social support, social connection, trust, and sense of belonging showed inverse associations with anxiety symptoms, whereas higher social participation was associated with greater anxiety severity. These findings suggest that social capital should not be treated as a uniformly beneficial resource and that its different dimensions may operate differently across educational contexts. When developing mental health initiatives, universities may consider distinguishing supportive social activities from mandatory or performance-oriented group activities and placing greater emphasis on the quality of social support rather than participation quantity alone. Institutions may also consider strengthening psychological safety in supervisor-student interactions, improving access to confidential mental health support, and paying attention to structural pressures and disparities in social resources among lower-income students. Further longitudinal and intervention research is needed to define the boundaries and mechanisms of these associations and to determine whether modifying specific dimensions of social capital can improve mental health outcomes.

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Original Article

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Selectivity-aware dual-target cholinesterase optimization using a novel model SPaR-GNN

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SUMMARY: We developed SPaR-GNN (Selectivity Pair-aware Ranking Graph Neural Network), a multitask molecular learning framework for dual-target cholinesterase optimization. SPaR-GNN jointly predicts acetylcholinesterase (AChE) activity, butyrylcholinesterase (BuChE) activity, and molecular selectivity while incorporating explicit pair-aware supervision from structurally similar analogs and selectivity-cliff pairs. In addition, we integrated SPaR-GNN into a goal-driven molecular optimization workflow for candidate generation and prioritization. Experimental results showed that SPaR-GNN performed satisfactorily in selectivity-oriented prediction, and particularly in local analog ranking and direction-aware pair-sensitive benchmarks. The optimization framework further demonstrated that template-based candidate generation combined with SPaR-GNN scoring can be used to prioritize candidate molecules according to predefined dual-target optimization objectives. Together, these results demonstrate that SPaR-GNN provides an effective and traceable computational framework for dual-target cholinesterase inhibitor discovery and optimization in Alzheimer's disease.

Keywords: Alzheimer's disease, acetylcholinesterase, butyrylcholinesterase, graph neural network, multitask learning, drug discovery, molecular optimization

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by progressive memory loss and cognitive decline (*1,2*). Its pathological features include amyloid-β deposition, Tau hyperphosphorylation, neuroinflammation, neuronal loss, and synaptic dysfunction (*1,3,4*). Despite advances in understanding AD pathology, effective therapies remain limited. Approved cholinesterase inhibitors, including donepezil, galantamine, and rivastigmine, primarily enhance cholinergic neurotransmission rather than directly modifying the underlying disease pathology, and their therapeutic efficacy may decline with disease progression (*5*). Both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are involved in cholinergic dysfunction during AD progression (*6,7*), motivating the development of dual-target cholinesterase inhibitors.

Conventional medicinal chemistry approaches to dual-target compound discovery and optimization often involve extensive trial-and-error and experimental costs (*8*). Computational approaches that involve molecular representations and prioritize promising candidates therefore provide an attractive strategy to improve screening efficiency (*9*). However, dual-target cholinesterase modeling requires simultaneous consideration of AChE activity, BuChE activity, and target selectivity, making candidate prioritization sensitive to structural variations. Conventional fingerprint-based machine learning models and multilayer perceptrons (MLPs) rely on predefined molecular representations, whereas graph neural networks (GNNs) directly represent molecules as atom–bond graphs and can incorporate structure-dependent molecular features (*10-13*).

Beyond molecule-level prediction, lead optimization frequently requires differentiation of closely related analogs. This is particularly challenging for activity and selectivity cliffs, where minor structural modifications can produce disproportionately large changes in biological activity or target preference (*14*). Consequently, good global prediction performance does not necessarily ensure reliable prioritization of structurally similar compounds.

To address these challenges, we developed SPaR-GNN (Selectivity Pair-aware Ranking Graph Neural Network), a multitask molecular learning framework for dual-target cholinesterase modeling. SPaR-GNN jointly

models AChE activity, BuChE activity, and selectivity while incorporating pair-aware supervision to include local structure–selectivity relationships among related analogs. This combines multitask learning across related prediction tasks with explicit learning of pairwise relationships relevant to analog prioritization (*15,16*).

In this study, we curated and standardized an AChE/BuChE dataset and created pair-sensitive benchmarks consisting of structurally similar pairs, selectivity cliffs, and strict selectivity cliffs (AChE–BuChE polarity reversals). We developed SPaR-GNN to evaluate both molecule-level prediction and local analog discrimination, and we also integrated the model into a goal-driven molecular optimization workflow to prioritize AChE-oriented or BuChE-oriented candidates. The curated dataset, benchmark splits, model implementation, and optimization workflow are publicly available through our GitHub repository (*https://github.com/Eucliwood-bsb/SPaR-GNN*).

2. Materials and Methods

2.1. Dataset construction

2.1.1. Dataset collection, standardization, and quality control

Bioactivity data for AChE and BuChE were collected from ChEMBL (*17*) version 36 and BindingDB (*18*). Only quantitative half-maximal inhibitory concentration (IC_{50}) measurements were retained. Records lacking valid molecular structures, target information, numerical activity values, or activity units were excluded. Target names and organism annotations were standardized, activity values were converted to IC_{50} (nM) and transformed into pIC_{50}, and molecular structures were standardized using RDKit (*19*) to generate canonical SMILES representations. For molecule–target pairs with multiple measurements, median pIC_{50} values were used as representative activity labels. To improve label reliability, replicated measurements were further evaluated using the pIC_{50} range, and labels were retained when only a single measurement was available or when range$|pIC_{50} \leq 1.5$. The resulting dataset was organized in a multitask format containing AChE activity, BuChE activity, and target-availability masks. Although this procedure reduces variability among replicated measurements, systematic differences arising from assay protocols, enzyme preparations, substrate conditions, or other experimental settings across public databases cannot be completely eliminated.

Molecules with activity measurements for both targets were used to construct the dual-target subset. Selectivity was defined as

$$Selectivity = pIC50_{AChE} - pIC50_{BuChE}$$

Molecules were classified as AChE-selective (Selectivity ≥ 1.0), BuChE-selective (Selectivity ≤ -1.0), or balanced ($-1.0 <$ Selectivity < 1.0). Because selectivity is derived from the difference between two independently measured activities, residual assay-to-assay variability may propagate into the selectivity labels even after activity standardization and quality filtering.

2.1.2. Identification of structurally similar pairs and creation of selectivity-cliff benchmarks

Pairwise molecular relationships were identified among molecules with dual-target activity labels. Molecular similarity was calculated using Morgan fingerprints and Tanimoto similarity. Molecule pairs with a Tanimoto similarity greater than or equal to 0.60 were defined as structurally similar pairs. This procedure produced 24,452 structurally similar pairs.

For each structurally similar pair (i,j), the pairwise selectivity difference was defined as

$$\Delta y_{ij}^{S} = y_i^{S} - y_j^{S}. \quad (1)$$

Each pair was additionally annotated according to the selectivity classes of its two endpoints. The 24,452 structurally similar pairs consisted of 4,104 AChE-selective–AChE-selective pairs, 4,344 AChE-selective–balanced pairs, 705 AChE-selective–BuChE-selective pairs, 1,539 BuChE-selective–BuChE-selective pairs, 2,928 BuChE-selective–balanced pairs, and 10,832 balanced–balanced pairs.

Selectivity cliffs were defined as structurally similar pairs with endpoints that belonged to different selectivity classes and with an absolute selectivity difference greater than or equal to 1.0 log unit:

$$\left|\Delta y_{ij}^{S}\right| \geq 1.0. \quad (2)$$

The eligible cross-class combinations were AChE-selective–balanced, BuChE-selective–balanced, and AChE-selective–BuChE-selective. A total of 7,977 structurally similar pairs satisfied these endpoint-class and selectivity-difference criteria and constituted the selectivity-cliff benchmark. These included 4,344 AChE-selective–balanced pairs, 2,928 BuChE-selective–balanced pairs, and 705 AChE-selective–BuChE-selective pairs.

Strict selectivity cliffs were defined as the AChE-selective–BuChE-selective subset of the selectivity-cliff benchmark. These pairs represent reversals between the two opposing target-selectivity classes. A total of 705 pairs met this definition and constituted the strict selectivity-cliff subset. Because an AChE-selective endpoint has $y^{S} \geq 1.0$ and a BuChE-selective endpoint has $y^{S} \leq -1.0$, every strict selectivity cliff necessarily has an absolute selectivity difference of at least 2.0 log units.

The full set of structurally similar pairs was used as the basic pair set for pair-aware learning and similar-pair

evaluation. Pair-type-dependent weights were assigned hierarchically: structurally similar pairs that were not selectivity cliffs were given a weight of 1.0, selectivity cliffs were given a weight of 3.0, and strict selectivity cliffs were given a weight of 5.0. Strict-cliff weights replaced, rather than accumulated with, the lower pair-type weights.

Activity cliffs were annotated separately for AChE and BuChE. Structurally similar pairs exhibiting an activity difference greater than or equal to 1.0 log unit for the corresponding target were labeled as target-specific activity cliffs and used only in the relevant downstream analyses.

2.1.3. Dataset splitting strategy and pairwise leakage control

A pair-first scaffold-closure splitting strategy was used for the pair-sensitive experiments. First, 24,452 structurally similar molecular pairs were identified from the standardized dual-target dataset using a Morgan fingerprint Tanimoto similarity threshold of 0.60. Each pair was then annotated as an ordinary structurally similar pair, a selectivity cliff, or a strict selectivity cliff according to the definitions provided in Section 2.1.2. Pair identification and pair-type annotation were completed before assigning molecules to the training, validation, and test partitions.

To prevent the two endpoints of a structurally similar pair from being assigned to different partitions, molecules connected through the retained pair relationships were grouped into connected components. For the scaffold-closure split, Bemis–Murcko scaffold relationships were additionally incorporated into component construction. Molecules sharing the same Bemis–Murcko scaffold, as well as molecules connected through retained structurally similar pair relationships, were placed in the same closure component.

The resulting closure components were assigned exclusively to the training, validation, or test partition. Molecules inherited the partition assignment of their corresponding component, and each molecular pair was assigned to the same partition as its two endpoints. Only pairs with two endpoints in the same partition were retained in the corresponding pair subset.

In accordance with this protocol, the selectivity-cliff benchmark contained 5,863 training pairs, 1,060 validation pairs, and 1,054 test pairs. The strict selectivity-cliff subset contained 424 training pairs, 147 validation pairs, and 134 test pairs. The complete similar-pair test subset contained 3,373 pairs and was used to calculate similar-pair direction accuracy.

This procedure ensured that no retained molecular pair spanned different data partitions. In the scaffold-closure setting, it additionally ensured that no Bemis–Murcko scaffold was shared among the training, validation, and test sets, thereby providing a more stringent assessment of generalization to unseen chemotypes. This was used as the primary protocol for the pair-sensitive results.

2.2. Model: SPaR-GNN (Selectivity Pair-aware Ranking GNN)

As shown in Figure 1, we propose SPaR-GNN to predict the dual-target molecular properties of cholinesterase-related compounds (*20*). Let a molecule be represented as a graph $G = (V,E)$, where atoms are nodes and bonds are edges. For molecule i, the target values are denoted by y_i^A and y_i^B, corresponding to the pIC_{50} values measured against AChE and BuChE, respectively. For molecules with both target labels, we define the selectivity as $y_i^S = y_i^A - y_i^B$.

This formula reflects the degree to which a compound is biased towards AChE over BuChE. In practice, not every molecule has complete annotations for both targets. Therefore, we introduced the binary masks $m_i^A \in \{0,1\}$ and $m_i^B \in \{0,1\}$ to indicate whether the corresponding activity labels are observed. The selectivity mask is defined as $m_i^S = m_i^A \cdot m_i^B$ so that selectivity supervision is only applied to dual-labeled molecules.

The learning objective in our setting was not limited to accurate prediction of absolute activity. Instead, we sought to simultaneously model: (i) AChE activity, (ii) BuChE activity, and (iii) selectivity-related differences between the two targets. This multitask formulation is motivated by the fact that selective inhibitor design depends on both shared structure–activity patterns and target-specific deviations. Moreover, medicinal chemistry decisions often rely on relative comparisons among structurally similar analogs rather than isolated point estimates for individual molecules (*21*).

To better align training with this optimization-oriented setting, SPaR-GNN combines single-molecule regression with pair-level supervision derived from structurally similar molecular pairs (*22*), including selectivity cliff pairs. As summarized in the overall architecture in Figure 1, this design enables the model to learn not only global activity trends but also local structure–selectivity relationships that are especially important to analog prioritization.

2.3. Selectivity-aware multitask GINE

The molecular encoder of SPaR-GNN is based on a residual Graph Isomorphism Network with Edge features (GINE) (*23*). Atom and bond features are first projected into a shared hidden space and processed through stacked residual GINE layers (*24*). Representations from multiple encoder layers are subsequently integrated using an attention-like layer-fusion mechanism, followed by global mean and max pooling to obtain a shared molecular embedding. Detailed encoder formulations, including feature projection, residual message passing,

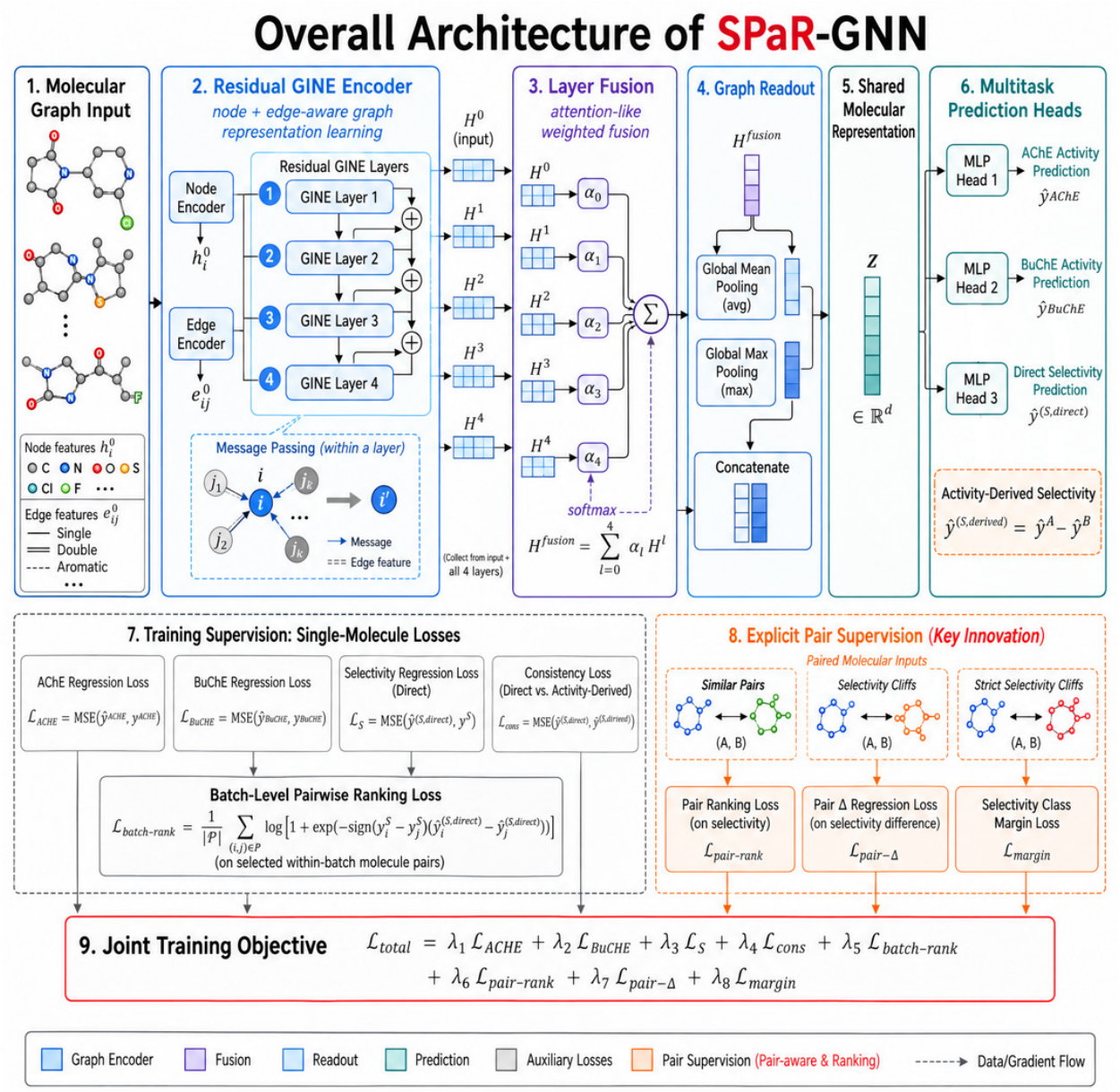


Figure 1. Overall architecture of SPaR-GNN.

