

The Multidimensional Regulatory Network of Osteoblast Differentiation and Targeted Therapeutic Strategies for OP

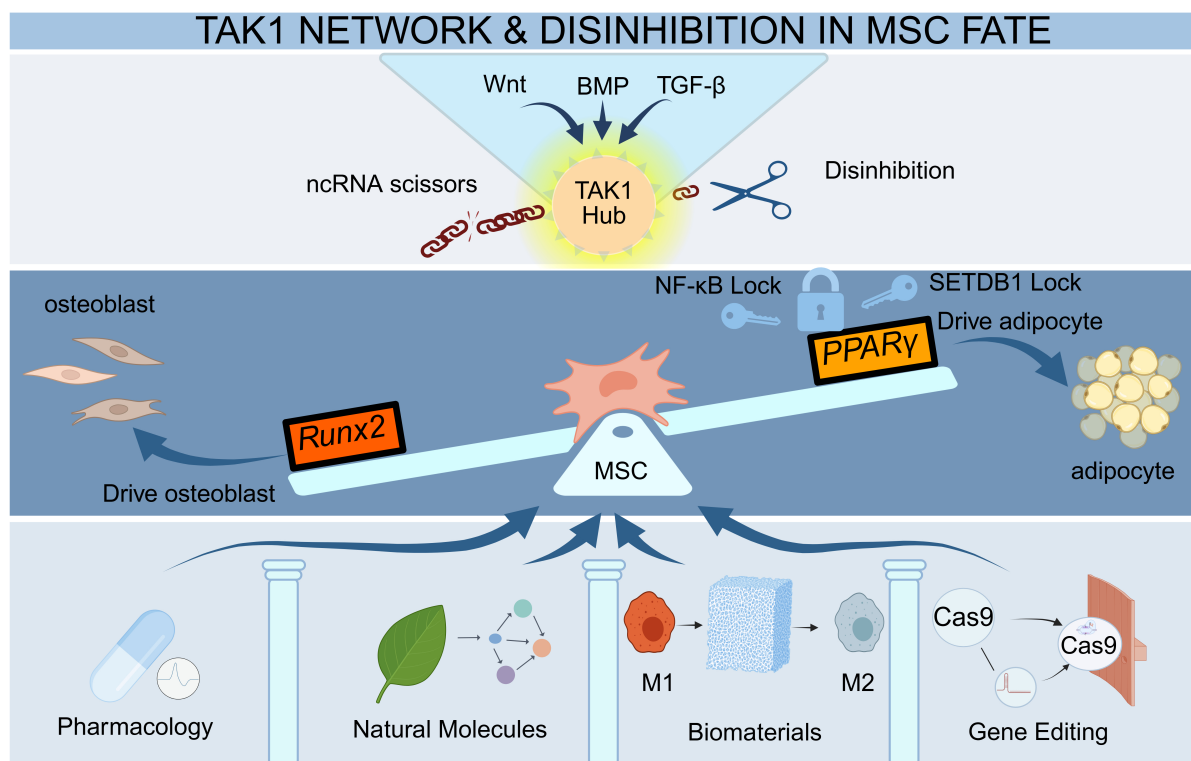
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Graphical Abstract



<https://doi.org/10.71321/rqm4ns12>

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The Multidimensional Regulatory Network of Osteoblast Differentiation and Targeted Therapeutic Strategies for OP

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Received: 2025-12-01 | Accepted: 2026-01-29 | Published online: 2026-03-09

Abstract

Osteoporosis (OP) represents a systemic failure of bone homeostasis, necessitating a therapeutic paradigm shift from broad-spectrum anti-resorptive agents to precision osteo-anabolic strategies. This review analyzes the multidimensional regulatory network governing osteoblast differentiation, emphasizing the transition from linear pathway descriptions to systems biology. We first elucidate the hierarchical integration of core signaling cascades—specifically the "context-dependent" crosstalk among Wnt, BMP, and TGF- β pathways—and their convergence on the master transcriptional hub, Runx2. A critical focus is placed on the pathological collapse of the Runx2-PPAR γ equilibrium, which drives the inverse trajectory of osteogenic attenuation and marrow adiposity. Furthermore, we dissect the post-transcriptional "disinhibition" logic mediated by non-coding RNAs as a pivotal epigenetic layer. Bridging mechanistic insights with clinical translation, we classify emerging targeted strategies into three precision dimensions: pathology-dependent pharmacological reprogramming, osteoimmunomodulatory biomaterials with spatiotemporal responsiveness, and "scarless" gene editing technologies. Finally, we critically address pivotal translational bottlenecks—specifically the delivery barriers across the mineralized matrix and off-target safety risks—proposing that future therapeutics must evolve toward the precision homeostatic remodeling of the skeletal microenvironment.

Keywords: OP; osteoblast; differentiation; signaling; therapeutic paradigm

Introduction

The skeletal system transcends its role as a mere physical framework for mechanical support to function as a highly active metabolic organ. Its physiological homeostasis hinges on the delicate dynamic equilibrium of bone remodeling—specifically, the tight coupling between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The uncoupling of this balance, driven by aging or hormonal fluctuations, precipitates OP, a condition characterized by systemic bone loss [1-2]. Clinically defined by reduced bone mass, microarchitectural deterioration, and a significantly elevated risk of fragility fractures, OP presents persistent therapeutic challenges. Current pharmacotherapies, such as bisphosphonates, are hampered by a lack of cellular specificity, leading to severe complications including osteonecrosis of the jaw [2]. To overcome these limitations, the therapeutic paradigm must shift from broad phenotypic inhibition to the precise dissection of deep molecular networks, thereby facilitating the development of novel osteo-anabolic strategies.

Emerging evidence highlights the profound "context-dependency" of osteogenic regulation. The dualistic "double-edged sword" nature of TGF- β —balancing homeostasis against pathological induction [3]—and the strict reliance of Wnt5a signaling flow on specific receptor combinations [4] underscore the inadequacy of traditional linear intervention strategies. Furthermore, extensive pathway crosstalk and endogenous feedback loops establish a highly intricate regulatory topology [5].

Consequently, this review systematically deconstructs the multidimensional network of osteogenic differentiation across three critical dimensions: core signaling integration, transcriptional hubs, and non-coding RNA regulation. Our objective is to provide a theoretical foundation for the next generation of precision bone regenerative therapies.

Pathogenesis of OP

The core of OP lies in the disruption of the balance between bone formation and bone resorption. This is not only due to the disorder of osteoblast differentiation, function and progen-

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itor cell pool, but also involves the vicious cycle such as the dysregulation of osteogenic and osteoclasts coupling and adipogenesis.

Overview of Osteoblast Differentiation and Osteogenic Function

Osteoblasts, the pivotal effectors of bone formation, originate from multipotent MSCs [6]. The differentiation of osteoblasts is a highly orchestrated, multi-stage cascade. Initiated by signaling pathways such as Bone Morphogenetic Protein (BMP) and Wnt, MSCs commit to the osteoprogenitor lineage and proliferate into pre-osteoblasts. Under the command of master transcriptional regulators Runx2 and Osterix, these cells ultimately mature into highly secretory osteoblasts [7]. Following completion of bone matrix synthesis, a subset of osteoblasts becomes embedded within the mineralized matrix and differentiates into osteocytes, which constitute the majority of bone cells. These osteocytes function as primary mechanosensors and central coordinators of signaling networks within bone tissue. The remaining osteoblasts are either eliminated through apoptosis or undergo phenotypic conversion into quiescent bone lining cells that cover the bone surface [8].

Functionally, osteoblasts are primarily responsible for synthesizing the organic matrix—predominantly Type I collagen—to construct the osteoid scaffold, while concurrently secreting non-collagenous proteins, such as osteocalcin and osteopontin, to modulate the local microenvironment. Subsequently, they drive mineralization by releasing calcium- and phosphate-rich matrix vesicles and expressing phosphatases, notably alkaline phosphatase (ALP). These mechanisms catalyze the ordered deposition of hydroxyapatite crystals within collagen fibrils, thereby transforming soft osteoid into rigid osseous tissue [9].

Dysregulation of Osteoblast-Osteoclast Homeostasis and Mechanisms of Bone Loss

The pathological essence of OP lies in the dismantling of the "osteoblast-osteoclast" coupling mechanism and a deviation in the "osteogenic-versus-adipogenic" differentiation trajectory. Collectively, these factors establish a catabolic dominance within the bone microenvironment.

The decline in osteogenic capacity stems not merely from compromised terminal differentiation efficiency, but more fundamentally from the exhaustion of the upstream progenitor reservoir. The canonical Wnt ligand, Wnt10b, has been identified as a pivotal regulator maintaining the homeostasis of adult bone marrow mesenchymal progenitor cells (MPCs). Deficiency in Wnt10b confers an instructive "biphasic phenotype" in mice: an initial, transient increase in bone mass during juvenility driven by accelerated differentiation, followed by accelerated physiological bone decay as the progenitor pool is prematurely depleted, failing to replenish functional osteoblasts [1]. This quantitative depletion is frequently accompanied by qualitative deterioration—specifically, a pathological drift in the MSC differentiation trajectory. As the antagonistic equilibrium between Runx2 and the adipogenic factor PPAR γ collapses, ectopic accumulation of bone marrow adipocytes occurs. Far from being metabolically inert bystanders, these adipocytes actively secrete RANKL to drive osteoclastogenesis, establishing a vicious feedback loop of "lipid infiltration aggravating bone resorption" [10].

Communication mismatch between osteoblasts and osteoclasts represents another dimensional mechanism underlying bone loss. Beyond the classical imbalance of the RANKL/OPG axis, non-canonical Wnt signaling exerts a more cryptic yet critical influence. Osteoblast-lineage-derived Wnt5a acts via a paracrine mechanism to engage the Ror2 receptor on osteoclast precursors, upregulating RANK transcription, thereby significantly amplifying osteoclastic activity [11]. This stands in sharp contrast to Wnt5a's osteogenic role under specific receptor contexts, suggesting that the Wnt5a-Ror2 axis may function as a pathological "accelerator" of bone resorption. Such signaling exhibits high context-dependency: within the multiple myeloma (MM) microenvironment, tumor cells inversely suppress Ror2 expression in osteoprogenitors to blockade non-canonical Wnt signaling, thereby exacerbating osteogenic suppression within osteolytic lesions [12]. These diametrically opposed molecular consequences profoundly underscore the functional plasticity of signaling pathways across distinct microenvironmental configurations.

Key Signaling Pathways Governing Osteoblast Differentiation

A schematic overview of the interconnected Wnt, BMP/TGF- α , and downstream transcriptional regulatory modules governing osteoblast differentiation is presented in Figure 1.

Wnt Signaling Pathway: Crosstalk between Canonical and Non-canonical Cascades

Wnt signaling functions not as a simple linear directive, but as a dynamic logic matrix governing cell fate. The canonical pathway operates primarily by disrupting the homeostatic degradation of β -catenin. Upon binding of Wnt ligands to the Frizzled receptors and LRP5/6 co-receptors, the signal is transduced via the key adaptor protein Dishevelled (Dvl). This disrupts the functionality of the Axin/GSK-3 β /APC destruction complex, allowing β -catenin to accumulate in the cytoplasm, translocate to the nucleus, and associate with TCF/LEF transcription factors to initiate target gene expression [13-15]. Conversely, the non-canonical pathway, with Ror2 acting as a central hub, circumvents β -catenin reliance, instead directly orchestrating cytoskeletal remodeling via PKC activation and calcium flux [16-17]. Although these pathways generally function independently, they often converge through deep signal crosstalk within the multidimensional regulatory networks of cell differentiation to dictate the final cellular response.

Distinct ligands have evolved highly specific regulatory strategies centered on the " β -catenin stability" node. Wnt3a not only directly activates canonical signaling but also alleviates the repression of osteogenesis by downregulating the ErbB3/Src axis [18]. However, as this finding relies predominantly on ALP activity—an early differentiation marker—its actual contribution to terminal events, such as mineralized nodule formation, necessitates multidimensional validation. Meanwhile, Wnt10b has been established as a pivotal factor maintaining the MPC pool via the canonical pathway; its deficiency precipitates progenitor exhaustion and age-related bone loss [1, 19]. While the molecular nuances of Wnt10b-driven self-renewal await complete dissection via in vivo lineage tracing, evidence suggests that Wnt10b-induced, VEGF-mediated angiogenesis may serve

a mechanistic role beyond mere blood supply, potentially coupling vascular niche signals to stem cell maintenance [20]. The functional paradigm of the non-canonical ligand Wnt5a profoundly illustrates the principle of signaling "context-dependence." The qualitative nature of Wnt5a signaling is contingent upon the cell surface receptor profile: when bound to the receptor tyrosine kinase-like orphan receptor 2 (Ror2), it inhibits TCF-mediated transcription following β -catenin stabilization; conversely, in cells co-expressing FZD4 and LRP5, it switches roles to become a canonical activator [4]. During MSC osteogenic differentiation, Wnt5a activates the CaMKII-TAK1-NLK axis via Ror2/FZD, thereby suppressing adipogenesis and promoting osteogenesis [21]. While this mechanism has been elucidated in cell lines, the suppression of PPAR γ poses a risk of systemic metabolic perturbation, highlighting the need for tissue-specific targeting strategies in future translational research. This signaling plasticity is further evident in MM, where tumor cells blockade osteogenesis by suppressing Ror2 expression in osteoprogenitors; restoring this pathway can reverse the inhibition [12]. However, given the *in vitro* basis of these findings, their validity and therapeutic potential within the complex *in vivo* bone marrow microenvironment remain to be verified in animal models.

In vivo studies further underscore the dual signaling activity of Wnt5a. Systemic overexpression leads to skeletal deformities in mice yet activates β -catenin reporters in the cranial dura mater [22]. This suggests that Wnt5a can convert into a canonical agonist under specific conditions, although whether this causes delayed cranial ossification requires confirmation via conditional knockout models. Developmentally, Wnt5a and Wnt5b coordinate the rhythm of chondrocyte proliferation and differentiation through the differential and antagonistic regulation of Sox9 and the cell cycle [23]. Furthermore, the effects of Wnt5a are not confined to the osteogenic lineage; osteoblast-derived Wnt5a can be captured via paracrine signaling by Ror2 on osteoclast precursors, activating the JNK-c-Jun pathway and upregulating RANK expression [11]. This interaction between osteoblasts and osteoclasts significantly amplifies the complexity of therapeutic targeting.

While the Wnt network constitutes the cornerstone of bone remodeling regulation, it does not operate in a hermetic vacuum. To achieve the precise spatiotemporal coupling of bone metabolism, Wnt signaling must undergo deep synergy and integration with other critical signaling clusters, such as the BMP/TGF- β superfamily.

BMP/TGF- β Signaling Pathways

As central pillars of the TGF- β superfamily, the Transforming Growth Factor- β (TGF- β) and BMP signaling pathways orchestrate MSC osteogenesis and differentiation. They operate through a dualistic mechanism involving the canonical Smad-dependent axes (TGF- β /Smad2/3 and BMP/Smad1/5/8) and non-canonical cascades, such as p38 MAPK and JNK, playing distinct yet critical roles [5, 24].

BMP signaling functions as a potent osteo-inductive force through the synergy of these pathways. Evidence suggests a temporal dichotomy in regulation: p38 preferentially modulates early osteogenic markers, whereas JNK governs the expression of late-stage markers [25]. This intricate spatiotemporal specificity underscores the primary challenge in clinical translation: biological efficacy is strictly governed by local con-

centration and signaling kinetics. Physiological BMP signaling operates with precise spatiotemporal confinement, a pattern that traditional high-dose bolus administration fails to recapitulate, frequently resulting in adverse effects such as ectopic ossification [26]. To circumvent this delivery dilemma, intervention at endogenous regulatory nodes within the BMP pathway has emerged as a critical strategy. The output intensity of BMP signaling is constrained by a sophisticated endogenous

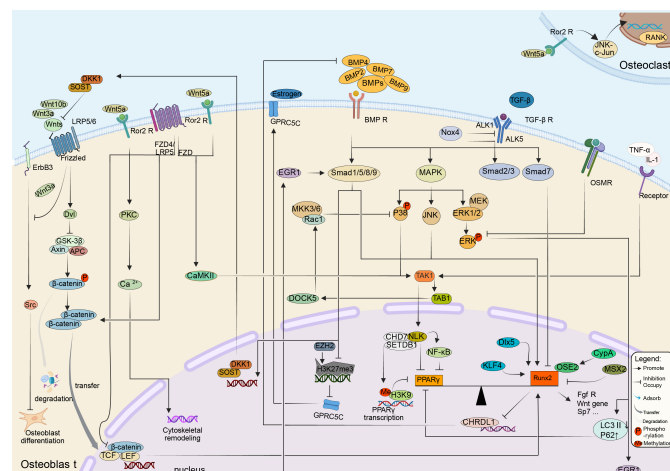


Figure 1. The multidimensional signaling topology and hierarchical integration of the osteoblast differentiation network.