layer fusion, and graph-level pooling, are provided in Supplementary Figure S1 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

The resulting shared molecular representation is passed to three task-specific prediction heads for AChE activity, BuChE activity, and direct selectivity. SPaR-GNN therefore provides two distinct selectivity estimates. The direct selectivity estimate is generated by the dedicated selectivity head $\hat{y}^{S,\mathrm{direct}}$. In contrast, the activity-derived selectivity estimate is calculated deterministically from the two target-specific activity predictions $\hat{y}^{S,\mathrm{derived}}$. Thus, the activity-derived estimate is not generated from the direct selectivity output. The dedicated direct-selectivity branch explicitly learns the measured selectivity endpoint, whereas the activity-derived branch preserves the relationship between selectivity and the two target-specific activity predictions. The two estimates are connected during training through the selectivity-consistency objective described below. This dual-view approach is intended to facilitate selectivity-oriented prediction and downstream molecular optimization. Detailed structures of the prediction heads are provided in Supplementary Figure S2 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

2.4. Explicit pair supervision and the training objective

The explicit pair supervision module of SPaR-GNN is shown in Figure 2. The training objective combines single-molecule multitask regression, selectivity consistency, batch-level ranking, and explicit pair supervision to jointly model absolute activity and local structure–selectivity relationships.

For individual molecules, masked mean squared error losses are applied to AChE, BuChE, and direct selectivity predictions:

$$L_A = \frac{\sum_i m_i^A \left(\hat{y}_i^A - y_i^A\right)^2}{\sum_i m_i^A},$$
$$L_B = \frac{\sum_i m_i^B \left(\hat{y}_i^B - y_i^B\right)^2}{\sum_i m_i^B}, \quad (3)$$
$$L_S = \frac{\sum_i m_i^S \left(\hat{y}_i^{S,direct} - y_i^S\right)^2}{\sum_i m_i^S}.$$

Consistency between direct selectivity prediction and the activity-derived estimate $\hat{y}_i^{S,derived}$ is enforced by

$$L_{cons} = \frac{\sum_i m_i^S \left(\hat{y}_i^{S,direct} - \hat{y}_i^{S,derived}\right)^2}{\sum_i m_i^S}. \quad (4)$$
$$\hat{y}_i^{S,derived} = \hat{y}_i^A - \hat{y}_i^B$$

A symmetric stop-gradient formulation is used in implementation to reduce the domination of either prediction branch.

For batch-level selectivity ranking (*25*), dual-labeled molecule pairs *(i,j)* are selected when

$$|y_i^S - y_j^S| \geq \gamma. \quad (5)$$

The corresponding ranking loss is

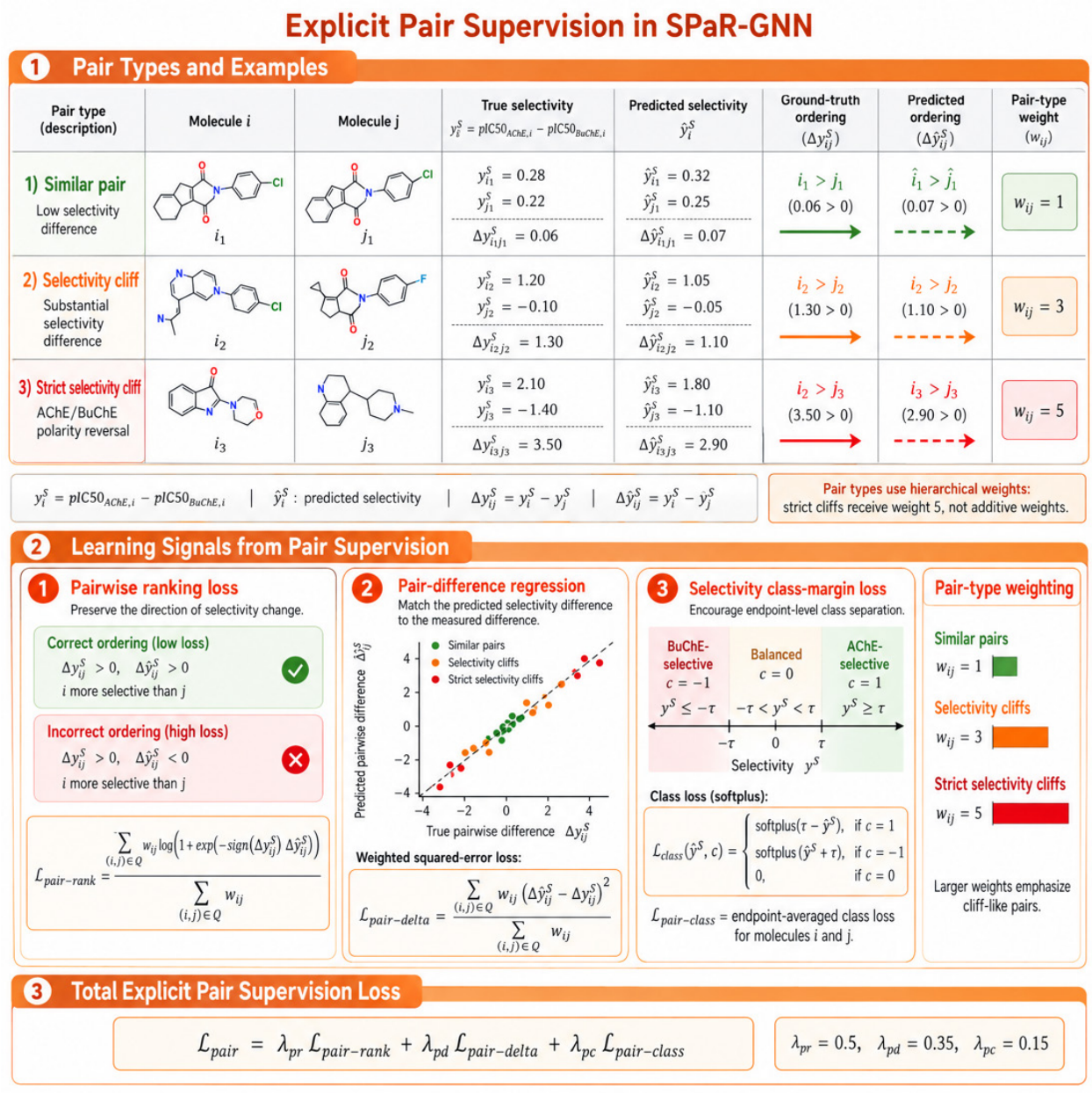


Figure 2. Explicit pair supervision in SPaR-GNN. Structurally similar pairs, selectivity cliffs, and strict selectivity cliffs are used for pairwise ranking, selectivity-difference regression, and endpoint-level class-margin supervision. Pair-type weights of 1.0, 3.0, and 5.0 are assigned hierarchically to ordinary structurally similar pairs, selectivity cliffs, and strict selectivity cliffs, respectively.

$$L_{\text{batch_rank}} = \frac{1}{|P|}\sum_{(i,j)\in P}\log\left(1+\exp\left[-\text{sign}(y_i^S-y_j^S)(\hat{y}_i^{S,direct}-\hat{y}_j^{S,direct})\right]\right), \quad (6)$$

where P denotes the selected within-batch pairs.

Explicit pair supervision is applied to the complete structurally similar pair set, with selectivity cliffs and strict selectivity cliffs treated as increasingly difficult nested subsets (*26*). Let Q denote the explicit training-pair set. Ordinary structurally similar pairs, selectivity cliffs, and strict selectivity cliffs are given hierarchical pair weights of 1.0, 3.0, and 5.0, respectively. For each pair *(i,j)*,

$$\Delta y_{ij}^S = y_i^S - y_j^S, \quad (7)$$

and

$$\Delta\hat{y}_{ij}^{S,direct} = \hat{y}_i^{S,direct} - \hat{y}_j^{S,direct}. \quad (8)$$

The pairwise ranking loss is defined as

$$L_{\text{pair_rank}} = \frac{\sum_{(i,j)\in Q} w_{ij}\log\left(1+\exp\left[-\text{sign}(\Delta y_{ij}^S)\Delta\hat{y}_{ij}^{S,direct}\right]\right)}{\sum_{(i,j)\in Q} w_{ij}}, \quad (9)$$

where Q denotes the explicit training-pair set and w_{ij} is the hierarchical pair-type weight. Specifically, $w_{ij}=1.0$ for structurally similar pairs that are not selectivity cliffs, $w_{ij}=3.0$ for selectivity cliffs that are not strict cliffs, and $w_{ij}=5.0$ for strict selectivity cliffs. The weights are mutually exclusive and are not accumulated across nested pair categories.

Pairwise selectivity differences are additionally optimized through

$$L_{\text{pair_delta}} = \frac{\sum_{(i,j)\in Q} w_{ij}\left(\Delta\hat{y}_{ij}^{S,direct}-\Delta y_{ij}^S\right)^2}{\sum_{(i,j)\in Q} w_{ij}}. \quad (10)$$

A class-margin loss is further used to distinguish AChE-selective, balanced, and BuChE-selective compounds according to threshold τ:

$$L_{\text{class}} = \begin{cases}\log\left(1+\exp(\tau-\hat{y}^{S,direct})\right), & c=1,\\ \log\left(1+\exp(\hat{y}^{S,direct}+\tau)\right), & c=-1,\\ 0, & c=0,\end{cases} \quad (11)$$

where $c\in\{-1,0,1\}$ denotes BuChE-selective, balanced, and AChE-selective classes, respectively.

The explicit pair supervision term is

$$L_{\text{pair}} = \lambda_{\text{pr}}L_{\text{pair_rank}}+\lambda_{\text{pd}}L_{\text{pair_delta}}+\lambda_{\text{pc}}L_{\text{pair_class}}, \quad (12)$$

Where $L_{\text{pair_class}}$ is the endpoint-averaged class-margin loss for each explicit pair.

Finally, the overall objective is

$$L_{\text{total}} = \lambda_A L_A+\lambda_B L_B+\lambda_S L_S+\lambda_C L_{\text{cons}}+\lambda_R L_{\text{batch_rank}}+L_{\text{pair}}, \quad (13)$$

Thus, SPaR-GNN jointly optimizes molecule-level activity and selectivity prediction, relative selectivity ordering, and pairwise discrimination of structurally related analogs.

2.5. Goal-driven molecular optimization framework

To extend SPaR-GNN from predictive modeling to analog prioritization, we developed a goal-driven molecular optimization framework that combines template-based local molecular editing with SPaR-GNN-based scoring (Figure 3) (*27*). Given an input molecule and a user-defined optimization goal G, the framework first generates structurally related candidates and applies prediction-independent structural and physicochemical filters. SPaR-GNN is then used to evaluate the retained candidates and the input molecule. The resulting predictions are used to apply prediction-dependent goal-specific constraints, after which feasible candidates are scored and ranked according to the specified objective.

Rather than performing unrestricted de novo generation, the framework searches a finite set of locally edited analogs $C(x_0)=\{x_1,x_2,\ldots,x_n\}$ while preserving structural proximity to the starting scaffold (*28*). The goal specification G defines optimization preferences and constraints, including target activity, selectivity, structural similarity, and physicochemical properties (*29*).

For each candidate x, SPaR-GNN provides predicted AChE activity $\hat{y}^A(x)$, BuChE activity $\hat{y}^B(x)$, derived selectivity $\hat{y}^{S,\text{derived}}(x)$, and direct selectivity $\hat{y}^{S,\text{direct}}(x)$. Structural similarity to the starting molecule is quantified using the Morgan fingerprint Tanimoto similarity, $\text{Sim}(x,x_0)$ (*30*).

Candidate optimization is formulated as a constrained ranking problem in which hard constraints determine candidate admissibility and soft preferences define goal-dependent prioritization. This formulation allows AChE-oriented, BuChE-oriented, or balanced dual-target objectives to be specified by changing the goal configuration without retraining the predictive model. Candidate generation, filtering, and ranking are described in the following section.

2.6. Template-based candidate generation, filtering, and ranking

To ensure chemical interpretability and controllability, candidate generation was performed through template-based local editing rather than unconstrained molecular generation (*31*). Given an input molecule x_0, a predefined library of medicinal chemistry-inspired transformation rules was applied to generate a structurally related candidate pool $C(x_0)$ (*32,33*). The transformations included commonly used local modifications such as

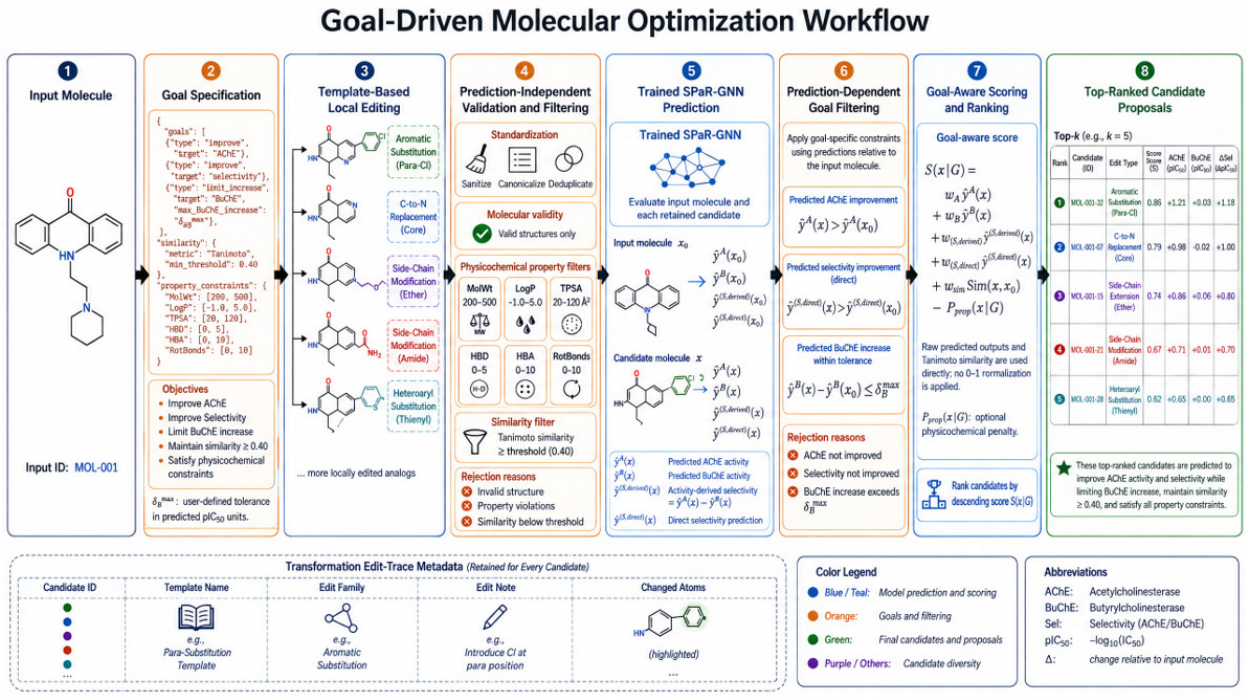


Figure 3. Goal-driven molecular optimization workflow based on SPaR-GNN. Starting from an input molecule and a user-defined objective, locally edited analogs are generated and screened using prediction-independent structural and physicochemical constraints. SPaR-GNN then evaluates the retained candidates and the input molecule. The resulting predictions are used for prediction-dependent goal-specific filtering, followed by direct weighted scoring and final candidate ranking.

aromatic substitution, bioisosteric replacement, and side chain modification. Each generated candidate was standardized, sanitized, deduplicated, and associated with an edit trace describing the corresponding structural modification. Detailed transformation rules and examples are provided in Supplementary Figure S3 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

After generation, candidate molecules were standardized, sanitized, and deduplicated. Prediction-independent filtering was then applied using molecular validity, structural similarity, and physicochemical property constraints. These filters did not require SPaR-GNN outputs.