This schematic illustrates the systems-level logic governing osteogenic fate, highlighting the transition from extracellular signal integration to nuclear transcriptional reprogramming and epigenetic locking. **(A)** Membrane Reception and Context-Dependent Crosstalk: The network initiates with the spatiotemporal integration of core signals. Canonical Wnt signaling (left) is triggered by ligands such as Wnt10b and Wnt3a, which stabilize β -catenin to drive progenitor maintenance. In contrast, non-canonical Wnt5a exhibits profound "context-dependency": it engages the Ror2/FZD receptor complex to activate the osteogenic PKC and CaMKII axes, while acting via a paracrine mechanism on osteoclast precursors (top right) to upregulate RANK and amplify resorption. Meanwhile, the BMP and TGF- β pathways (in the middle) not only activate their respective pathways but also converge at the MAPK effector factor, among which the BMP2-activated pathway is strictly regulated by auxiliary sensors (such as GPRC5C), which is epigenetically derepressed upon the removal of EZH2-mediated H3K27me3 marks. **(B)** Cytoplasmic Convergence Hubs: Distinct signals converge on TAK1, a pivotal kinase nexus that integrates inputs from Wnt (via CaMKII), BMP/TGF- β , and inflammatory cytokines (TNF- α /IL-1) to prioritize cell survival and differentiation. Signal flux is further fine-tuned by intracellular feedback loops: EGR1 acts as a "cytosolic adapter" bridging ERK to Smad activation (positive feedback), whereas OSMR functions as a metabolic brake by suppressing ERK phosphorylation and blocking autophagic flux (indicated by LC3II reduction and P62 accumulation). **(C)** The Runx2-PPAR γ Equilibrium and Epigenetic Blockade: In the nucleus, Runx2 serves as the master transcriptional hub, synergized by co-factors and self-reinforced by repressing the antagonist CHRD1. The ultimate lineage commitment hinges on the reciprocal equilibrium between Runx2 and the adipogenic regulator PPAR γ . The diagram highlights a critical pathological mechanism: the collapse of this balance is prevented by a Wnt5a-induced epigenetic blockade, where the NLK-CHD7-SETDB1 complex catalyzes H3K9 methylation at the PPAR γ promoter, thereby silencing adipogenesis to consolidate the osteogenic program.

network; for instance, DOCK5 has been identified as a key negative checkpoint for BMP2 signaling. It selectively attenuates p38 activity via the inhibition of the Rac1-MKK3/6 axis, while notably sparing the canonical Smad1/5/9 phosphorylation [27]. The delineation of such negative feedback mechanisms suggests that targeting endogenous inhibitory nodes could sensitize cells to BMP signals, offering a novel paradigm to enhance safety by reducing the requisite therapeutic dosage of exogenous BMP.

In contrast to the unequivocal osteogenic potency of BMP, TGF- β exerts a quintessential "double-edged sword" effect on bone metabolism, with terminal outcomes highly contingent upon cell type and microenvironmental context [3, 5]. For instance, TGF- β 1 suppresses osteogenic differentiation in dental follicle cells [28], whereas the blockade of the TGF- β Type I receptor ALK5 downregulates the osteogenic capacity of human MSCs [29], implying that a basal level of TGF- β signaling is indispensable for osteogenesis. These seemingly contradictory findings reflect the profound lineage specificity of the pathway. Consequently, single-dimensional observations are often prone to bias; resolving these discrepancies necessitates the establishment of a unified validation framework utilizing rigorous Gain- and Loss-of-function assays. This complexity is further highlighted by Nox4-associated paradoxes: while Nox4 deficiency leads to TGF- β receptor downregulation, it is counterintuitively accompanied by elevated Smad2 phosphorylation [30]. Such contradictions likely stem from the differential engagement of distinct receptor subtypes, such as the balance between ALK5 and ALK1 activation [3].

Integration of Wnt, BMP, and TGF- β Signaling Networks

The Wnt, BMP, and TGF- β signaling pathways do not operate in isolation; rather, they coalesce into a highly dynamic and "context-dependent" regulatory network facilitated by receptor sharing, nodal convergence, and transcriptional integration. This networked interactivity constitutes the central logic governing the precise orchestration of bone formation and homeostasis [2, 5].

A pivotal locus for this multi-pathway integration is the shared intracellular signaling hub, most notably Transforming Growth Factor- β Activated Kinase 1 (TAK1). TAK1 functions not only as the requisite upstream kinase for the non-canonical BMP/TGF- β pathways [24] but also as the nexus integrating Wnt5a signaling (via the CaMKII-TAK1-NLK axis) with inflammatory cascades (TNF- α /IL-1) [21, 27]. Consequently, TAK1 acts as a molecular crossroad, synthesizing inputs from Wnt, BMP/TGF- β , and inflammatory stimuli to dictate decisive cell fate outcomes.

Inter-pathway crosstalk initiates at the level of ligand expression and activity modulation. BMP signaling is known to upregulate the Wnt antagonists SOST and DKK1, a negative feedback mechanism designed to prevent aberrant bone overgrowth [5, 24]. Conversely, Runx2 transcriptionally represses the BMP antagonist CHRDL1, establishing a Runx2/CHRDL1/BMP4 positive feedback loop [31]. Within the nucleus, Runx2 serves as the master transcriptional nexus: it is a direct target of BMP/Smad1/5/8, is synergistically promoted by Wnt/ β -catenin, and is negatively regulated by TGF- β -induced Smad7 [28]. This intricate integration explains why a single molecular entity can elicit diametrically opposed biological outputs depending on the specific signaling milieu.

Existing research paradigms, largely constrained by linear analysis of single pathways, are increasingly insufficient to address this biological reality. Future breakthroughs necessitate the adoption of systems biology approaches to elucidate the mechanism of signal "priority sorting" at critical nodes like TAK1 under pathological conditions. Such insights will define the theoretical foundation for the next generation of precision therapies for skeletal diseases [2-3].

Transcriptional Regulation and Key Factors in Osteogenesis

Runx2 as the Master Regulator of Osteoblast Differentiation

Runx-related transcription factor 2 (Runx2) serves as a quintessential "master regulator" of MSC osteogenic lineage commitment. Its orchestration spans the entire continuum of cell proliferation, matrix synthesis, and signaling remodeling, exerting precise spatiotemporal control over the osteogenic program [28, 31-33].

During early differentiation, Runx2 promotes progenitor proliferation by modulating FGF receptors and Wnt genes, subsequently inducing Sp7 (Osterix) expression to drive maturation. Remarkably, during endochondral ossification, it is capable of inducing the transdifferentiation of hypertrophic chondrocytes into osteoblasts [32]. However, this potency exhibits a distinct tissue-specific dichotomy: while Runx2 activity protects against OP in the osteogenic lineage, its aberrant expression in articular cartilage acts as a pathogenic driver of osteoarthritis. To consolidate differentiation signals, Runx2 actively engineers positive feedback loops to remodel the microenvironment. Research has uncovered a "Runx2/CHRDL1/BMP4" axis, wherein Runx2 transcriptionally represses the BMP antagonist CHRDL1, thereby unleashing BMP4/Smad signaling [31]. However, chromatin immunoprecipitation (ChIP) assays have demonstrated relatively low enrichment of Runx2 at the CHRDL1 promoter, failing to conclusively verify direct binding. This suggests that the observed repression may be indirect; future investigations should incorporate high-resolution techniques, such as Chromatin Conformation Capture (Hi-C), to elucidate the physical interaction mechanisms underlying this regulation.

Beyond its expression levels, the transcriptional efficacy of Runx2 is contingent upon the dynamic assembly of transcriptional complexes. Kruppel-like factor 4 (KLF4) has been identified to co-occupy over 80% of osteogenic enhancer regions with Runx2, synergistically driving downstream gene expression [34]. Similarly, Cyclophilin A (CypA) significantly potentiates the DNA-binding affinity of Runx2 to OSE2 elements via direct protein interaction [35]. A critical knowledge gap with high translational relevance remains in this field: it is currently undefined whether the peptidyl-prolyl cis-trans isomerase (PPIase) activity of CypA is substantively involved in the conformational regulation of Runx2. Resolving this ambiguity is pivotal for drug discovery, as it will determine whether small-molecule design should target the enzymatic active site of CypA or its protein-protein interaction interface with Runx2. On the counter-regulatory side of the network, Runx2 is subject to rigorous competitive constraints to maintain homeostasis. During development, the homeobox proteins Dlx5 and Msx2 exert activating and repressive functions, respectively, through

competitive binding to the Runx2 promoter [36]. In pathological contexts, TGF- β -induced Smad7 functions as a potent molecular "brake," blocking Runx2-mediated activation of downstream osteogenic genes [28].

Other Regulatory Factors

Although Runx2 constitutes the central nexus of osteogenic differentiation, the ultimate determination of osteogenic fate within complex pathophysiological milieus hinges on the "fine-tuning" and "remodeling" of core signals by auxiliary factors.

To surmount the activation threshold required for differentiation, cells exploit cross-pathway crosstalk to achieve cascade amplification. Early Growth Response 1 (EGR1) has been identified not merely as a transcription factor, but as a pivotal "cytosolic adapter" bridging the non-canonical and canonical BMP pathways. Research demonstrates that the potent osteo-inductive factor BMP9 rapidly induces EGR1 expression via the MEK/ERK axis. Newly synthesized EGR1 physically associates with SMAD1/5 in the cytoplasm, maintaining SMAD activity by sterically hindering phosphatase-mediated dephosphorylation, thereby establishing an "ERK-EGR1-SMAD" positive feedback loop [37]. While this mechanism elegantly elucidates the source of BMP9 signaling persistence, it leaves a significant knowledge gap: the specific identity of the phosphatase competitively inhibited by EGR1 remains unknown.

Beyond signal amplification, osteoblasts require the capacity to interrogate the microenvironment and epigenetic landscape; the orphan receptor GPRC5C functions as this "environmental sensor." In the quiescent state, GPRC5C expression is stringently repressed by EZH2-mediated H3K27me3 modifications. Upon the alleviation of this repression by BMP2, GPRC5C rapidly drives matrix mineralization by responding to estrogen signaling [38]. Currently, the "orphan receptor" status of GPRC5C constitutes a formidable bottleneck for clinical translation. Research must shift from phenotypic description to mechanistic dissection, utilizing high-throughput CRISPR screening or ligand-fishing strategies to identify endogenous ligands and downstream G-protein coupling. This will delineate specific signal transduction pathways, laying the foundation for the development of agonists that mimic osteo-anabolic metabolism. On the negative regulatory spectrum, the Oncostatin M Receptor (OSMR) functions as a critical "brake," underscoring the intricacy of the osteogenic network through the inverse utilization of shared signaling nodes. Diametrically opposed to the reliance of EGR1 on ERK phosphorylation for activation, aberrantly elevated OSMR in OP models suppresses autophagic flux (manifested by reduced LC3II and accumulated P62) by downregulating p-ERK levels, thereby blocking osteogenesis and promoting adipogenesis [39]. This profoundly reveals the duality and context-dependence of ERK signaling: it serves as the requisite "engine" for EGR1-mediated amplification yet acts as a key node "extinguished" by OSMR to arrest differentiation. Although targeting OSMR silencing has achieved an "osteo-anabolic and anti-adipogenic" effect in ovariectomized (OVX) models, this contradicts earlier findings of impaired bone formation in systemic knockout mice. This discrepancy strongly suggests a compensatory interplay between the local microenvironment and systemic regulation.

In synthesis, the cytosolic signal maintenance by EGR1, the epigenetic derepression of GPRC5C, and the metabolic auto-

phagy regulation by OSMR collectively construct a multidimensional auxiliary regulatory matrix beyond the core Runx2 axis. This serves as a caveat that therapeutic strategies for skeletal diseases must transcend the linear reductionism of single-target approaches and shift towards the systemic remodeling of network topology.

The Runx2-PPAR γ Axis: A Critical Switch between Osteogenesis and Adipogenesis

The lineage commitment of bone marrow MSCs adheres to a zero-sum logic of mutual exclusivity, a balance primarily maintained by the reciprocal inhibition between the master osteogenic transcription factor, Runx2, and the core adipogenic regulator, PPAR γ . In the context of OP and skeletal aging, the collapse of this equilibrium—characterized by the arrest of the Runx2-driven osteogenic program and the concurrent onset of PPAR γ -mediated marrow adiposity—constitutes the fundamental pathological basis of bone loss.

Regulation of this equilibrium by canonical Wnt signaling exhibits complex "bidirectional effects." Although Wnt10b theoretically consolidates osteogenic dominance by suppressing PPAR γ and its downstream adipogenic targets [20], in vivo and in vitro experiments point to a significant mechanistic dichotomy. While Wnt10b deficiency precipitates severe bone loss in mice, the corresponding bone marrow stromal cells paradoxically exhibit diminished adipogenic potential in culture [1]. This phenomenon suggests that the core in vivo function of Wnt10b may not be merely to drive differentiation polarity, but to maintain the quantity and vitality of the undifferentiated progenitor pool. Consequently, the exhaustion of the MSC reservoir following Wnt10b loss yields consequences far more severe than a simple shift in differentiation bias. Future research must therefore transcend the limitations of single-marker detection, employing lineage tracing technologies to precisely delineate the temporal switching mechanism by which Wnt signaling governs the transition between "stemness maintenance" and "differentiation initiation."

In contrast to the direct transcriptional regulation by the canonical pathway, non-canonical Wnt signaling imposes "gene silencing" on PPAR γ through sophisticated epigenetic mechanisms. Wnt5a-activated NLK kinase functions as a nuclear molecular scaffold, recruiting the histone methyltransferase SETDB1 and the chromatin remodeler CHD7 to the PPAR γ promoter. This complex catalyzes H3K9 methylation, leading to chromatin compaction and transcriptional silencing [21]. This "epigenetic blockade" arrests the adipogenic program at its source. Given that PPAR γ is also a systemic hub for insulin sensitivity, the breakthrough for clinical translation lies in developing interfering molecules that specifically deconstruct the "NLK-SETDB1-PPAR γ " ternary complex. The objective is to achieve a safe "functional decoupling" that preserves osteogenic benefits while mitigating systemic metabolic risks, rather than blindly ablating the entire PPAR γ pathway.

Acting as a terminal checkpoint linking microenvironmental stress to cell fate, metabolic autophagic flux plays an equally decisive role. Pathologically elevated OSMR blocks autophagic flux by inhibiting ERK activity, thereby preventing the clearance of intracellular lipid droplets via "lipophagy." The resulting accumulation of lipotoxicity exerts a suppressive effect on Runx2 activity, inversely solidifying the dominance of PPAR γ [39].

In summary, the competition between Runx2 and PPAR γ is

substantively a systemic confrontation spanning transcriptional, epigenetic, and metabolic dimensions. Future therapeutic strategies must evolve from a simplistic view of "reciprocal trade-offs" between single factors toward a holistic remodeling of the entire regulatory chain: "Progenitor Pool – Epigenetic Blockade – Autophagic Flux."

Non-coding RNA-mediated Post-transcriptional Regulatory Networks

A schematic overview of the miRNA/lncRNA-centered post-transcriptional regulatory network and its major signaling/transcriptional nodes in osteoblast differentiation is presented in Figure 2.

miRNA-mediated Targeting of Signaling Nodes and Transcription Factors

MicroRNAs (miRNAs) function as highly efficient molecular switches that safeguard osteogenic signal flux through the mechanism of "disinhibition"—the precise clearance of endogenous inhibitors within signaling networks. Research indicates that miRNAs specifically target the molecular "brakes" of signaling pathways, thereby alleviating blockades on core cascades including BMP, MAPK, and PI3K/AKT. Specifically, miR-218-5p directly binds and suppresses the Suppressor of Cytokine Signaling 3 (SOCS3). This interaction not only releases the inhibition of SMAD1 downstream of BMP signaling but also disrupts the negative feedback loop promoting osteoclastogenesis, effectively remodeling metabolic balance in postmenopausal OP (PMOP) models [40]. Similarly, at the level of kinase regulation, miR-497-5p targets High Mobility Group AT-Hook 2 (HMG2) to significantly attenuate JNK pathway phosphorylation, inhibiting apoptosis and promoting differentiation [41]. In the context of Traumatic Brain Injury (TBI) stress, miR-92a-3p activates repressed PI3K/AKT signaling by inhibiting Integrin Binding Sialoprotein (IBSP), thereby accelerating osteogenesis [42].

While existing studies have outlined this regulatory framework, their explanatory power remains constrained by the limitations of a reductionist perspective. The preponderance of evidence is derived from isolated precursor cell models [41–42], which artificially strip away the essential paracrine interactions with osteoclasts and vascular endothelial cells inherent to the bone microenvironment. More paradoxically, the inverse expression trends of SOCS3—high in bone tissue versus low in peripheral blood [40]—strongly suggest a mechanistic decoupling between local microenvironments and systemic regulation. Consequently, future mechanistic dissection must transcend single-dimensional expression screening. By leveraging spatial transcriptomics to map in situ interaction landscapes under pathological conditions, combined with multicellular co-culture systems, research can define the authentic efficacy of miRNAs while respecting the complexity of tissue communication. Beyond modulating upstream signal flux, miRNAs directly dictate the abundance of transcription factors and membrane receptors, serving as conduits linking extracellular physical fields to intracellular genetic programs. At the transcriptional terminus, miR-151b has been identified as a potent repressor of osteogenic differentiation. Its aberrant upregulation in osteoporotic environments cascades to shut down the BMP-6-

induced OSX/ALP/Runx2 expression program by specifically clamping the 3'UTR of the homeobox transcription factor Msx2 [43]. At the membrane receptor interface, miRNAs mediate rapid mechanotransductive responses: mechanical stretching alleviates the blockade on Cannabinoid Receptor 2 (CNR2) by downregulating miR-187-3p, subsequently activating the "Stress-CNR2-Runx2" axis to drive bone formation [44]. Although these findings have pinpointed critical targets, current mechanistic resolutions remain incomplete. It remains inconclusive whether specific MAPK isoforms are involved in the downstream cascade of Msx2 [43]. Furthermore, studies on CNR2 have simulated only tensile stress, neglecting the

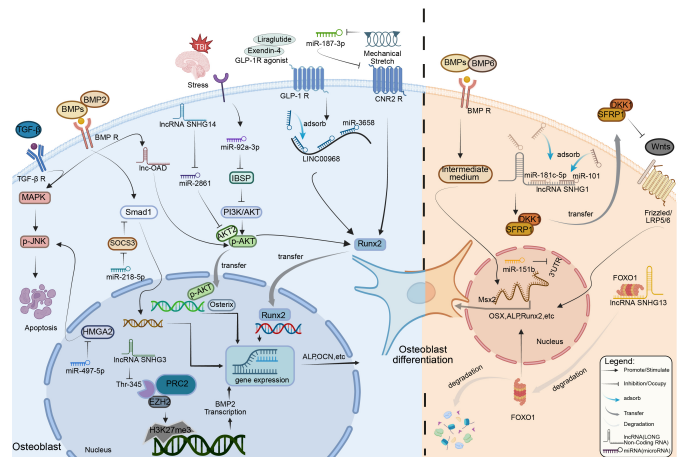


Figure 2. The post-transcriptional logic and epigenetic architecture of non-coding RNA-mediated osteogenic fate determination.