SPaR-GNN was subsequently applied to the candidates retained after prediction-independent filtering and to the input molecule. The model provided predicted AChE activity, predicted BuChE activity, derived selectivity, and direct selectivity. Goal-specific, prediction-dependent constraints were then evaluated relative to the corresponding predictions for the input molecule. Depending on the optimization objective, candidates could be required to improve predicted AChE activity, improve predicted selectivity, limit an undesirable increase in predicted BuChE activity, or satisfy the corresponding BuChE-oriented criteria (Supplementary Figure S4 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

Candidates satisfying both the prediction-independent and prediction-dependent constraints were considered feasible. A goal-dependent score was then calculated to integrate the model predictions with structural similarity and optional physicochemical penalties:

$$S(x|G) = w_A\hat{y}^A(x) + w_B\hat{y}^B(x) + w_{S,\text{derived}}\hat{y}^{S,\text{derived}}(x) + w_{S,\text{direct}}\hat{y}^{S,\text{direct}}(x) + w_{\text{sim}}\text{Sim}(x,x_0) - P_{\text{prop}}(x|G). \quad (14)$$

The user-defined optimization objective is denoted, and the relative contributions of the corresponding prediction and similarity terms are controlled. Here, $P_{\text{prop}}(x|G)$ represents optional penalties for deviations from preferred physicochemical ranges.

The feasible candidates were ranked in descending order according to $S(x|G)$, and the top-ranked molecules were retained as optimization proposals. Because the score weights and filtering criteria can be adjusted according to the desired target preference, the same candidate-generation framework can support AChE-oriented, BuChE-oriented, or balanced dual-target optimization without retraining the predictive model. Detailed goal configurations, physicochemical thresholds, candidate-level scores, and edit-trace information are provided in the Supplementary Materials (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

2.7. Implementation details and hyperparameter settings

All experiments were implemented in Python using

PyTorch and PyTorch Geometric and trained on an NVIDIA RTX 5070 GPU. To assess robustness to random initialization and stochastic training, key neural-model experiments were repeated using multiple independent random seeds. Key implementation details and hyperparameter settings are summarized in Supplementary Table S1 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=320*).

Each molecule was represented as a graph with atoms as nodes and bonds as edges. Atom features included atomic number, hybridization, degree, formal charge, hydrogen count, aromaticity, and ring membership, while bond features included bond type, conjugation, ring membership, and stereochemistry. Categorical descriptors were one-hot encoded with an additional "unknown" category when necessary.

The SPaR-GNN encoder used four residual GINE layers with a hidden dimension of 128 and a dropout rate of 0.2. Node and edge features were projected into a shared hidden space, processed through stacked residual GINE blocks, fused using the attention-like layer fusion module, and aggregated by concatenated global mean and max pooling before being passed to three task-specific prediction heads.

Training used the AdamW optimizer with a learning rate of 1×10^{-3} and a weight decay of 1×10^{-5}. Molecule and pair batch sizes were 64 and 128, respectively. Training was performed for up to 300 epochs with early stopping patience of 35 epochs.

The loss weights were set to $\lambda_A = 1.0$, $\lambda_B = 1.0$, $\lambda_S = 0.8$, $\lambda_C = 0.5$, and $\lambda_R = 0.5$. For explicit pair supervision, $\lambda_{pr} = 0.5$, $\lambda_{pd} = 0.35$, and $\lambda_{pc} = 0.15$. Batch-level ranking used a margin threshold of $\gamma=0.5$ with up to 512 informative within-batch pairs. The selectivity class threshold was $\tau = 1.0$. Structurally similar pairs were defined using a Tanimoto similarity threshold of 0.60. Pair-type weights were assigned hierarchically: ordinary structurally similar pairs received a weight of 1.0, selectivity cliffs received a weight of 3.0, and strict selectivity cliffs received a weight of 5.0.

To ensure consistent validation-based checkpoint selection across neural models, a common selectivity-oriented validation framework was used for MLP, GNN, GINE, and SPaR-GNN. For models without a dedicated direct-selectivity head, the unavailable direct-selectivity term was omitted and the remaining terms were re-normalized. At each epoch, a mixed validation score was calculated based on direct selectivity Spearman correlation, activity-derived selectivity Spearman correlation, selectivity-cliff Spearman correlation, and strict selectivity-cliff direction accuracy:

$$S_{\mathrm{val}} = \frac{0.35\rho_{\mathrm{sel}}^{\mathrm{direct}} + 0.25\rho_{\mathrm{sel}}^{\mathrm{activity}} + 0.25\rho_{\mathrm{cliff}} + 0.15Acc_{\mathrm{dir}}^{\mathrm{strict}}}{w_{\mathrm{used}}}.$$

Here, w_{used} denotes the sum of weights corresponding to non-missing validation terms. For models without a direct selectivity prediction head, the corresponding term was omitted and the remaining terms were re-normalized. The checkpoint with the highest S_{val} was selected for final test-set evaluation.

For non-neural baselines such as XGBoost or random forest, no epoch-wise checkpoint selection was used. These models were trained with fixed hyperparameter settings and evaluated using the same validation and test partitions. Unless otherwise stated, all hyperparameters were fixed for the scaffold-closure experiments.

The selectivity-related metrics reported in tables were calculated using the direct selectivity prediction $\hat{y}^{S,\mathrm{direct}}$ in SPaR-GNN, and those metrics were calculated as $\hat{y}^{S,\mathrm{derived}}$ in the other baseline models without a dedicated selectivity head based on the predicted target activity.

3. Results

3.1. Dataset construction and characteristics

The resulting dual-target dataset contained 3,085 compounds, including 864 AChE-selective compounds (28.0%), 1,783 balanced compounds (57.8%), and 438 BuChE-selective compounds (14.2%). At the molecule level, 2,289, 397, and 399 compounds were assigned to the training, validation, and test partitions, respectively.

For molecules with activity measurements against both targets, selectivity was defined as:

$$y_i^S = pIC50_{\mathrm{AChE},i} - pIC50_{\mathrm{BuChE},i}.$$

To characterize local selectivity relationships, we enumerated all molecular pairs with a Morgan fingerprint Tanimoto similarity greater than or equal to 0.60. This procedure produced 24,452 structurally similar pairs. Each pair was annotated according to the selectivity classes of its two endpoints and its pairwise selectivity difference. The complete similar-pair set contained 4,104 AChE-selective–AChE-selective pairs, 4,344 AChE-selective–balanced pairs, 705 AChE-selective–BuChE-selective pairs, 1,539 BuChE-selective–BuChE-selective pairs, 2,928 BuChE-selective–balanced pairs, and 10,832 balanced–balanced pairs.

Selectivity cliffs were defined as structurally similar pairs with endpoints in different selectivity classes and with an absolute selectivity difference greater than or equal to 1.0 log unit. The eligible endpoint-class combinations were AChE-selective–balanced, BuChE-selective–balanced, and AChE-selective–BuChE-selective. A total of 7,977 pairs satisfied these criteria and constituted the selectivity-cliff benchmark. These included 4,344 AChE-selective–balanced pairs, 2,928 BuChE-selective–balanced pairs, and 705 AChE-selective–BuChE-selective pairs.

The 705 AChE-selective–BuChE-selective pairs were defined as strict selectivity cliffs because they represented polarity reversals between the two opposing

selectivity classes. These pairs form a nested subset of the 7,977 selectivity cliffs. No additional Tanimoto threshold beyond the general structurally similar-pair threshold of 0.60 was imposed for strict selectivity cliffs.

Under the final pair-first scaffold-closure protocol, the structurally similar pair relationships, together with Bemis–Murcko scaffold relationships, were used to form closure components before train/validation/test assignment. A total of 101 closure components were obtained and assigned exclusively to the training, validation, or test partition. Molecules inherited the split assignment of their corresponding component, and pairs were assigned to the same partition as their endpoints.

The selectivity-cliff benchmark contained 5,863 training pairs, 1,060 validation pairs, and 1,054 test pairs. The strict selectivity-cliff subset contained 424 training pairs, 147 validation pairs, and 134 test pairs. After component-level split assignment, all structurally similar pairs were filtered according to endpoint membership, yielding 3,373 structurally similar pairs with both endpoints in the test partition.

Accordingly, the three groups of pair-sensitive metrics were evaluated for distinct but nested test subsets. Similar-pair direction accuracy was calculated for all 3,373 structurally similar test pairs. Selectivity-cliff RMSE, Spearman correlation, and direction accuracy were calculated for all 1,054 test selectivity cliffs. Strict-cliff RMSE, Spearman correlation, and direction accuracy were calculated for the 134 test AChE-selective–BuChE-selective polarity-reversal pairs (Figure 4).

Table 1 summarizes the definitions, sizes, and uses of the pair subsets. The 7,977 selectivity cliffs can be divided into two mutually exclusive endpoint-class groups: 7,272 balanced-involving selectivity cliffs and 705 strict AChE-selective–BuChE-selective polarity-reversal pairs. The balanced-involving subset consists of 4,344 AChE-selective–balanced pairs and 2,928 BuChE-selective–balanced pairs. Thus, strict selectivity cliffs form a nested subset of the selectivity-cliff benchmark, whereas the three endpoint-class combinations form a mutually exclusive partition of all 7,977 selectivity cliffs.

Table 2 shows the endpoint-class distribution of the 7,977 selectivity cliffs after pair-first scaffold-closure splitting. AChE-selective–balanced cliffs were the most frequent, accounting for 4,344 pairs (54.46%), followed by BuChE-selective–balanced cliffs with 2,928 pairs (36.71%) and strict AChE-selective–BuChE-selective polarity reversals with 705 pairs (8.84%).

Because the structurally similar pair relationships

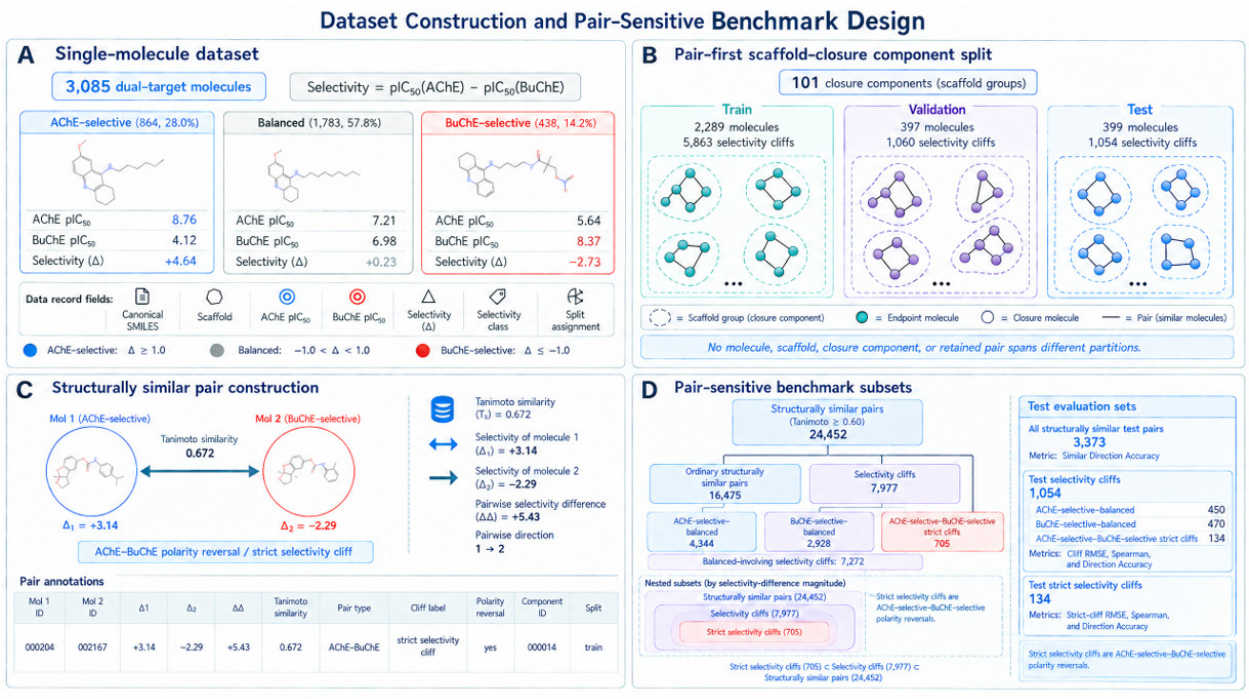


Figure 4. Overview of the cholinesterase selectivity dataset and pair-sensitive benchmark. A, The single-molecule dataset contains 3,085 compounds with dual-target AChE and BuChE potency labels. Molecular selectivity is defined as the difference between AChE and BuChE potency and is used to assign AChE-selective, balanced, or BuChE-selective classes. **B,** Pair-first scaffold-closure splitting based on 101 closure components. The selectivity-cliff benchmark contains 5,863 training, 1,060 validation, and 1,054 test pairs while preventing scaffold-, component-, and pair-endpoint-level leakage. **C,** Structurally similar pairs are identified using a Morgan fingerprint Tanimoto similarity threshold of 0.60 and are annotated with endpoint selectivity classes, pairwise selectivity differences, selectivity-cliff labels, and strict polarity-reversal labels. **D,** Relationships among the pair-sensitive subsets. The complete dataset contains 24,452 structurally similar pairs. Of these, 7,977 cross-class pairs with an absolute selectivity difference greater than or equal to 1.0 log unit are defined as selectivity cliffs. The 705 AChE-selective–BuChE-selective polarity-reversal pairs form the strict selectivity-cliff subset. The test subsets contain 3,373 structurally similar pairs, 1,054 selectivity cliffs, and 134 strict selectivity cliffs.

were identified before split assignment and subsequently incorporated into scaffold-closure component construction, we further assessed potential information leakage across the resulting data partitions (Table 3). With a scaffold-closure split, no train–test overlap was detected at the molecule, canonical or standardized SMILES, Bemis–Murcko scaffold, or pair-endpoint level. Pair assignments were fully consistent with the molecule-level split, and no endpoints of test pairs appeared among the training molecules, confirming the absence of pair-level leakage. This split therefore provided a more stringent assessment of generalization to unseen chemotypes.