This schematic delineates the dichotomous regulatory landscape governed by non-coding RNAs, contrasting the logic of "disinhibition" (Left, Blue panel) against "suppression" (Right, Orange panel) in the control of osteoblast differentiation. (A) The Osteo-Anabolic Network: Disinhibition and Epigenetic Derepression. The left panel illustrates how osteogenic signals are safeguarded through the neutralization of endogenous inhibitors. Signaling Disinhibition: MicroRNAs act as checkpoints to release signaling blockades. miR-218-5p targets the inhibitor SOCS3, thereby unleashing Smad1 activity; miR-92a-3p, triggered by TBI stress, suppresses IBSP to reactivate the PI3K/AKT axis; and mechanical stretch downregulates miR-187-3p, relieving the repression on the CNR2 receptor. ceRNA & Epigenetic Scaffolds: Long non-coding RNAs (lncRNAs) function through different structural mechanisms. In the cytoplasm, SNHG14 and LINC00968 act as competitive endogenous RNAs to protect the transcripts of AKT2 and Runx2 by inhibiting miR-2861 and adsorbing miR-3658, respectively. In the nucleus, SNHG3 acts as an epigenetic decoy, anchoring EZH2 (of the PRC2 complex) to prevent H3K27me3 deposition at the BMP2 promoter, thus maintaining transcriptional accessibility. (B) The Osteo-Suppressive Network: Feedback Paradox and Degradation. The right panel depicts mechanisms leading to differentiation arrest. The ceRNA Paradox: Highlighting functional divergence, SNHG1 adsorbs miRNAs such as miR-181c-5p and miR-101, preventing the inhibition of Wnt pathway antagonists DKK1 and SFRP1 (thereby stabilizing them), and ultimately leading to the blocking of the classical Wnt signaling pathway. Targeted Degradation: SNHG13 operates via a nucleic-acid-independent mechanism, directly binding FOXO1 to induce its ubiquitin-proteasomal degradation. Concurrently, miR-151b clamps the 3'UTR of Msx2, severing the downstream transcriptional cascade essential for mineralization.

physiological compressive forces primarily borne by the skeleton [44]. In light of this, subsequent work must delineate the kinase interaction networks downstream of *Mx2* and incorporate 3D bioreactor models capable of simulating complex loading environments—integrating composite tension-compression and fluid shear stress—to comprehensively evaluate the therapeutic potential of these miRNA targets under authentic physiological mechanical conditions.

Roles of lncRNAs in Determining Osteogenic Fate

In contrast to microRNAs, which predominantly fine-tune gene expression at the post-transcriptional level, long non-coding RNAs (lncRNAs) leverage their complex secondary structures and diverse subcellular compartmentalization to orchestrate a multidimensional regulatory architecture spanning from epigenetics to protein modification. By functioning as molecular sponges, scaffolds, and guides, lncRNAs establish a sophisticated layer of control over cellular fate.

As the canonical paradigm of lncRNA-mediated osteogenesis, the competing endogenous RNA (ceRNA) mechanism operates by competitively sequestering miRNAs to alleviate the repression of downstream osteogenic mRNAs. In osteogenic regeneration models, LINC00968 exhibits distinct positive regulatory functionality: under GLP-1R agonist induction, it specifically absorbs miR-3658, releasing the repressed core factor *Runx2* and thereby driving the mineralization and regeneration of human dental pulp stem cells (hDPSCs) [45]. However, functional divergence within the SNHG family reveals the high nonlinearity of this network. While SNHG14 activates the AKT2 pathway by blocking miR-2861, SNHG1 conversely releases Wnt inhibitors such as *SFRP1* and *DKK1* by sequestering miR-181c-5p or miR-101, leading to unexpected osteogenic arrest [46]. This intra-familial functional dichotomy indicates that ceRNA efficacy is not dictated solely by the expression fluctuations of a single molecule, but is strictly governed by the rigorous stoichiometry between the lncRNA, the miRNA, and their targets.

Beyond RNA-RNA interactions, lncRNAs exert control through independent dimensions by physically associating with proteins to modulate epigenetic modifications and signal transduction. In the nucleus, lncRNAs can serve as molecular decoys for epigenetic complexes. SNHG3 has been confirmed to directly anchor EZH2, the catalytic subunit of the PRC2 complex. By occupying the Thr-345 phosphorylation site, SNHG3 imposes steric hindrance that blocks EZH2-mediated H3K27me3 repressive methylation at the BMP2 promoter region [47]. The dissolution of this "SNHG3-EZH2-H3K27me3" axis directly reactivates the transcriptional competency of BMP2-Smad1/5 signaling. Conversely, cytosolic lncRNAs function more as signaling rheostats. lnc-OAD has been identified as an essential component of the BMP-2 cascade, directly governing *Osterix* expression by maintaining AKT phosphorylation levels; its depletion results in the severe impairment of the mineralization program [48]. Meanwhile, SNHG13 (DANCR) suppresses osteogenic differentiation via a nucleic acid-independent mechanism, directly binding FOXO1 and inducing its ubiquitin-proteasomal degradation [46].

Although these mechanisms illuminate the multifaceted potential of lncRNAs, the current chain of evidence faces two formidable challenges. First is the issue of model specificity versus universality: the epigenetic mechanism of SNHG3 was primar-

ily established in cardiovascular valve calcification models, while lnc-OAD validation remains confined to in vitro assays. Given the unique mechanical and metabolic signatures of the bone microenvironment, the reproducibility of these mechanisms must be verified in orthotopic animal models of OP or osseous defects. Second, transcript heterogeneity constitutes a cryptic barrier to functional resolution. Distinct splice variants of SNHG2 (GAS5) have been shown to exert diametrically opposed biological effects [46], implying that traditional whole-gene loss-of-function approaches may obscure the authentic roles of specific transcripts. Future intervention strategies must advance toward precision splicing modulation, utilizing systems like CRISPR-Cas13d to target exon junctions. This will enable the point-to-point regulation of specific transcript isoforms, moving beyond broad-spectrum gene silencing.

Targeted treatment strategies for OP

A schematic overview of the targeted intervention landscape—spanning pharmacological agents, natural small molecules, materials/mechanical strategies, and gene/RNA targeting—within the osteogenic regulatory network is presented in Figure 3.

With the deepening understanding of the multidimensional regulatory networks governing osteogenic differentiation, the treatment of OP should transition from traditional approaches such as hormone replacement or broad-spectrum anti-resorptive therapies toward precise modifications targeting critical signaling nodes, microenvironmental homeostasis, and genetic information.

Pharmaceutical Interventions

Consequently, next-generation pharmacological strategies are moving beyond the binary paradigm of simple pathway activation or blockade. Instead, the focus has shifted toward bidirectional fine-tuning and spatiotemporal optimization tailored to specific pathological states.

Pathology-Dependent Bidirectional Regulation: The TGF- β signaling pathway exhibits a dichotomous nature, manifesting diametrically opposed roles across different stages of bone pathology. This necessitates a "divide-and-conquer" strategy for precision intervention.

In conditions driven by aberrant signaling and matrix disarray, such as OI, the angiotensin II receptor antagonist Losartan exerts a non-canonical osteoprotective function. By specifically curbing the aberrant phosphorylation of intracellular SMAD2 and ligand secretion, Losartan has been shown to ameliorate cortical bone microarchitecture [49]. Conversely, in scenarios of differentiation arrest characterized by signaling deficiency, the anticancer agent Elesclomol functions as an agonist for the orphan nuclear receptor NR4A1. By reconstructing the "NR4A1-TGF- β -RAF-1" cascade, it functions to reactivate the dormant osteogenic program [50]. However, such interventions must be vigilant against off-target effects. For instance, while the chemotherapeutic Bleomycin suppresses tumors, it paradoxically induces SMAD7 expression, thereby blocking *Runx2* activity and potentially contributing to iatrogenic bone loss [28].

Non-canonical Pathways and Spatiotemporal Kinetics: To circumvent the tumorigenic risks associated with canonical signaling and enhance therapeutic efficiency, the exploitation

of non-canonical pathways and the optimization of pharmacokinetics constitute a critical pivot point.

In myeloma-associated bone disease, targeting Ror2-mediated non-canonical Wnt signaling may offer a more favorable safety profile. By activating the Wnt5a/PKC/NFATc1 axis, this approach precisely counteracts the osteogenic inhibition inherent to the tumor microenvironment without perturbing oncogenic networks [12]. Spatiotemporal Rhythm: In combinatorial therapy, the superposition of growth factors is not a matter of simple arithmetic but a rigorous calculation of "spatiotemporal rhythm." The synergistic efficacy of recombinant human BMP-7 and PDGF-BB is strictly contingent upon a low-frequency pulsatile regimen; continuous high-frequency stimulation paradoxically induces receptor desensitization or signal antagonism, leading to ectopic ossification [10]. This discovery profoundly revises conventional drug administration paradigms. Future interventions must incorporate smart bioinspired carriers that mimic physiological pulsatile release rhythms, in order to develop strategies for mitigating off-target effects.

The Opportunities and Complexity of Drug Repurposing: The "drug repurposing" of systemic agents has significantly expanded the landscape of osteogenic intervention, yet it introduces the complexity of cross-system regulation.

Metabolic agents, specifically GLP-1 receptor agonists, have been confirmed to ameliorate the bone microenvironment by enhancing Cx43 gap junctions or mediating lncRNA-driven epigenetic derepression [45, 51]. In stark contrast, the neurological agent Dextromethorphan, while blocking NMDA receptors, significantly suppresses Runx2 expression as a form of "collateral damage" [52]. Given the universality of signaling pathways across tissues, existing repurposing strategies urgently require the establishment of robust bone safety assessment frameworks. When utilizing non-orthopedic drugs for chronic conditions, it is imperative to synchronously monitor bone metabolic indices or develop bone-targeted delivery systems to mitigate the risk of off-target skeletal toxicity.

Precision Modulation of Signaling Nodes by Natural Small Molecules

Natural products represent more than mere nutritional supplements; they constitute a vast chemical library for identifying high-selectivity modulators of signaling pathways. Distinct from strategies relying on crude extracts, phytochemical small molecules with defined structures function as allosteric modulators or enzymatic inhibitors to precisely intervene at critical kinase nodes and epigenetic enzymes within the osteogenic network. This inherent "structural diversity" offers unique solutions to circumvent the off-target toxicities often associated with conventional synthetic pharmacotherapies.

Redirection of Kinase Nodes: Targeting the TAK1 Hub As mentioned earlier, TAK1 serves as a pivotal crossroad integrating inflammatory signals with osteogenic cues. Within the chronic inflammatory microenvironment of OP, Phaseol (also known as Phaseollin) has been identified as a natural small molecule capable of specifically binding and modulating TAK1 activity. Rather than simply blockading TAK1, Phaseol suppresses the hyperactivation of the downstream NF- κ B cascade, effectively redirecting the signaling flux toward the p38 MAPK and Smad1/5/8 pathways to favor osteogenesis [53]. This strategy of "redirection rather than occlusion" effectively converts inflammatory stress within the microenvironment into an osteo-

genic driver, providing a paradigm for the precise management of inflammatory bone loss.

Maintenance of Homeostasis via Epigenetic and Post-Translational Modifications: Beyond the direct activation of signaling pathways, natural small molecules enhance osteogenic efficacy by modulating the post-translational modifications

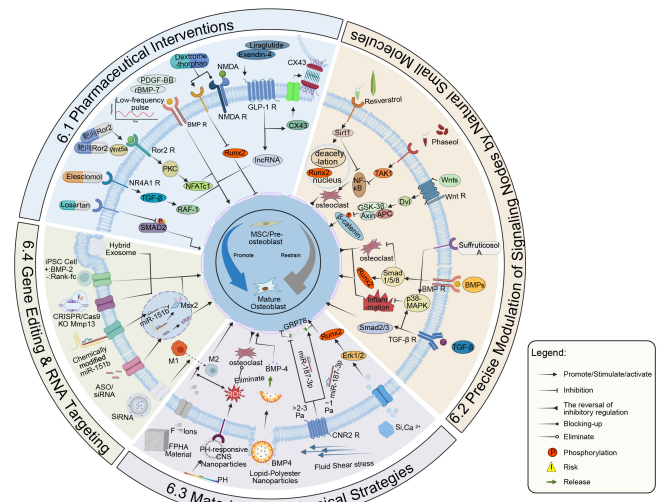


Figure 3. The four-dimensional landscape of precision therapeutic strategies targeting the osteogenic regulatory network.

This radial diagram synthesizes next-generation intervention paradigms, categorizing strategies into four precision dimensions that converge to restore the homeostatic MSC-to-osteoblast differentiation trajectory (Center). **(6.1) Pathology-Dependent Pharmacological Reprogramming (Top Left):** Moving beyond broad-spectrum agents, this quadrant highlights context-specific modulation. Losartan acts as a non-canonical protector to rectify hyperactive TGF- β signaling in OI, whereas Elesclomol functions as an agonist to reactivate dormant pathways via NR4A1. The schematic also emphasizes "spatiotemporal rhythm," illustrating how low-frequency pulsatile delivery of BMP-7 (inset graph) prevents receptor desensitization, contrasting with the risks of continuous dosing. Notably, the yellow hazard icon warns against collateral damage, such as Dextromethorphan-induced Runx2 suppression. **(6.2) Natural Small Molecule Node Modulation (Top Right):** Phytochemicals serve as precise allosteric modulators. Phaseol binds the TAK1 hub, redirecting signaling flux from the inflammatory NF- κ B cascade to the osteogenic p38/BMP pathway. Resveratrol activates the deacetylase SIRT1, which stabilizes Runx2 post-translationally to bypass inflammatory suppression. Suffruticosol A synergistically reactivates canonical and non-canonical BMP signaling. **(6.3) Biomaterial and Mechanical Engineering (Bottom):** Constructing a biomimetic microenvironment. Strategies include FPHA biomaterials that drive immunomodulatory M2 macrophage polarization and pH-responsive CNS nanoparticles that scavenge ROS in the acidic osteoporotic niche. The mechanotransduction pathway demonstrates the "biphasic window," where fluid shear stress (~ 1 Pa) activates the CNR2 receptor to promote anabolism, whereas excessive force ($>2-3$ Pa) induces damage. **(6.4) Gene Editing and RNA Targeting (Bottom Left):** Genetic source reprogramming. This dimension utilizes Hybrid Exosomes to penetrate the mineralized matrix barrier for CRISPR/Cas9 delivery, enabling "scarless" repair. Concurrently, ASOs and chemically modified miRNA inhibitors provide precise post-transcriptional silencing to derepress osteogenic factors like Mx2.

(PTMs) of transcription factors. Resveratrol, a specific agonist of Sirtuin 1 (SIRT1), functions through a deacetylation mechanism. Research demonstrates that activated SIRT1 directly deacetylates Runx2, protecting it from ubiquitin-proteasome degradation. This significantly enhances the protein stability and transcriptional activity of Runx2 without necessitating an increase in its mRNA levels [54]. Furthermore, SIRT1 suppresses NF-κB acetylation, thereby alleviating the inhibition of osteogenesis by inflammatory cytokines. This dual mechanism suggests that targeting deacetylases is a potent strategy for reversing the "functional silence" of transcription factors in senile OP.

Synergistic Activation and Translational Perspectives: Addressing the issues of resistance or compensation associated with single-pathway targeting, certain natural molecules exhibit synergistic multi-target effects. Suffruticosol A has been confirmed to synchronously activate both the canonical BMP2/Smad1/5/8 pathway and the non-canonical MAPK cascade, reigniting the differentiation program under stress-induced suppression [55]. However, clinical translation is currently hampered by poor bioavailability and target specificity drift. Future research must transcend simple phenotypic screening; rather, these molecules should be regarded as lead compounds. By integrating Structure-Based Drug Design (SBDD), future efforts should aim to develop next-generation small molecules that combine high bone tissue affinity with precise molecular targeting (Table 1).

Materials & Mechanical Strategies

The core philosophy of material and mechanical strategies lies in constructing a biomimetic local microenvironment. By leveraging physicochemical cues as "solid-state signaling sources" that transcend the cell membrane, these approaches effectively circumvent the targeting limitations inherent to systemic drug administration.

Osteoimmunomodulation: Modern biomaterial design has evolved beyond the mere activation of integrin signaling via nanotopography [56] toward the active remodeling of the immune microenvironment—a concept known as osteoimmunomodulation. Inorganic ions have been validated as independent signaling inducers; functionalized scaffolds releasing silicon and calcium ions are capable of promoting osteogenic differentiation via the Erk1/2-Runx2 axis, reducing the reliance

on exogenous growth factors [57]. Material-mediated metabolic reprogramming represents a novel therapeutic frontier. Fluorine-doped hydroxyapatite (FPHA), by releasing trace fluoride, switches macrophage metabolism from pro-inflammatory glycolysis to oxidative phosphorylation (OxPhos), thereby fostering an M2-phenotype-dominant, pro-osteogenic microenvironment [58]. Addressing the acidic pathological milieu of OP, pH-responsive cerium nanoparticles (CNS) sense local acidity and generate ROS in situ, achieving selective depletion of mature osteoclasts while preserving osteogenic precursors [59]. Current strategies predominantly focus on singular functions, failing to address the complex pathological cascade of OP. Future intelligent materials should aim to construct "logic gate-like" multi-stage response systems. These would integrate sequential functionalities—"early osteoclast clearance → intermediate immune tolerance → late bone regeneration"—within a single vector.

Biochemical Factors: Kinetics and Intracellular Delivery: The therapeutic efficacy of biochemical factors is not dictated solely by dosage but is strictly governed by their release kinetics. Evidence confirms that mimicking physiological sustained release is critical for initiating anabolism, whereas high-dose burst release is not only ineffective but may precipitate catabolism or systemic adverse events [26]. To resolve this paradox, nanodelivery technology is evolving from simple physical encapsulation to "intracellular programmability." Utilizing a dual mode of "extracellular receptor binding + intracellular degradation," lipid-polyester nanoparticles have shown the potential to achieve efficient intracellular delivery and sustained release of BMP4 [60]. Most controlled-release systems are evaluated under static in vitro conditions, neglecting the dynamic washout effects of body fluid flow. Consequently, translational research must incorporate microfluidic chip technology to simulate the physiological hemodynamic environment, thereby calibrating the in vivo degradation rates and release kinetics of nanocarriers to improve the translational predictive value.