3.2. SPaR-GNN improves selectivity prediction

To evaluate the predictive capability of SPaR-GNN, we compared SPaR-GNN against four representative baselines on the standard single-molecule multitask prediction task. The benchmark included a tree-based model using molecular fingerprints (XGB) (*34*), a fingerprint-based neural baseline (MLP) (*35*), a message-passing GNN (*12*), and a GINE (*36*) model with edge-aware graph convolutions. All models were evaluated using the same benchmark dataset with a scaffold split. Following the multitask setting described earlier, predictive performance in terms of AChE activity, BuChE activity, and selectivity are described below.

Table 4 summarizes the single-molecule multitask prediction results with a scaffold-closure split. As expected, prediction performance varied substantially across models when evaluated using unseen chemotypes. In the individual target tasks, XGB resulted in the best AChE performance, with the lowest RMSE (1.138 ± 0.019) and the highest Spearman correlation (0.723 ± 0.018). XGB also resulted in the lowest BuChE RMSE (1.149 ± 0.023), whereas MLP resulted in the highest BuChE Spearman correlation (0.642 ± 0.022). In contrast, SPaR-GNN did not outperform the baseline models in single-target activity prediction, indicating that its primary advantage does not lie in optimizing isolated AChE or BuChE regression accuracy.

A different pattern emerged for selectivity prediction. SPaR-GNN resulted in both the lowest selectivity RMSE

Table 1. Definitions, counts, and uses in evaluation for the molecular-pair subsets. Selectivity cliffs and strict selectivity cliffs are nested subsets of the structurally similar pair set

Pair subset	Definition	Total	Test	Use in the study
Structurally similar pairs	Morgan fingerprint Tanimoto similarity ≥ 0.60	24,452	3,373	Basic pair-aware learning set and Similar Dir. Acc. evaluation
Ordinary similar pairs	Structurally similar pairs that do not meet the selectivity-cliff definition	16,475	2,319	Explicit pair supervision with a pair weight of 1.0
Selectivity cliffs	Structurally similar cross-class pairs with $\lvert\Delta y^{S}_{ij}\rvert \geq 1.0$	7,977	1,054	Explicit pair supervision with a weight of 3.0 and cliff RMSE, Spearman, and direction accuracy
Balanced-involving selectivity cliffs	AChE-selective—balanced or BuChE-selective—balanced selectivity cliffs	7,272	920	Endpoint-class characterization of the cliff benchmark
Strict selectivity cliffs	AChE-selective—BuChE-selective polarity-reversal pairs within the selectivity-cliff set	705	134	Explicit pair supervision with a weight of 5.0 and strict-cliff RMSE, Spearman, and direction accuracy

Table 2. Distribution of the 7,977 selectivity cliffs after pair-first scaffold-closure splitting. Selectivity cliffs were defined as structurally similar cross-class pairs with an absolute selectivity difference greater than or equal to 1.0 log unit. Ratios within each split were calculated relative to the number of selectivity cliffs in the corresponding partition, whereas ratios of all pairs were calculated relative to all 7,977 selectivity cliffs

Split	Selectivity-cliff type	Count	Ratio within split	Ratio of all pairs
All	AChE-selective—balanced	4,344	54.46%	54.46%
All	BuChE-selective—balanced	2,928	36.71%	36.71%
All	AChE-selective—BuChE-selective polarity reversal	705	8.84%	8.84%
Train	AChE-selective—balanced	3,298	56.25%	41.34%
Train	BuChE-selective—balanced	2,141	36.52%	26.84%
Train	AChE-selective—BuChE-selective polarity reversal	424	7.23%	5.32%
Validation	AChE-selective—balanced	596	56.23%	7.47%
Validation	BuChE-selective—balanced	317	29.91%	3.97%
Validation	AChE-selective—BuChE-selective polarity reversal	147	13.87%	1.84%
Test	BuChE-selective—balanced	470	44.59%	5.89%
Test	AChE-selective—balanced	450	42.69%	5.64%
Test	AChE-selective—BuChE-selective polarity reversal	134	12.71%	1.68%

(1.071 ± 0.044) and the highest selectivity Spearman correlation (0.442 ± 0.054), outperforming all baseline models with the scaffold-closure split. MLP resulted in the next-best selectivity RMSE (1.145 ± 0.031), while XGB resulted in the second-highest selectivity Spearman correlation (0.366 ± 0.042). These results indicate that SPaR-GNN better preserves the relative relationship between AChE and BuChE activities when generalizing to unseen chemotypes, despite its weaker performance on the individual target regression tasks.

This distinction is important because accurate prediction of AChE or BuChE activity alone does not necessarily translate into accurate estimation of their relative selectivity. By incorporating selectivity-aware and pair-aware objectives during training, SPaR-GNN is designed to prioritize dual-target relationships rather than solely minimizing independent single-target prediction errors. The scaffold-split results therefore support the intended role of SPaR-GNN as a selectivity-oriented model, with its clearest advantage observed in selectivity prediction rather than individual target activity prediction.

Overall, these results demonstrate that the primary strength of SPaR-GNN lies in modeling selectivity across unseen chemotypes. This property is particularly relevant to downstream lead optimization, where reliable ranking and prioritization of compounds according to their selectivity profiles may be more informative than marginal improvements in single-target prediction accuracy.

3.3. SPaR-GNN captures pairwise selectivity relationships

Single-molecule metrics do not fully assess a model's ability to distinguish structurally related analogs with different selectivity profiles. We therefore evaluated all models on three nested pair-sensitive test subsets: structurally similar pairs, selectivity cliffs, and strict selectivity cliffs. Structurally similar pairs were defined using a Morgan fingerprint Tanimoto similarity threshold of 0.60. Selectivity cliffs were structurally similar cross-class pairs with an absolute selectivity difference greater than or equal to 1.0 log unit, whereas strict selectivity cliffs were the AChE-selective–BuChE-selective polarity-reversal subset.

The Similar Dir. Acc. metric was evaluated for all 3,373 structurally similar test pairs. The cliff RMSE, Spearman correlation, and direction accuracy metrics were evaluated for the 1,054 test selectivity cliffs. The strict-cliff RMSE, Spearman correlation, and direction accuracy metrics were evaluated for the 134 test strict selectivity cliffs.

To assess robustness to random initialization and stochastic training, all models were evaluated using multiple independent random seeds. The resulting mean ± standard deviation values are summarized in Table 5. With a scaffold-closure split, SPaR-GNN achieved the best mean performance across all seven pair-sensitive metrics. With the 3,373 structurally similar test pairs, SPaR-GNN achieved the highest direction accuracy (0.708 ± 0.021). With the 1,054 selectivity cliffs, it resulted in the lowest RMSE (2.083 ± 0.076), the highest Spearman correlation (0.646 ± 0.053), and the highest direction accuracy (0.742 ± 0.023). The same advantage was retained with the 134

Table 3. Leakage checks for the pair-first scaffold-closure split. All checks passed, indicating no molecule-, SMILES-, scaffold-, or pair-endpoint-level leakage between the training and test partitions

Level	Check	Key	Total/ Train	Test	Leakage/ Inconsistent	Status
Molecule	Cross-split uniqueness	mol_id	3085	-	0	PASS
Molecule	Train—test intersection	mol_id	2289	399	0	PASS
Molecule	Cross-split uniqueness	smiles_can	3085	-	0	PASS
Molecule	Train—test intersection	smiles_can	2289	399	0	PASS
Molecule	Cross-split uniqueness	smiles_std	3085	-	0	PASS
Molecule	Train—test intersection	smiles_std	2289	399	0	PASS
Scaffold	Cross-split uniqueness	scaffold	1318	-	0	PASS
Scaffold	Train—test intersection	scaffold	1077	138	0	PASS
Pair	Similar-pair split consistency with molecule split	-	24452	-	0	PASS
Pair endpoint	Test similar-pair endpoints in training molecules	mol_id	-	3373 pairs/397 endpoints	0	PASS
Selectivity cliff	Cliff split consistency with molecule split	-	7977	-	0	PASS
Cliff endpoint	Test-cliff endpoints in training molecules	mol_id	-	1054 pairs/230 endpoints	0	PASS

Table 4. Single-molecule multitask prediction results with a scaffold split. Results are expressed as the mean ± standard deviation on the test set over ten independent seeds. A lower RMSE indicates better regression accuracy, while a higher Spearman correlation indicates better rank consistency. The best result in each column is shown in bold

Model	AChE RMSE	AChE Spearman	BuChE RMSE	BuChE Spearman	Selectivity RMSE	Selectivity Spearman
XGB	**1.138 ± 0.019**	**0.723 ± 0.018**	**1.149 ± 0.023**	0.619 ± 0.029	1.152 ± 0.029	0.366 ± 0.042
MLP	1.208 ± 0.061	0.646 ± 0.025	1.180 ± 0.039	**0.642 ± 0.022**	1.145 ± 0.031	0.345 ± 0.044
GNN	1.471 ± 0.090	0.416 ± 0.067	1.231 ± 0.084	0.613 ± 0.051	1.247 ± 0.088	0.206 ± 0.115
GINE	1.488 ± 0.099	0.397 ± 0.064	1.278 ± 0.091	0.594 ± 0.051	1.214 ± 0.094	0.293 ± 0.089
SPaR-GNN	1.889 ± 0.221	0.523 ± 0.073	1.851 ± 0.225	0.590 ± 0.060	**1.071 ± 0.044**	**0.442 ± 0.054**

Table 5. Multi-seed comparison of baseline models and SPaR-GNN for pair-sensitive benchmarks with a scaffold-closure split. Similar direction accuracy was evaluated for all 3,373 structurally similar test pairs with a Morgan fingerprint Tanimoto similarity greater than or equal to 0.60. Cliff RMSE, Spearman correlation, and direction accuracy were evaluated for the 1,054 test selectivity cliffs. Strict-cliff metrics were evaluated for the 134 AChE-selective–BuChE-selective polarity-reversal pairs. Results are expressed as the mean ± standard deviation over independent random seeds. A lower RMSE indicates better pairwise regression accuracy, whereas a higher Spearman correlation and direction accuracy indicate better preservation of pairwise selectivity ordering. The best result in each metric column is shown in bold

Model	Similar Dir. Acc.	Cliff RMSE	Cliff Spearman	Cliff Dir. Acc.	Strict Cliff RMSE	Strict Cliff Spearman	Strict Cliff Dir. Acc.
XGB	0.618 ± 0.044	2.319 ± 0.051	0.380 ± 0.088	0.636 ± 0.053	3.063 ± 0.077	0.340 ± 0.076	0.775 ± 0.064
MLP	0.644 ± 0.029	2.282 ± 0.043	0.457 ± 0.061	0.659 ± 0.036	3.021 ± 0.075	0.413 ± 0.068	0.824 ± 0.044
GNN	0.560 ± 0.072	2.435 ± 0.091	0.208 ± 0.195	0.565 ± 0.085	3.409 ± 0.147	0.069 ± 0.201	0.609 ± 0.107
GINE	0.569 ± 0.068	2.408 ± 0.088	0.233 ± 0.167	0.573 ± 0.080	3.343 ± 0.147	0.136 ± 0.185	0.637 ± 0.107
SPaR-GNN	**0.708 ± 0.021**	**2.083 ± 0.076**	**0.646 ± 0.053**	**0.742 ± 0.023**	**2.813 ± 0.115**	**0.592 ± 0.076**	**0.880 ± 0.021**

Table 6. Ablation and baseline comparison of pair-sensitive benchmarks with a scaffold-closure split. All results are expressed as the mean ± standard deviation over 100 random seeds. Similar direction accuracy was evaluated for all 3,373 structurally similar test pairs. Cliff metrics were evaluated for the 1,054 test selectivity cliffs, and strict-cliff metrics were evaluated for the 134 test AChE-selective–BuChE-selective polarity-reversal pairs. Independent single-task GINE trains separate AChE and BuChE predictors and computes selectivity from their prediction difference. Parameter-matched GINE uses the same encoder and prediction heads as SPaR-GNN but removes pair-aware losses. Pairwise Morgan-XGB directly predicts pairwise selectivity differences from Morgan fingerprint pair features. A lower RMSE indicates better pairwise selectivity-difference regression, whereas a higher Spearman correlation and direction accuracy indicate better preservation of pairwise selectivity ordering. The best result in each metric column is shown in bold

Model/ Variant	Similar Dir. Acc.	Cliff RMSE	Cliff Spearman	Cliff Dir. Acc.	Strict Cliff RMSE	Strict Cliff Spearman	Strict Cliff Dir. Acc.
Full model SPaR-GNN	**0.708 ± 0.021**	**2.083 ± 0.076**	**0.646 ± 0.053**	**0.742 ± 0.023**	**2.813 ± 0.115**	**0.592 ± 0.076**	**0.880 ± 0.021**
Reviewer-requested baselines							
Independent single-task GINE	0.615 ± 0.048	2.368 ± 0.092	0.328 ± 0.163	0.630 ± 0.057	3.284 ± 0.134	0.216 ± 0.183	0.699 ± 0.105
Parameter-matched GINE w/o pair-aware loss	0.636 ± 0.054	2.307 ± 0.093	0.419 ± 0.156	0.658 ± 0.067	3.150 ± 0.152	0.306 ± 0.170	0.744 ± 0.107
Pairwise Morgan-XGB	0.596 ± 0.020	2.579 ± 0.026	0.053 ± 0.020	0.623 ± 0.024	3.443 ± 0.039	-0.095 ± 0.034	0.710 ± 0.039
SPaR-GNN component ablations							
w/o direct selectivity supervision	0.604 ± 0.059	2.286 ± 0.141	0.366 ± 0.175	0.617 ± 0.071	3.126 ± 0.212	0.283 ± 0.215	0.698 ± 0.117
w/o selectivity consistency loss	0.658 ± 0.047	2.196 ± 0.120	0.517 ± 0.139	0.684 ± 0.056	2.996 ± 0.171	0.451 ± 0.144	0.795 ± 0.073
w/o batch-level ranking loss	0.661 ± 0.045	2.195 ± 0.114	0.522 ± 0.139	0.686 ± 0.055	2.992 ± 0.167	0.470 ± 0.152	0.803 ± 0.074
w/o explicit pair ranking loss	0.661 ± 0.042	2.192 ± 0.108	0.524 ± 0.125	0.687 ± 0.049	2.981 ± 0.162	0.467 ± 0.152	0.798 ± 0.075
w/o pair-difference regression	0.631 ± 0.050	2.287 ± 0.093	0.409 ± 0.137	0.652 ± 0.060	3.083 ± 0.157	0.321 ± 0.153	0.751 ± 0.094
w/o cliff-pair weighting	0.649 ± 0.044	2.242 ± 0.098	0.478 ± 0.131	0.672 ± 0.054	3.058 ± 0.143	0.400 ± 0.143	0.774 ± 0.076

strict selectivity cliffs, as indicated by the lowest RMSE (2.813 ± 0.115), the highest Spearman correlation (0.592 ± 0.076), and the highest direction accuracy (0.880 ± 0.021).