Physical Therapy: The Biphasic Window of Mechanotransduction: The essence of physical therapy lies in the precision control of mechanical stimulus intensity. Fluid shear stress regulates osteogenic metabolism through a strict "biphasic window": it promotes anabolism only within a specific intensity range (~1 Pa), whereas exceeding this threshold (>2-3 Pa) converts the stimulus into a damage signal [61]. This mecha-

Table 1. Representative Natural Small Molecules Targeting Osteogenic Signaling Nodes.

Compound	Classification	Primary Molecular Target	Mechanism of Action (Signaling Modulation)	Reference
Phaseol	Coumestan	TAK1 (Kinase Hub)	Allosterically modulates TAK1 to inhibit NF-κB signaling while redirecting flux to p38 MAPK/BMP pathways.	[53]
Resveratrol	Polyphenol	SIRT1 (Deacetylase)	Activates SIRT1 to deacetylate Runx2, preventing its proteasomal degradation; concurrently inhibits NF-κB acetylation.	[54]
Suffruticosol A	Stilbene Oligomer	BMP / MAPK	Synergistically activates canonical Smad1/5/8 and non-canonical MAPK cascades to rescue differentiation arrest.	[55]

Note: Summary of representative natural small molecules and their molecular mechanisms in driving the osteogenic differentiation program via precise modulation of critical signaling hubs and remodeling of intracellular signaling fluxes.

no-chemical transduction is highly dependent on intracellular precision sensors, including the ER chaperone GRP78 and the miR-187-3p/CNR2 axis [44, 62]. These sensors function as "molecular switches," influencing whether physical signals activate osteogenic pathways or induce apoptosis. Existing rehabilitation strategies often employ "one-size-fits-all" mechanical parameters, ignoring the critical fact that the mechanosensitivity of cells in osteoporotic patients is likely altered. Future research should prioritize the development of companion diagnostic tools based on mechanosensitive markers. These would serve as the basis for customizing implant design parameters and postoperative rehabilitation protocols tailored to the patient's specific mechanobiological profile.

Gene Editing & RNA Targeting

Distinct from pharmacological and material interventions that operate downstream at the protein or cellular level, gene editing and RNA technologies aim to execute "precise reprogramming" directly at the genetic source, offering promising strategies for treating hereditary bone diseases and managing refractory OP.

At the post-transcriptional modification level, RNA-targeting strategies are considered to possess considerable potential for high specificity. Research on OI indicates that employing antisense oligonucleotides (ASOs) or siRNA to achieve "allele-specific silencing" can potentially correct the haploinsufficiency [63-64]. This precise silencing mechanism extends to the non-coding RNA regulatory network; for instance, chemically modified miR-151b inhibitors specifically relieve the repression of *Mx2*, thereby effectively restoring the transcriptional activity of osteogenic genes [43]. Concurrently, genome editing technology is undergoing a paradigm shift from "gene disruption" to "precision repair." While CRISPR/Cas9-mediated knockout of *Mmp13* has proven effective in delaying osteoarthritis progression [65], the field is accelerating toward Base Editing and Prime Editing. Theoretically, these novel technologies may mitigate the safety concerns of genomic instability associated with traditional editing by bypassing the reliance on DNA double-strand breaks (DSBs), thus enabling the "scarless repair" of point mutations [63]. Furthermore, "ex vivo gene enhancement" combined with iPSCs represents an alternative translational pathway. By re-infusing cells genetically modified to overexpress BMP-2 or knock down RANK-Fc, this approach achieves a synergistic potentiation of "cellular regeneration" and "genetic regulation" [66-67].

Despite clear therapeutic targets, the clinical translation of nucleic acid drugs has long been impeded by the instability of naked moieties and biological membrane barriers. Current delivery strategies are progressively pivoting away from viral vectors, which carry immunogenic risks, toward non-viral vectors dominated by Lipid Nanoparticles (LNPs) and functionalized biomaterials. These alternatives leverage superior biocompatibility and modification flexibility to shield the nucleic acid payload [68]. Addressing the unique barrier presented by the dense extracellular matrix (ECM) of bone tissue, engineered "hybrid exosomes" have presented a promising approach for delivering CRISPR systems via membrane fusion, facilitating the penetration of macromolecular drugs into the bone/cartilage matrix [65]. At the cellular targeting level, nanocarrier-mediated siRNA delivery has achieved specific modulation of macrophages within the osteoimmune microenvironment,

opening novel avenues for ameliorating the osteogenic niche [69].

Currently, while gene therapy exhibits vast prospects, the critical leap from "bench to bedside" hinges on the establishment of rigorous evaluation and quality control frameworks. Confronting potential off-target effects and long-term safety concerns, future translational research must not be satisfied solely with numerical improvements in editing efficiency. Instead, it is imperative to construct functional evaluation systems based on gold-standard markers such as *Runx2* and *COL1A1* [70]. This must be coupled with Whole Genome Sequencing (WGS) to comprehensively screen for off-target risks, facilitating the translation of precise modulation at the molecular level translates into functional benefits in histological bone regeneration, rather than merely transient perturbations in signaling pathways.

Future Perspectives

The therapeutic paradigm for OP is undergoing a fundamental shift from a singular reliance on anti-resorptive strategies toward "precision homeostatic remodeling" grounded in systems biology. This evolution is predicated on a profound comprehension of metabolic regulation mechanisms: key signaling pathways, such as Wnt and BMP, exhibit high context-dependency, with their biological effects rigorously contingent upon the specific microenvironment. This context-sensitivity elucidates a primary failure mode of traditional single-target therapies under pathological conditions. Central to systemic bone loss is the dysregulated competition between the *Runx2*-driven osteogenic and *PPAR γ* -driven adipogenic lineages. This imbalance is orchestrated by multidimensional regulation, notably progenitor pool exhaustion precipitated by *Wnt10b* attenuation and epigenetic silencing mediated by the NLK-SETDB1 complex. Concurrently, non-coding RNAs modulate differentiation thresholds predominantly via a logic of "disinhibition," functioning by dismantling endogenous checkpoints such as *SOC3*. Corresponding translational trajectories have crystallized into three distinct avenues. First, the design of "functionally decoupled" pharmacotherapies aims to precisely delineate and separate the osteo-anabolic benefits of signaling pathways from their deleterious off-target effects. Second, the development of smart, responsive biomaterials seeks to engineer chronologically orchestrated therapies that achieve "initial osteoclast inhibition followed by osteogenic induction." Third, the advancement of "scarless" gene editing—integrating Base Editing and Prime Editing with exosome-based delivery—offers a strategy to correct genetic defects while precluding the generation of hazardous DNA DSBs.

Nevertheless, the "bench-to-bedside" translation currently confronts three critical bottlenecks: the paucity of efficient delivery vectors capable of penetrating the dense mineralized matrix barrier of bone tissue; the lacuna in long-term safety evaluation frameworks, particularly regarding the surveillance of osteogenic hyper-activation and off-target editing risks; and the limitations of extant disease models, which necessitate the adoption of humanized organoids and large animal models to validate clinical predictive efficacy. Future breakthroughs will likely rely on overcoming a rigorous and fortified bridge spanning the chasm between these frontier mechanistic insights

and the pragmatic challenges of clinical translation.

Abbreviations

adipose-derived stem cells: ASCs; alkaline phosphatase: ALP; bone mineral density: BMD; Bone Morphogenetic Protein: BMP; bone marrow stromal cells: BMSCs; Cyclophilin A: CypA; competing endogenous RNA: ceRNA; dextromethorphan: DXM; Frizzled: FZD; fluid shear stress: FFSS; growth response protein 1 : EGR1; glucagon-like peptide-1 receptor: GLP-1R; Long non-coding RNAs: lncRNAs; lipopolysaccharide: LPS; lipid nanoparticle: LNP; mesenchymal stem cells: MSCs; microRNAs: miRNAs; mature osteoclasts: mOCs; Notch intracellular domain: NICD; Osteoporosis: OP; osteoporotic adipose-derived stem cells: OP-ASCs; osteopontin: OPN; Oncostatin M receptor: OSMR; osteogenesis imperfecta: OIM; osteocalcin: OCN; ovariectomized: OVX; protein kinase C: PKC; Phaseolamin: PHA; precursor osteoclasts: pOCs; recombinant human BMP7: rhBMP7; reactive oxygen species: ROS; SET domain bifurcation histone lysine methyltransferase 1: SETDB1; soy peptides: SOP; trabecular number: Tb.N; T-cell factor: TCF; type I collagen alpha 1: COL1A1; Transforming Growth Factor-beta: TGF- β ; traumatic brain injury: TBI; umbilical cord mesenchymal stem cells: UCMSCs; World Health Organization: WHO; 3' untranslated regions: 3'UTRs;

Author Contributions

Chuntao Liang: Writing-review & editing, Writing-original draft. Dongcheng Peng: Writing-review & editing, Validation. Hongkai Wang: Writing-review & editing, Conceptualization, Supervision. All authors read and approved the final manuscript.

Acknowledgements

Not Applicable

Funding Information

Not Applicable.

Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

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

Data Availability

Not Applicable.