These results show that SPaR-GNN improves both the numerical prediction and ordering of pairwise selectivity changes under scaffold-controlled generalization. In contrast to the conventional baselines, which exhibited larger performance losses with cliff-like pairs, SPaR-GNN maintained a stronger rank consistency and direction discrimination while also reducing pairwise regression error. The consistent advantage across RMSE, Spearman correlation, and direction accuracy indicates that the improvement is not restricted to a single aspect of pairwise prediction.

Notably, the fingerprint-based MLP was the strongest conventional baseline for most scaffold-split pair-sensitive metrics, outperforming the conventional GNN and GINE models. Nevertheless, SPaR-GNN consistently surpassed MLP across all seven metrics, including the more challenging strict selectivity-cliff benchmark. Together with the single-molecule results, these findings indicate that graph representation alone does not account for the performance of SPaR-GNN and they prompt further evaluation of the selectivity- and pair-aware components of the framework.

3.4. Ablation study of SPaR-GNN components

To assess the contribution of individual SPaR-GNN components, we performed a systematic ablation study with the scaffold-closure split using the same evaluation protocol and 100 random seeds. As summarized in Table 6, the full SPaR-GNN achieved the best mean performance across all seven pair-sensitive metrics, including a cliff RMSE of 2.083, a Spearman correlation of 0.646, and a direction accuracy of 0.742 and a strict-cliff RMSE of 2.813, a Spearman correlation of 0.592, and a direction accuracy of 0.880.

Among the evaluated components, direct selectivity supervision and pair-difference regression displayed the largest contributions. Removing direct selectivity supervision reduced cliff and strict-cliff Spearman

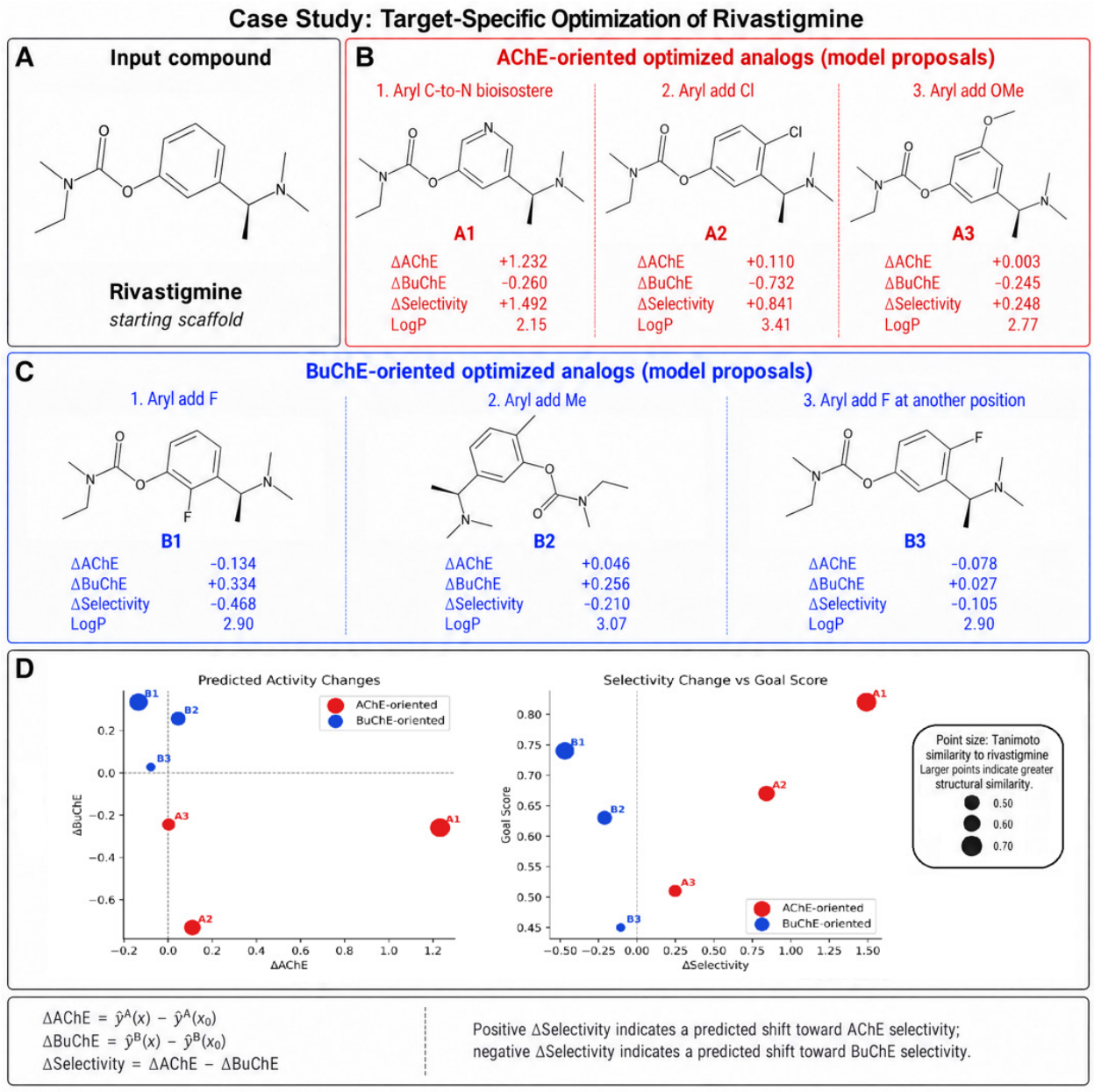


Figure 5. Optimization of compound rivastigmine. A, Rivastigmine was used as the input molecule for template-based local analog generation. **B,** Top-ranked candidates pursuant to the AChE-oriented optimization objective. **C,** Top-ranked candidates pursuant to the BuChE-oriented optimization objective. **D,** Distribution of prioritized candidates according to predicted changes in AChE and BuChE activity (left) and predicted selectivity change versus goal score (right). ΔAChE and ΔBuChE denote differences in predicted pIC_{50} relative to the input molecule, and ΔSelectivity = ΔAChE - ΔBuChE. Positive ΔSelectivity indicates a predicted shift toward AChE selectivity, whereas negative values indicate a predicted shift toward BuChE selectivity. Red and blue points represent candidates prioritized pursuant to the AChE- and BuChE-oriented objectives, respectively. Point size represents Tanimoto similarity to rivastigmine, with larger points indicating greater structural similarity.

correlations, as did removing pair-difference regression. Removal of the consistency, ranking, or cliff-weighting objectives also consistently degraded pair-sensitive performance, indicating complementary contributions from these components.

The full SPaR-GNN also substantially outperformed the independent single-task GINE, parameter-matched GINE, and Pairwise Morgan-XGB baselines, particularly in terms of rank consistency for the cliff and strict-cliff subsets. These results suggest that the performance gains are not attributable to model capacity or pairwise inputs alone but are associated with the combined contribution of the selectivity- and pair-aware objectives under scaffold-level generalization.

3.5. Goal-directed optimization of rivastigmine analogs

Rivastigmine was selected as a representative starting scaffold to demonstrate objective-dependent candidate generation and ranking under two predefined scoring configurations: AChE-oriented and BuChE-oriented objectives (Figure 5).

Under the AChE-oriented configuration, the highest-ranked analog was generated by an aryl C-to-N bioisosteric replacement and showed predicted changes of ΔAChE = +1.232, ΔBuChE = -0.260, and ΔSelectivity = +1.492. Aromatic chlorination and methoxy substitution also produced candidates with positive predicted selectivity shifts, although smaller than that of the top-ranked analog (Figure 5B).

Under the BuChE-oriented configuration, a different set of analogs was prioritized, including fluorine- and methyl-substituted candidates (Figure 5C). The highest-ranked candidate showed predicted changes of ΔBuChE = +0.334, ΔAChE = -0.134, and ΔSelectivity = -0.468. Other highly ranked candidates displayed similar model-predicted trends consistent with the specified objective.

The two scoring configurations produced clearly separated candidate rankings (Figure 5D). AChE-oriented candidates were characterized by positive predicted Δ AChE and selectivity shifts, whereas BuChE-oriented candidates tended toward positive predicted ΔBuChE and negative selectivity shifts, demonstrating objective-dependent prioritization from the same starting scaffold.

Top-ranked candidates retained moderate similarity to rivastigmine, with a Tanimoto similarity generally ranging from approximately 0.6 to 0.7, indicating local rather than extensive scaffold modification. This case study therefore serves as a demonstration of the workflow for objective-dependent candidate generation and prioritization. Because ranking was based on predictions from SPaR-GNN, these results do not constitute experimental evidence of improved activity, selectivity, pharmacokinetic properties, or therapeutic potential.

4. Discussion

Computational approaches for cholinesterase inhibitor discovery have conventionally focused on single-target activity, whereas AD lead optimization may require consideration of both AChE and BuChE activity and their relative selectivity. BuChE activity may become increasingly important during AD progression as AChE activity declines in later stages (*37*). We therefore developed SPaR-GNN to jointly model AChE activity, BuChE activity, and selectivity through multitask prediction combined with selectivity- and pair-aware supervision.

Results indicated that the main advantage of SPaR-GNN lies in selectivity modeling rather than isolated single-target prediction. Under scaffold-controlled evaluation, the fingerprint-based MLP remained competitive and outperformed the conventional GNN and GINE baselines on several single-molecule metrics, indicating that graph representation alone does not guarantee improved generalization to unseen chemotypes. SPaR-GNN likewise did not outperform conventional baselines on individual AChE or BuChE prediction but did achieve the best performance in terms of single-molecule selectivity. Together with the ablation results, this suggests that its advantage is more closely associated with the selectivity- and pair-aware learning framework than with the use of a graph architecture alone. This distinction is relevant to medicinal chemistry, where optimization often depends on shifting the relative activity between targets rather than maximizing either target independently.

The scaffold-closure split provided a stringent assessment of generalization by eliminating train-test overlap at the molecule, SMILES, scaffold, and pair-endpoint levels. This is particularly important for pair-sensitive evaluation because structural overlap can otherwise lead to optimistic estimates of performance (*10*). In this setting, SPaR-GNN displayed good pair-sensitive performance, substantiating its ability to prioritize selectivity changes among structurally related compounds beyond conventional pointwise regression.

The ablation study further corroborated the contribution of the combined framework. The full SPaR-GNN achieved the best mean performance across all evaluated pair-sensitive metrics, while removal of individual selectivity- or pair-aware objectives consistently reduced performance. Direct selectivity supervision and pair-difference regression produced particularly large effects on cliff and strict-cliff rank consistency, while consistency, ranking, and cliff-weighting objectives provided complementary contributions. The superiority of the full model over independent single-task GINE, parameter-matched GINE without pair-aware losses, and Pairwise Morgan-XGB further suggests that the observed gains arise from the combined selectivity- and pair-aware formulation rather than model capacity or pairwise input alone.

SPaR-GNN was further integrated into a goal-directed molecular optimization workflow combining template-based local editing, physicochemical filtering, goal-aware scoring, and candidate ranking. In a rivastigmine case study, AChE- and BuChE-oriented objectives produced distinct candidate rankings from the same starting scaffold while retaining relatively local structural modifications. Because candidate generation was restricted to a predefined template library and ranking relied on predictions from the same SPaR-GNN model, this analysis should be interpreted as a demonstration of the workflow for objective-dependent candidate generation and prioritization rather than evidence of improved pharmacological properties, experimentally validated structure-activity relationships, or superior compounds.

Nonetheless, there were several limitations. First, ChEMBL and BindingDB contain measurements obtained under heterogeneous experimental protocols and assay conditions (*17,18*). Although data standardization and quality control were implemented, residual variability may propagate into selectivity labels because selectivity is calculated based on both AChE and BuChE measurements. Experimentally harmonized dual-target datasets would therefore enhance future selectivity modeling. Second,

the proposed candidates are computational predictions and need to be validated experimentally. Third, the predefined medicinal chemistry template library improves interpretability and chemical plausibility but restricts accessible chemical space. Future work could expand this space using reaction-based generation, reinforcement learning, or large language model-guided molecular design (*38,39*).

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Original Article

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Clinicopathological characterization and prognostic factors of H3 K27M-altered diffuse midline gliomas: A real-world cohort study

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SUMMARY: Diffuse midline glioma (DMG) is a high-grade malignant brain tumor. The 5-year survival rate for DMG remains close to zero. It is a rare and highly aggressive tumor, which is more common in children than in adults. We here report our retrospective study of 210 cases of DMG. Surgical tumor samples were analyzed by routine histopathology and immunohistochemistry for H3 K27M, ATRX, p53, OLIG2 and Ki-67. The most frequent anatomic locations in patients were the thalamus and brainstem. During follow-up, 169/210 patients (80.5%) died from the disease, with a median survival time of 11 months (range: 0 to 87 months). Kaplan-Meier analysis indicated that the prognosis of adult patients was better than that of the pediatric cohort ($P = 0.006$). Multivariable Cox regression identified male sex, spinal cord tumor location, and a Ki-67 index of ≤ 10% as independent favorable prognostic factors. Also, meta-analysis indicated that the prognosis of adult patients was better than that of the pediatric cohort ($P = 0.002$), patients with brainstem tumors had a worse prognosis compared to those with tumors in other locations ($P = 0.003$). We highlight the aggressive nature of DMG and the critical role of clinical and molecular markers in prognosis. These findings demonstrate substantial clinicopathological heterogeneity in H3 K27M-altered DMG and support the combined consideration of age, tumor location, and proliferative activity in future prognostic stratification. Prospective multicenter validation and more comprehensive molecular profiling are required.

Keywords: diffuse midline glioma, prognostic factors, clinicopathological characteristics

1. Introduction

H3 K27-altered diffuse midline gliomas (DMG) are aggressive and currently incurable intracranial gliomas, characterized by high levels of intrinsic resistance to multiple therapies (*1*). In 2021, the 5th edition of the WHO Classification of Tumors of the Central Nervous System redefined these tumors as "Diffuse midline glioma, H3 K27-altered," reflecting the recognition of multiple underlying mechanisms beyond mutation. This classification incorporates various molecular alterations, primarily involving changes in H3 K27, TP53, ACVR1, PDGFRA, EGFR, and EZHIP (*2*). H3 K27-altered DMG predominantly occurs in pediatric populations and is infrequently observed in adults, comprising approximately 50% of all pediatric high-grade gliomas (HGGs). This malignancy is associated with a high mortality rate, characterized by a median overall survival (OS) of less than one year (*3*). In contrast, adult DMG represents a much smaller and less well-characterized subgroup, and its clinical behavior, molecular profile, and prognostic determinants remain incompletely understood. Despite increasing recognition of H3 K27-altered DMG, most published studies have focused on relatively small cohorts, predominantly pediatric populations, or isolated molecular features. Consequently, the integrated relationships among clinical presentation, histopathological characteristics, molecular markers, and patient outcomes remain insufficiently characterized, particularly in real-world clinical practice. This limitation hampers accurate prognostic stratification and the development of individualized management strategies.

The incidence of DMG is notably low, which often constrains the comprehensive analysis of its clinical characteristics and molecular mechanisms due to the limited number of cases. Consequently, it is crucial to conduct an in-depth examination of the key molecular

features of DMG to elucidate its pathogenesis and identify therapeutic targets, utilizing analyses derived from extensive case reports. Numerous therapeutic targets have been identified, with several undergoing rigorous preclinical investigation. Research has been conducted on targeting epigenetic modifications (*4*) (such as PRC2 and HDACs), growth factor receptors (*5*) (including EGFR and IGF1R), cell cycle checkpoints (*6*) (such as CDK4/6 and WEE1), DNA damage repair mechanisms (*7*) (including PARP and GD2), and immunotherapy. However, the efficacy of these targets remains uncertain. Importantly, the prognostic relevance of clinical and molecular factors may vary across patient subgroups, particularly between pediatric and adult DMG, potentially influencing therapeutic responsiveness and clinical trial stratification (*8,9*). Understanding the independent prognostic factors of DMG is essential for further exploration of personalized treatment efficacy targeting specific factors. This knowledge can guide the development of tailored therapeutic strategies for different patient populations.

In this study, we performed a comprehensive clinicopathological evaluation of 210 patients with H3 K27M-altered DMG using real-world clinical data collected from a large tertiary referral center. We systematically integrated clinical characteristics, histopathological findings, immunohistochemical profiles, survival analyses, and an external meta-analysis to comprehensively evaluate prognostic determinants of this rare tumor. By including both pediatric and adult patients within a single unified framework, this study provides clinically relevant evidence regarding age-related differences, molecular prognostic markers, and risk stratification, thereby helping bridge existing knowledge gaps and providing practical information for future personalized management of DMG.

2. Materials and Methods

2.1. Patient cohort

We retrospectively reviewed 210 patients with H3 K27M-altered DMG who were diagnosed and treated at Xiangya Hospital of Central South University between September 2016 and July 2023. Overall survival was defined as the interval from diagnosis to death or the last follow-up. The inclusion criteria were: 1. The tumor was located in a midline position. 2. The imaging and pathological diagnosis were consistent with DMG. The histopathologic findings were reviewed by 3 neuropathologists. This study retrospectively analyzed the clinical, imaging, pathological, and molecular characteristics of these patients. Among them, there were 102 male patients (48.6%) and 108 female patients (51.4%), with 122 adult patients (58.1%) and 88 pediatric patients (41.9%) (patients aged ≤ 18 years were classified as pediatric patients, whereas those aged > 18 years were classified as adults). A total of 199 patients (94.8%) underwent surgical resection, while the remaining 11 patients (5.2%) underwent stereotactic biopsy. All procedures involving human participants were conducted in accordance with the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (Approval number: 202210233). Informed consent was obtained from each patient or the patient's legal guardian for the conducted research. This study was reported in line with the PROCESS Guideline (*10*).

2.2. Immunohistochemistry

IHC was carried out as described previously (*11,12*). Tumor tissues were fixed in formalin, dehydrated, embedded in paraffin, and sectioned into 3–5 μm slices for hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining. After deparaffinization and rehydration, antigen retrieval was performed using sodium citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2). To minimize non-specific staining, sections were blocked with 10% goat serum at 37°C for 30 minutes. Subsequently, the sections were incubated with diluted primary antibodies overnight at 4°C (ZSGB-BIO, Beijing, China; GFAP: ZA-0529; Ki-67: ZM-0167; OLIG2: ZM-0169; ATRX: ZA-0016; p53: ZM-0408; H3 K27M: ZA-0321). The following day, biotin-labeled IgG secondary antibodies were applied for 30 minutes at room temperature. Staining was developed using DAB, followed by counterstaining with hematoxylin. Finally, slides were covered by coverslips using gelatin (*13*). Immunoreactivity was considered positive when nuclear staining of Ki-67, OLIG2, ATRX, p53, or H3 K27M was observed in tumor cells, whereas GFAP positivity was defined by cytoplasmic staining. Nuclear staining in tumor cells was assessed for both intensity and proportion. Tumors were considered p53-positive if ≥ 10% of tumor nuclei showed strong staining, and p53-negative if < 10% or absent staining. Ambiguous cases were independently reviewed by 3 neuropathologists.

2.3. MGMT methylation analysis

MGMT promoter methylation status was determined using methylation-specific PCR (MSP) on extracted tumor DNA (*14*). Tumors were classified as MGMT-methylated if the methylated allele was detectable according to the assay protocol, and unmethylated otherwise. Of the 210 cases, 97 cases were evaluable for MGMT methylation, with the remaining cases lacking sufficient DNA for analysis.

2.4. Meta-analysis

The literature search strategy was designed around the

PICOT (*15*) question format: Do DMG patients (Patient problem) with the H3 K27-altered, including sex, age and anatomic location of tumor, (Intervention) compared to those under different conditions (Comparator), have a different prognosis of survival (Outcome) in observational cohort studies (Type of study)? Electronic searches were performed using Ovid Embase, PubMed, and Web of Science from their database inception dates to June 2024. A complete record of search strings is provided in Supplementary Text 1 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*), and the PubMed search strategy is provided as an example. After excluding irrelevant studies, all data were extracted from article texts, tables, and figures with any estimates made based on the presented data and figures. Mean differences (MDs) in overall survival time and their corresponding 95% confidence intervals were extracted or estimated from the published text, tables, and figures and subsequently pooled in the meta-analysis. This was presented as a forest plot. The I^2 statistic was used to estimate the percentage of total variation across studies, owing to heterogeneity rather than chance, with values > 50% considered as substantial heterogeneity. Publication bias was assessed through the generation of funnel plots. All statistical analyses and bias in publication (Supplementary Figure S2, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*) were conducted with Review Manager (RevMan) version 5.2.

2.5. Statistical analysis

Survival data were evaluated utilizing Kaplan-Meier analysis in conjunction with the log-rank test. Differences between groups were assessed using the chi-square test and Fisher's exact test. Multivariable survival analysis was conducted employing the Cox proportional hazards model. For the Cox multivariable analysis, variables were selected based on their significance in univariable survival analyses. The proportional hazards assumption for all Cox regression models was assessed using Schoenfeld residuals tests. Both univariable and multivariable models were tested, with results reported as chi-square (χ^2) statistics with corresponding degrees of freedom (df) and *P*-values. A significance level of $P < 0.05$ was considered indicative of violation of the proportional hazards assumption (Supplementary Table S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). Continuous variables, including tumor diameter and Ki-67 labeling index, were converted into categorical variables to enhance clinical interpretability and facilitate risk stratification. Specifically, a tumor diameter cutoff of 3 cm was applied, consistent with its common use in prior clinical and radiological studies (*16*) to pragmatically distinguish smaller from larger lesions. Similarly, a Ki-67 labeling index cutoff of 10% (*17*). Statistical analyses were executed using SPSS version 26.0 and R software. Heatmap was plotted by *https://www.bioinformatics.com.cn* (last accessed on 10 Dec 2024), an online platform for data analysis and visualization. A p-value of less than 0.05 was deemed to indicate statistical significance.

3. Results

3.1. Clinical characteristics

The clinical characteristics of 210 patients diagnosed with H3 K27M-altered DMG are detailed in Table 1. The mean age of the cohort was 26.44 years, with a median age of 26.0 years, spanning a range from 3 to 68 years. The primary tumor sites included the brainstem (86 out of 210 patients, 41.0%), thalamus (78 out of 210 patients, 37.1%), and spinal cord (25 out of 210 patients, 11.9%) (Figure 1, Figure 4A). The sex distribution comprised 102 males (48.6%) and 108 females (51.4%), while the age distribution included 122 adults (58.1%) and 88 children (41.9%). A total of 199 patients (94.8%) underwent surgical resection, whereas the remaining 11 patients (5.2%) received stereotactic biopsy. In adult patients, the thalamus was identified as the most prevalent tumor location, while in pediatric patients, the brainstem was the most frequently affected site. Sex-based stratification revealed that the thalamus was the predominant tumor location in male patients, whereas in female patients, the brainstem was most involved. The mean tumor diameter was calculated to be 4.49 cm (Figure 2).

Prior research has identified a higher incidence rate among children as a notable clinical feature of DMG. In order to further investigate the clinical characteristics within this case series, we stratified and summarized the cases according to sex and age. Statistical analyses demonstrated that tumors in adults were primarily located in the thalamus, whereas pediatric tumors were predominantly situated in the brainstem. Additionally, the incidence of DMG in the spinal cord was greater in adults compared to children (Table 2). From a sex-based perspective, tumors in females were predominantly located in the brainstem, whereas those in males were primarily concentrated in the thalamus (Table 3).

3.2. Molecular characteristics of H3 K27M- altered DMG

In most cases (129 out of 209, representing 61.7%), the Ki-67 proliferation index exceeded 10%. ATRX immunohistochemistry was conducted in 161 of the 210 patients, revealing that 115 cases (71.4%) were positive, and 46 cases (28.6%) were negative. Regarding p53 immunohistochemistry (n=208), 103 tumors (49.5%) tested positive, while 105 tumors (50.5%) tested negative. H3 K27M immunohistochemistry (n = 208) demonstrated that 198 tumors (95.2%) were positive,

Table 1. Clinical and Molecular Characteristics of 210 H3 K27M-mt DMGs

Character	Number
Age	
Adult (age above 18 y)	122 (58.1%, *n* = 210)
Pediatric (age 18 y or younger)	88 (41.9%, *n* = 210)
Sex	
Female	108 (51.4%, *n* = 210)
Male	102 (48.6%, *n* = 210)
Anatomic location	
Brainstem	86 (41.0%, *n* = 210)
Thalamus	78 (37.1%, *n* = 210)
Spinal cord	25 (11.9%, *n* = 210)
Other	21 (10.0%, *n* = 210)
Tumor diameter (cm)	
≤ 3	42 (20.5%, *n* = 205)
> 3	163 (79.5%, *n* = 205)
Molecular characteristics	
ATRX expression	
Positive	115 (71.4%, *n* = 161)
Negative	46 (28.6%, *n* = 161)
P53	
Positive	103 (49.5%, *n* = 208)
Negative	105 (50.5%, *n* = 208)
H3 K27M	
Positive	198 (95.2%, *n* = 208)
Negative	10 (4.8%, *n* = 208)
Ki-67(%)	
≤ 10	80 (38.3%, *n* = 209)
> 10	129 (61.7%, *n* = 209)
GFAP expression	
Positive	205 (98.0%, *n* = 209)
Negative	4 (2.0%, *n* = 209)
IDH1 expression	
Positive	7 (3.3%, *n* = 208)
Negative	201 (96.7%, *n* = 208)
OLIG2 expression	
Positive	198 (97.5%, *n* = 203)
Negative	5 (2.5%, *n* = 203)
MGMT promoter	
Methylated	7 (7.2%, *n* = 97)
Unmethylated	90 (92.8%, *n* = 97)
Median overall survival(months)	11

with 10 tumors (4.8%) being negative. Lastly, GFAP immunohistochemistry (n = 209) indicated that 205 tumors (98.0%) were positive, while 4 tumors (2.0%) were negative. In contrast, OLIG2 immunohistochemistry conducted on 203 tumors revealed that 198 tumors (97.5%) were positive, with only 5 tumors (2.5%) showing negative results (Figure 3, Figure 4A).

3.3. Survival data and prognostic factors

Comprehensive follow-up data were obtained for the entire cohort of 210 patients. During follow-up, 169 patients (80.5%) died. The median follow-up duration among surviving patients was 34 months. The median survival time was calculated to be 11.0 months, while the mean survival time was 16.5 months. The overall 1-year survival rate for the cohort was 46.2%, and the 2-year survival rate was 22.9%. The age-stratified analysis indicated that adults exhibited a more favorable prognosis

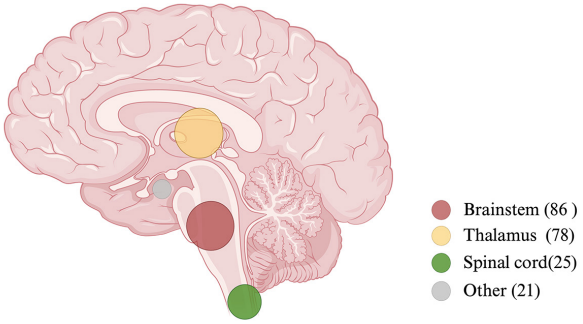


Figure 1. Anatomic and age distribution of H3 K27M-altered DMG.

than children (P = 0.006). No statistically significant difference in prognosis was observed between patients with tumor diameters greater than 3 cm and those with tumor diameters of 3 cm or less (P = 0.466). Analysis of tumor location demonstrated that individuals with brainstem tumors had a significantly poorer prognosis compared to those with tumors in other locations (P < 0.001). Furthermore, it was determined that males had a significantly better prognosis than females across the entire cohort (P = 0.014).

The data demonstrate that patients with p53-positive tumors exhibit a significantly poorer prognosis compared to those with p53-negative tumors (P = 0.003). Similarly, individuals with a Ki-67 index exceeding 10% have a worse prognosis than those with a lower Ki-67 index (P = 0.001). In contrast, no significant prognostic differences were identified based on ATRX status (positive or negative), H3 K27M status (positive or negative), OLIG2 status (positive or negative), GFAP status (positive or negative), or MGMT methylation status (methylated or unmethylated) (Figure 4B, Figure 5). Cases with missing data have been excluded from the analysis, and the missing ATRX and MGMT status data do not affect the conclusions (Supplementary Table S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). In addition, we analyzed the associations between age and sex and prognosis in patients with different tumor locations; however, no statistically significant results were observed (Supplementary Figure S3, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*).

Schoenfeld residuals tests supported the proportional hazards assumption for all Cox regression models. No significant violations were detected in univariable analyses (all P > 0.05), and the multivariable model global test was non-significant (χ^2 = 11.78, df = 7, P = 0.11) (Supplementary Table S2, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). In the multivariable Cox regression model, male sex (P = 0.023), spinal cord tumor location (P = 0.003), and a Ki-67 index of ≤ 10% (P = 0.039) were independently associated with favorable survival,

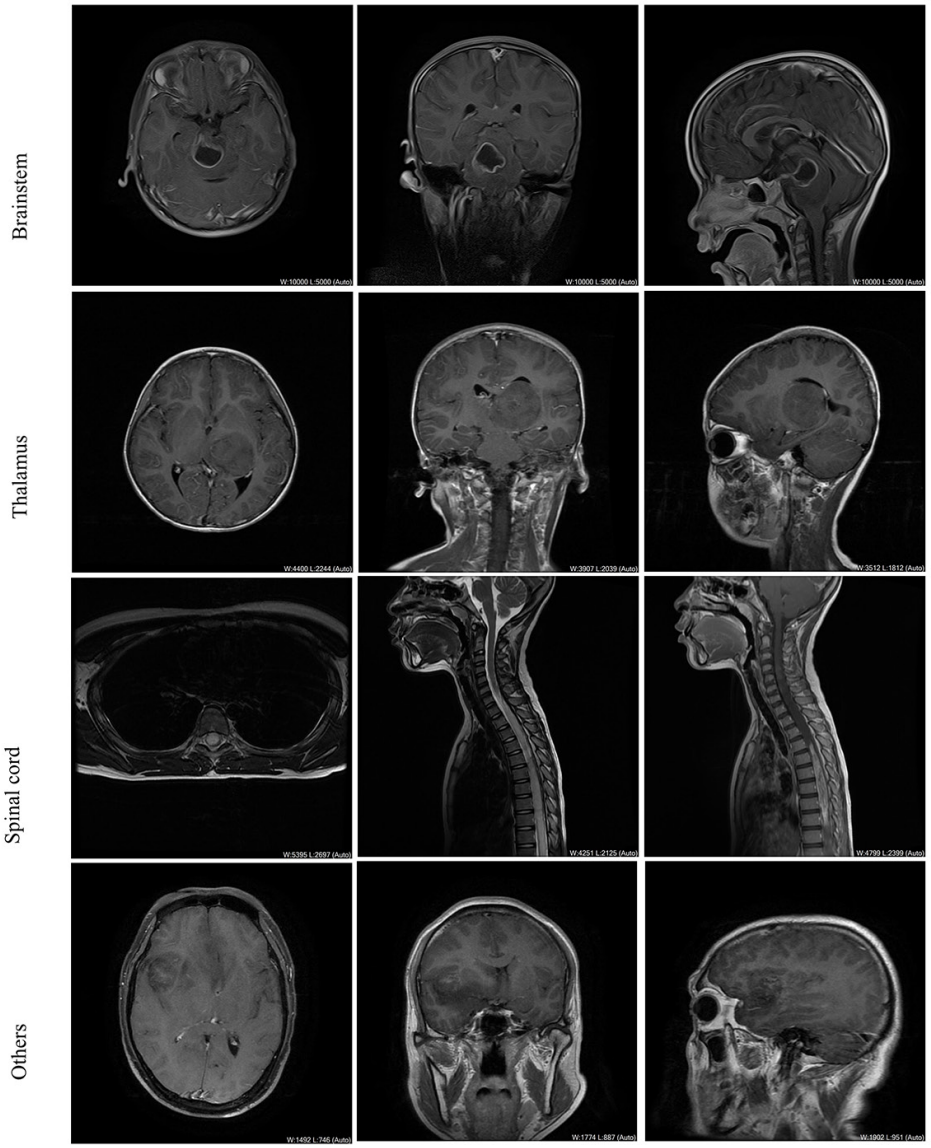


Figure 2. Representative imaging features of H3 K27M-altered DMG at different anatomical locations.

Table 2. Comparison of Clinical Characteristics Between Pediatric and Adult H3 K27M-altered DMG

	Adult (age above 18 y)	Pediatric (age 18 y or younger)	*P*-value
Case	122 (58.1%)	88 (41.9%)	0.069
Sex			
Female	56 (46.0%)	52 (59.1%)	
Male	66 (54.0%)	36 (40.9%)	
Anatomic location			< 0.001
Brainstem	32 (26.2%)	54 (61.4%)	0.014
Thalamus	54 (44.3%)	24 (27.3%)	0.029
Spinal cord	20 (16.4%)	5 (5.7%)	0.102
Other	16 (13.1%)	5 (5.7%)	0.157
Tumor diameter (cm)			
≤ 3	28 (23.7%)	13 (14.9%)	
> 3	90 (76.3%)	74 (85.1%)	

Table 3. Comparison of Clinical Characteristics Between Male and Female H3 K27M-altered DMG

	Female	Male	*P*-value
Case	108 (51.4%)	102 (48.6%)	
Age			0.069
Adult	56 (51.9%)	36 (35.3%)	
Pediatric	52 (48.1%)	66 (64.7%)	
Anatomic location			
Brainstem	54 (50.0%)	32 (31.4%)	0.008
Thalamus	33 (30.6%)	45 (44.1%)	0.046
Spinal cord	13 (12.0%)	12 (11.8%)	1.000
Other	8 (7.4%)	13 (12.7%)	0.251
Tumor diameter (cm)			0.862
≤ 3	22 (21.0%)	19 (19.0%)	
> 3	83 (79.0%)	81 (81.0%)	

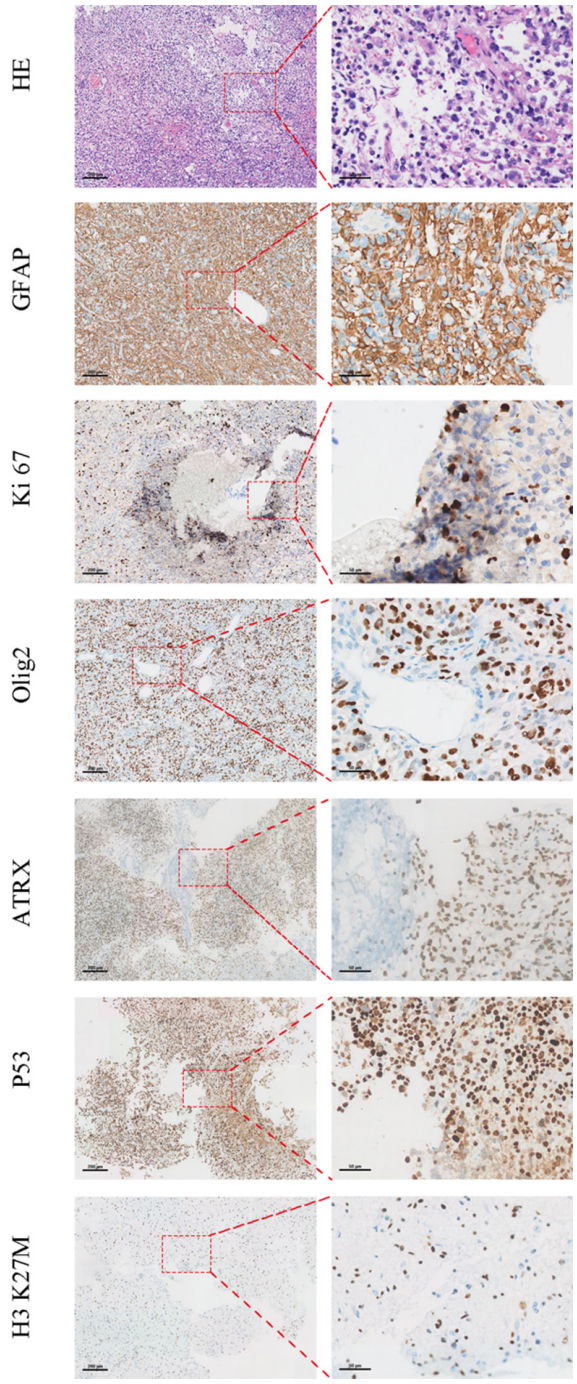


Figure 3. Representative hematoxylin and eosin (H&E) and immunohistochemical staining for GFAP, Ki-67, OLIG2, ATRX, p53, and H3 K27M.

whereas age and p53 status were not independently significant (Figure 6). To provide an exploratory visualization of the combined prognostic effects of the variables included in the multivariable Cox model, we constructed an overall survival (OS) nomogram. A higher total score, calculated as the sum of the scores assigned to each factor, corresponds to lower estimated 1-, 3-, and 5-year OS probabilities (Supplementary Figure S4, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). Because the predictive performance of this nomogram has not been independently validated, it should be regarded as a preliminary prognostic tool rather than a model ready for routine clinical use.

3.4. Meta-analysis

Although this study constructed the largest known cohort of DMG to date to explore its clinical prognostic factors, the single-center nature of the research may introduce potential bias. To assess the consistency of our cohort findings with previously published evidence, we performed a supportive meta-analysis of relevant studies. A comprehensive retrieval process yielded a total of 8,180 articles. Following a rigorous screening procedure, 21 articles were selected for inclusion in the meta-analysis (Supplementary Figure S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). The findings indicated that an age-stratified analysis demonstrated a significantly better prognosis for adults compared to children ($P = 0.002$, $I^2 = 47\%$). Furthermore, an analysis of tumor location revealed that patients with brainstem tumors exhibited a poorer prognosis relative to those with tumors situated in other locations ($P = 0.003$, $I^2 = 0\%$). No significant prognostic difference was observed between male and female patients ($P = 0.22$, $I^2 = 33\%$). The results of the analysis were illustrated using a forest plot (An I^2 value of less than 50% was considered indicative of acceptable heterogeneity, in which case the fixed effects model was employed; otherwise, the random effects model was adopted), and potential publication bias was evaluated through the construction of a funnel plot (Figure 7). Overall, the meta-analysis supported age and tumor location, but not sex, as comparatively reproducible prognostic factors across published cohorts.

4. Discussion

Diffuse midline gliomas constitute a heterogeneous category of tumors originating from the midbrain, pons, medulla, spinal cord, and thalamus and affect both pediatric and adult populations. These tumors exhibit diverse histological characteristics (*18*) and varying degrees of resectability, leading to heterogeneous clinical trajectories. The present study extends the current understanding of this heterogeneity by integrating clinicopathological variables, immunohistochemical markers, and survival outcomes in a large real-world cohort spanning both pediatric and adult patients. Rather than suggesting that prognosis is determined by any single factor, our findings indicate that survival may reflect the combined influence of patient characteristics, anatomical location, and tumor proliferative activity. Notably, the concordant associations of age and tumor location observed in both our cohort and the meta-analysis suggest that these variables may represent

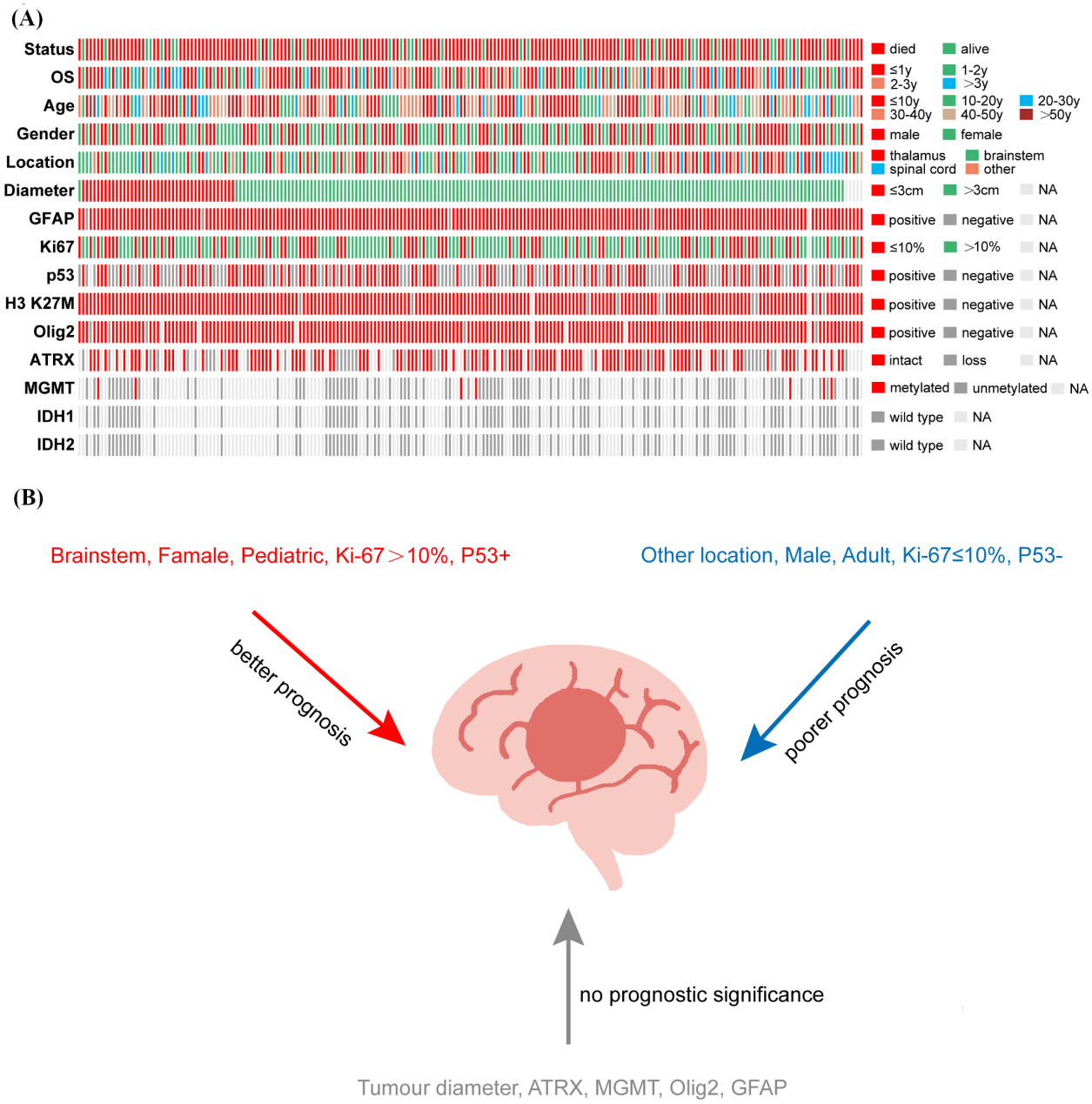


Figure 4. (A) Schematic summary of the clinical and pathological characteristics of 210 H3 K27M-altered DMG. (B) Impact of different variables on prognosis.

relatively robust prognostic indicators across different populations. In contrast, the sex-related association identified in the single-center cohort requires cautious interpretation and independent validation. These observations support a multidimensional approach to prognostic assessment and provide a rationale for incorporating routinely available clinicopathological variables into future risk-stratification frameworks.

Pediatric DMG has been closely associated with diffuse intrinsic pontine glioma, whereas advances in molecular classification have broadened the recognition of H3 K27-altered DMG across a wider age range and anatomical spectrum. Consistent with previous reports (*16*), tumors in adult patients in our cohort were more frequently located in the thalamus or spinal cord, whereas pediatric tumors were predominantly located in the brainstem. The more favorable survival observed in adults may therefore partly reflect differences in anatomical distribution, particularly the higher proportion of brainstem tumors among pediatric patients. However, because the location-stratified survival analyses did not reach statistical significance, the extent to which tumor location accounts for the age-related survival difference remains uncertain. Other age-associated biological or molecular differences may also contribute to the distinct clinical outcomes and warrant further investigation. These findings support the joint consideration of age and tumor location in prognostic assessment, while avoiding attribution of the survival difference to anatomical distribution alone.

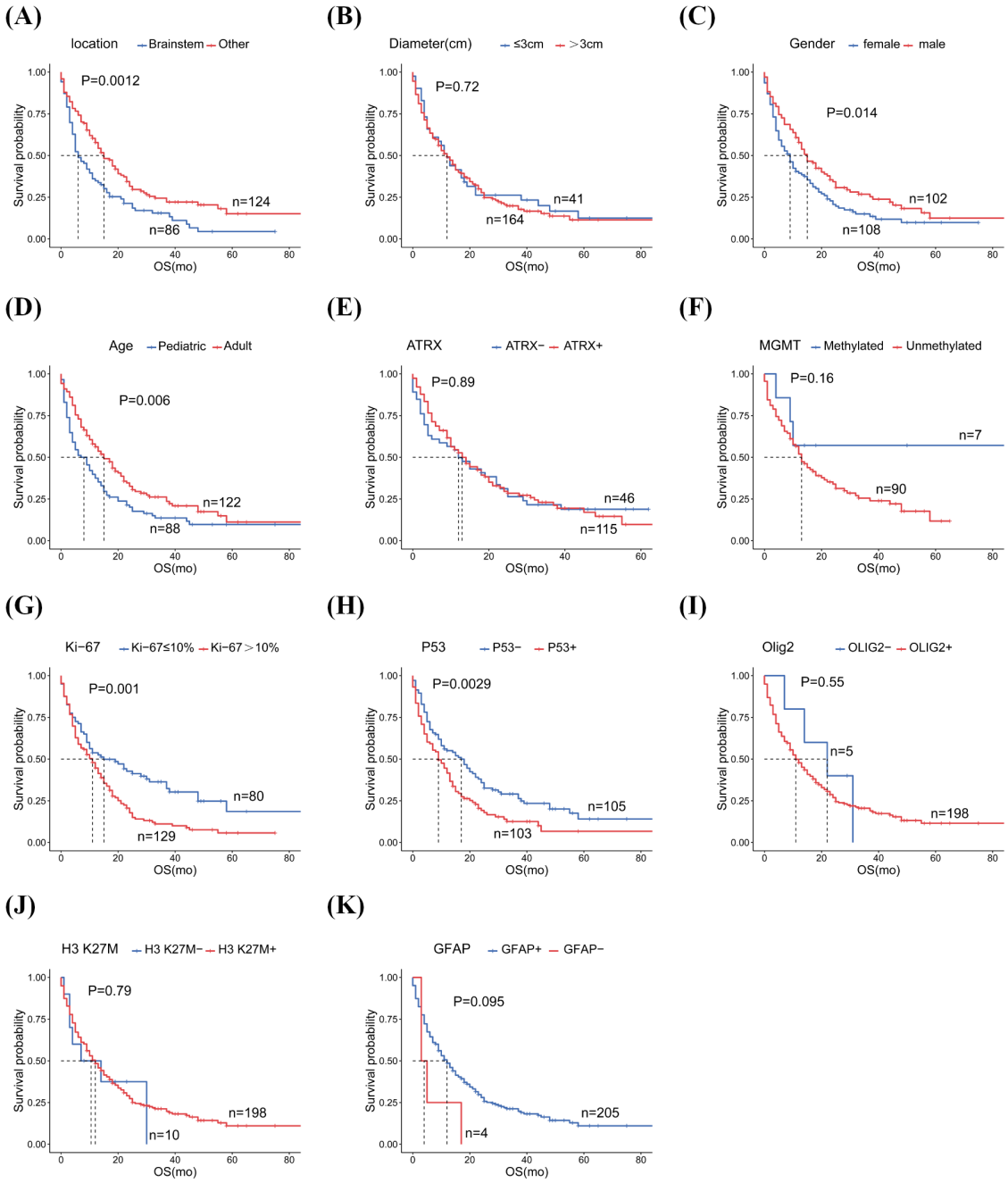


Figure 5. Kaplan–Meier curves for overall survival according to clinicopathological factors. Brainstem location **(A)**, female sex **(C)**, age ≤ 18 years **(D)**, a Ki-67 index > 10% **(G)**, and p53 positivity **(H)** were associated with poorer overall survival. Tumor diameter **(B)**, ATRX status **(E)**, MGMT promoter methylation status **(F)**, OLIG2 status **(I)**, H3 K27M status **(J)**, and GFAP status **(K)** were not significantly associated with overall survival.

Previous research did not recognize sex as a prognostic factor (*19*). However, our study found that males exhibited better overall survival than females. The observed survival advantage in males may be multifactorial. Male patients in our cohort more frequently presented with thalamic tumors, whereas females had a higher proportion of brainstem tumors, which are associated with poorer outcomes. This spatial distribution could partly explain the sex disparity in prognosis, although no significant association between sex and prognosis was observed among patients with tumors at different locations in our cohort (Supplementary Figure S3, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=311*). Prior studies have indicated a poorer prognosis for female pediatric patients compared to their male counterparts (*20*), and in our study, this observation extends across the entire age spectrum. Regarding sex differences in the incidence of gliomas and other cancers, extensive literature has documented these patterns (*21-23*), with some differences even linked to germline genetic variations (*24*). Previous studies in mouse models of glioma driven by Trp53 and

(A)

Variable	HR(95% CI)	*P* value
Age		
>18y	Reference	
≤18y	1.53 (1.13 to 2.07)	**0.006**
Gender		
Female	Reference	
Male	0.69 (0.51 to 0.93)	**0.015**
Tumor location		
Brainstem	Reference	
Thalamus	0.72 (0.52 to 1.01)	0.056
Spinal cord	0.32 (0.18 to 0.58)	**<0.001**
Other	0.66 (0.39 to 1.13)	0.129
Diameter		
>3cm	Reference	
≤3cm	0.93 (0.64 to 1.37)	0.727
ATRX expression		
Loss	Reference	
Intact	0.97 (0.66 to 1.44)	0.884
Ki67		
>10%	Reference	
≤10%	0.58 (0.42 to 0.81)	**0.001**
p53 positivity		
Negative	Reference	
Positive	1.59 (1.17 to 2.16)	**0.003**



(B)

Variable	HR(95% CI)	*P* value
Age		
>18y	Reference	
≤18y	1.14 (0.82 to 1.59)	0.442
Gender		
Female	Reference	
Male	0.69 (0.51 to 0.95)	**0.023**
Tumor location		
Brainstem	Reference	
Thalamus	0.81 (0.57 to 1.16)	0.251
Spinal cord	0.40 (0.22 to 0.74)	**0.003**
Other	0.75 (0.44 to 1.29)	0.299
Ki67		
>10%	Reference	
≤10%	0.70 (0.49 to 0.98)	**0.039**
p53 positivity		
Negative	Reference	
Positive	1.32 (0.95 to 1.83)	0.097

Figure 6. Univariable and multivariable Cox regression analyses of clinicopathological factors in H3 K27M-altered DMG. (A) Univariable analysis. **(B)** Multivariable analysis. Bold *P* values indicate statistical significance ($P < 0.05$).

Nf1 gene deletions reported longer survival in female mice, reflecting a sex-dimorphic survival pattern (*25*). In our study, sex as an independent prognostic factor may therefore have meaningful implications, though further validation in larger prospective cohorts is warranted.

In addition to demographic and anatomical factors, we evaluated the prognostic relevance of routinely available pathological markers. Among the pathological markers evaluated, Ki-67 provided the most consistent prognostic signal. This association is biologically plausible because a higher Ki-67 index reflects increased proliferative activity and may identify tumors with more aggressive growth (*16,26*). In contrast, p53 immunopositivity was associated with survival in univariable analysis but did not retain independent significance after adjustment. Because p53 staining is an indirect surrogate of TP53 pathway disturbance rather than a direct genomic measurement, its prognostic interpretation should remain cautious. ATRX, GFAP, and OLIG2 were not associated with survival, suggesting that markers useful for diagnostic or lineage characterization do not necessarily provide independent prognostic information.

Furthermore, the prognostic value of MGMT promoter methylation in H3 K27M-altered DMG remains inconclusive in the present study. Notably, MGMT promoter methylation was detected in only a small proportion of cases (7.2%), which substantially limits the statistical power to identify a potential survival difference between methylated and unmethylated tumors. Therefore, the absence of a significant association between MGMT methylation status and overall survival in our cohort should be interpreted with caution and should not be considered definitive evidence of a lack of prognostic relevance. Given the low prevalence of MGMT methylation in DMG and the limited sample size of methylated cases, larger multicenter cohorts or pooled molecular datasets will be required to more reliably evaluate its prognostic and therapeutic implications.

Currently, the primary treatment modalities for DMG include surgical resection and a combination of radiotherapy and chemotherapy (*27*). While surgical resection and focal radiotherapy can temporarily alleviate symptoms associated with DMG, they do not significantly extend patient survival (*28*). Nevertheless, a multicenter retrospective study conducted at Bellaria Hospital in Bologna, Italy, indicated that the concurrent administration of radiotherapy and chemotherapy following surgery is associated with improved patient survival outcomes (*29*). A subset of recently identified targeted therapies or modified drug regimens demonstrates potential therapeutic efficacy (*30*). Although immunotherapy has shown promise in treating non-central nervous system solid tumors, its effectiveness in intracranial tumors remains limited (*31*). To date, only therapies such as CAR-T-cell therapy (*32-34*) and virotherapy (*35,36*) have yielded promising outcomes in preliminary clinical trials. However, to substantiate these findings, further multicenter clinical trials are necessary, necessitating additional research to identify effective pharmacological interventions.

From a clinical perspective, age, tumor location, Ki-67 index, and p53 status are readily available variables that may complement molecular classification and clinical judgment when estimating prognosis; sex may be retained as an exploratory factor pending validation. Their combined use may support patient counseling, follow-up planning, and more balanced stratification in future biomarker-driven trials. Compared with

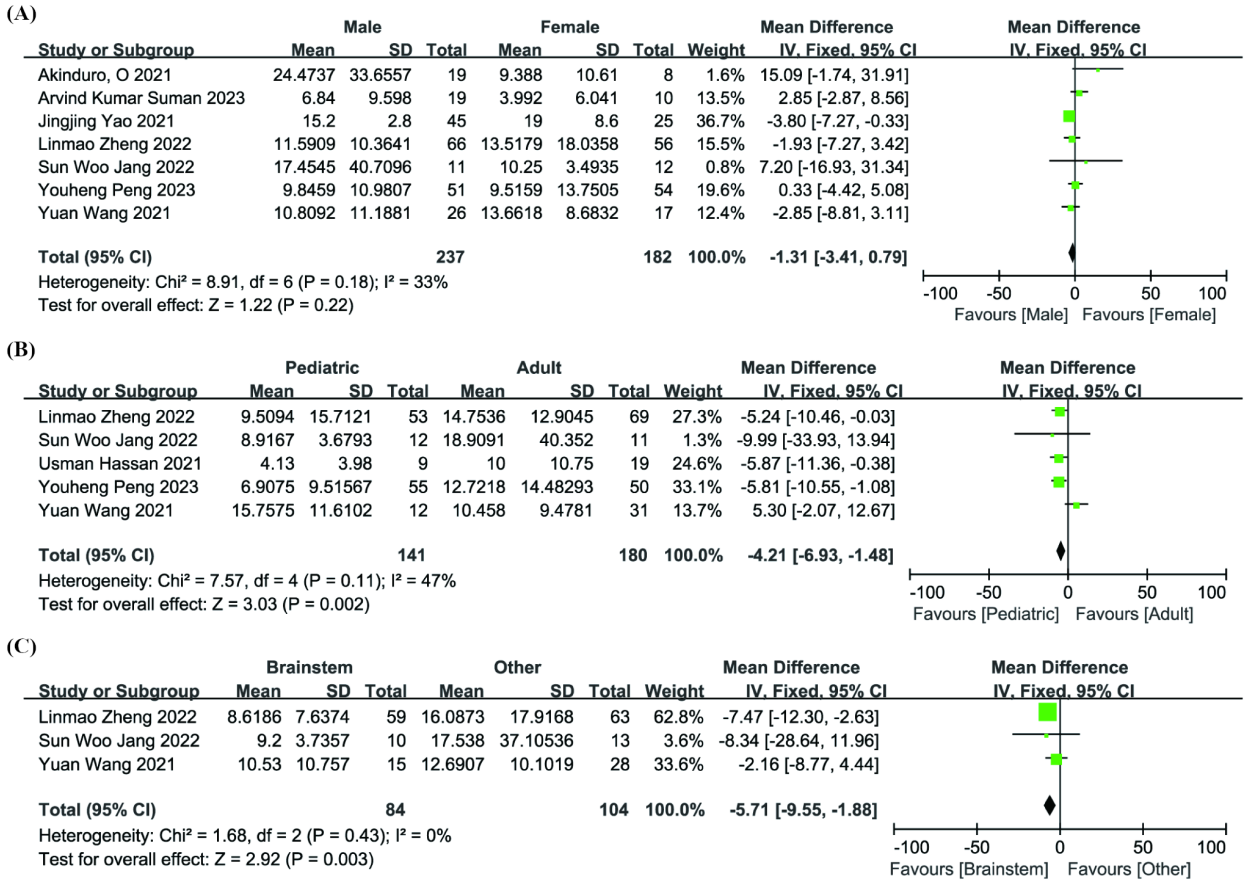


Figure 7. Forest plots of pooled mean differences in mean overall survival time according to sex (A), age group (B), and tumor location (C) in patients with H3 K27M-altered DMG.

previous reports (*16,19*), the major contribution of the present work lies not only in the relatively large cohort but also in the integration of clinicopathological characteristics, molecular marker evaluation, and long-term survival analyses within a real-world clinical setting. This comprehensive approach provides a more complete characterization of H3 K27-altered DMG across both pediatric and adult populations and offers clinically relevant evidence for prognostic assessment and risk stratification. Furthermore, the identified clinicopathological prognostic factors may facilitate risk-adapted patient management, support individualized clinical decision-making, and provide a practical foundation for future biomarker-driven clinical trials.

Nonetheless, our study is limited by its single-center retrospective design, the predominance of surgically resected tumors, limited statistical power for subgroup analyses, and the absence of more comprehensive genomic and epigenomic data, such as ACVR1 or PDGFRA mutations and histone modification profiles. Because next-generation sequencing and DNA methylation profiling were not available, the molecular characterization remained primarily limited to immunohistochemical markers. Consequently, we were unable to distinguish H3 molecular subtypes or comprehensively assess key co-occurring alterations such as ACVR1, FGFR1, TERT, and PDGFRA. Future studies incorporating comprehensive molecular profiling are warranted to address these limitations. In addition, detailed treatment-related variables, including extent of resection (biopsy/STR/GTR), postoperative radiotherapy, and chemotherapy, were not systematically available and therefore could not be incorporated into the multivariable Cox model. Consequently, residual confounding related to treatment modalities cannot be completely excluded and may have influenced the observed survival outcomes. The nomogram was also developed from the same single-center cohort, and its discrimination, calibration, and generalizability have not yet been fully evaluated. Prospective validation and independent external validation are therefore required before the model can be considered for routine clinical implementation. Furthermore, the meta-analysis was intended primarily as supportive validation of the single-center cohort rather than as an independent confirmatory analysis. Because it was conducted in a simplified manner and did not include formal quality assessment or detailed study-level summary tables, its findings should be interpreted as supportive rather than definitive evidence. This work is primarily based on clinical and statistical analyses, and no mechanistic experiments were performed to elucidate the underlying biological

pathways. Future multicenter studies integrating molecular, genomic, and therapeutic data are warranted to validate and expand upon these findings.

5. Conclusion

This study advances the current understanding of H3 K27-altered diffuse midline glioma by providing an integrated clinicopathological and prognostic evaluation of a large real-world cohort spanning pediatric and adult patients. Our findings highlight the combined prognostic relevance of patient age, tumor location, sex, p53 expression, and proliferative activity, while indicating that the strength and independence of these associations differ across variables. In particular, the concordant findings from the single-center cohort and meta-analysis support age and tumor location as comparatively reproducible prognostic factors, whereas the sex-related association should remain exploratory pending further validation. The identified clinicopathological factors are routinely available and may contribute to future clinical risk stratification, patient counseling, follow-up planning, and the design of more balanced biomarker-driven clinical trials. However, their clinical implementation requires validation in independent multicenter and prospective cohorts. Future studies integrating comprehensive genomic, epigenomic, and treatment-related data will be essential to refine prognostic models, clarify the biological basis of clinical heterogeneity, and support the development and evaluation of risk-adapted therapeutic strategies for H3 K27-altered DMG.

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

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Xiangya Hospital of Central South University (Approval number: 202210233). The studies were conducted following the institutional guidelines and local laws.

Data Availability statement: The data generated in this study are available within the article and its supplementary data files.

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