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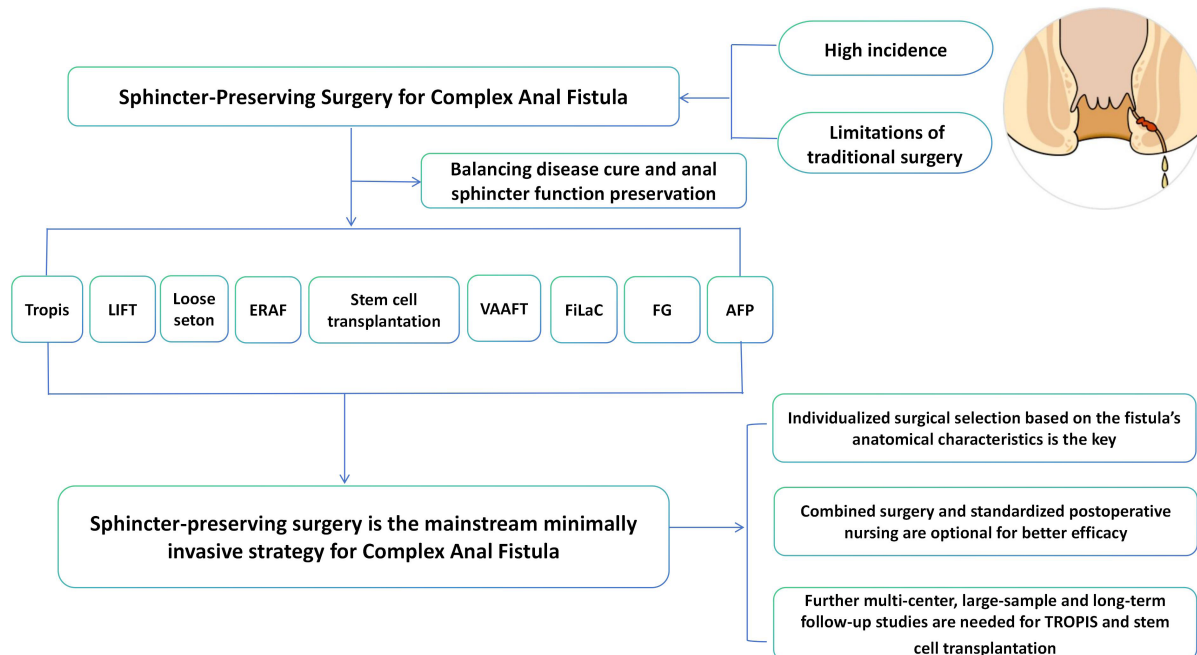
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Graphical Abstract



<https://doi.org/10.71321/y8d3rn20>

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A Systematic Review of Sphincter-Preserving Surgery for Complex Anal Fistula

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Received: 2025-08-19 | Accepted: 2025-09-06 | Published online: 2026-03-21

Abstract

Complex anal fistula is a common and refractory anorectal disease in clinical practice. Surgery is currently the main treatment method, and the sphincter-preserving surgical approach is the current surgical trend for complex anal fistula. This article reviews the surgical methods for complex anal fistulas that preserve the sphincter, including transanal opening of intersphincteric space, ligation of the intersphincteric fistula tract, loose-seton technique, endorectal advancement flap, mesenchymal adipose-stem cell transplantation, fistula-tract laser closure, video assisted anal fistula treatment, fibrin glue and anal fistula plug. This article reviews the articles on minimally invasive treatment for anal fistula in PubMed, Cochrane Library CENTRAL and Embase, and introduces the current research progress. The aim is to strive to maintain a balance between curing complex anal fistulas and protecting the function of the sphincter.

Keywords: Anal Fistula; Sphincter Preservation; Mesenchymal Stem Cells; Laser Therapy; Endoscopic Surgery

Introduction

Complex anal fistula refers to an anal fistula disease with two or more external and internal openings and two or more branch pipes or fistulas. It is one of the common anorectal surgical diseases in clinical practice. With the increase of work pressure and the change of living habits, the incidence of complex anal fistula has been increasing year by year. Data show that the incidence of complex anal fistula accounts for 1.67% to 3.6% of the incidence of anorectal fistula in China [1], and 8% to 25% abroad. Its main manifestations are repeated ulceration and discharge of pus from the anus, pain, itching, and prolonged non-healing, which affects the quality of life of patients. At present, surgery is still the preferred method for the clinical treatment of complex anal fistula. Conventional anal fistula resection has problems such as many postoperative complications and a high recurrence rate [2]. Therefore, how to cure complex anal fistulas while protecting the anal function of patients has important clinical significance. With the rise of the concept of minimally invasive treatment, the sphincter-preserving surgical approach has become the mainstream surgical method for treating complex anal fistulas. The surgical

methods for preserving the sphincter in complex anal fistulas are summarized as follows:

Tropis: Transanal opening of intersphincteric space

Transsphincter fistula incision was first proposed by Indian scholar Grag in 2017. The key points of this surgery lie in incising the internal orifice and the internal sphincter, opening the sphincter space, and maintaining smooth incision drainage. After long-term follow-up of 61 patients with high and complex anal fistulas, Grag successfully cured 47 patients in the end. The overall cure rate after the operation was 90.4% [3]. Pankaj Garg found that by applying the Tropis procedure to high and complex anal fistulas, the Tropis procedure could maintain the shape of the anal canal and the function of the sphincter to the greatest extent [4]. The postoperative pain was mild, the wound recovery was fast, and the postoperative scar was small, achieving a balance between curing anal fistulas and protecting anal function. It is worthy of clinical application and promotion. Shrivats Mishra treated 35 patients with anal fistulas with Tropis surgery and conducted a 3-month follow-up. Among them, 29 patients achieved complete healing, with a cure rate as high as 82.89% [5]. At present, the specific effica-

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cy of Tropis surgery in the treatment of complex anal fistulas still lacks multi-center and large-sample data support for verification. However, due to its advantage of effectively preserving the sphincter, it is still favored by surgeons.

LIFT: Ligation of the intersphincteric fistula tract

Ligation of the intersphincteric fistula tract was first proposed by Thai doctor Rojanasakul in 2007 [6]. The main operational points are to make an incision between the sphincters, ligate the fistula near the internal sphincter, separate the external opening to the external sphincter fistula, close the defect of the external sphincter, and suture the intersphincter incision. Rojanasakul was the first to apply this surgical method to 18 patients with anal fistula. After a 3-month follow-up, the cure rate was as high as 94.4%. Hariprasad compared the clinical efficacy of 40 patients with anal fistula treated by LIFT and fistula resection and found that LIFT surgery for complex anal fistula has the advantages of less surgical trauma, faster recovery, less pain and lower recurrence rate [7]. Zhang conducted a clinical efficacy analysis on 82 patients with complex anal fistula treated with LIFT [8]. After 4 to 120 months follow-up, 6 cases recurred and formed intersphincter fistulas after the operation, and 9 cases developed persistent or recurrent fistulas after the operation. The total clinical cure rate was 80.5%. For colorectal surgeons, the key to the success of LIFT surgery lies in the correct judgment of the fistula shape. The reasons for its failure or recurrence can be attributed to: (1) incomplete treatment of the internal opening; (2) complex branched sinus tracts; (3) multiple anal surgical treatments have been performed; (4) the infected glands and some sources of infection have not been cleared. However, compared with traditional surgical methods, the LIFT procedure, as a surgical approach through the intersphincter, causes less damage to the sphincter, can protect the anal function to the greatest extent, has a lower probability of postoperative complications, and has a high application value in the treatment of complex anal fistulas.

Furthermore, the combination of biological dressings (BioLIFT) and anal fistula plugs (Lift-plug) with the traditional LIFT surgical method has also been innovatively applied in the surgical treatment of complex anal fistulas with sphincter preservation. Although studies have shown that these two surgical methods have a high cure rate, can effectively protect the anal sphincter, and reduce the recurrence rate, due to the high surgical cost, the therapeutic effect has not been verified by large sample data, so it has not been widely used in clinical practice.

Loose-seton technique

During the operation, the internal orifice and internal sphincter are incised, the primary infection focus is removed, the external sphincter is not resected, and the fistula outside the sphincter is removed for loose-seton [9]. During the process, the external sphincter is not severed. It only serves the functions of drainage, marking, and promoting fibrosis through foreign body stimulation. Therefore, this surgical method has both the characteristics of drainage and the preservation of the sphincter. Kennedy and Zegarra treated 32 patients with relaxed suture drainage [10]. The time for removing the rubber band was 2 to 16 weeks (with an average of 7 weeks), and the average follow-up was 36 months. The cure rate was 78%. Some patients had mild air leakage or overflow, and only 1

case (3%) had occasional loose stool incontinence. No patients had solid stool incontinence.

The loose-seton technique reduces the damage to the sphincter and ensures smooth drainage at the same time. It has a good therapeutic effect on the upper sphincter and the high transsphincter anal fistula. However, there is still some controversy over the cure rate of this surgery at present. When this surgical method is used in clinical practice, it is found that: (1) The timing of removing the double-stranded rubber bands is particularly crucial. Observe that there is no secretion from the wound, the fluid flowing out of the incision is clean after rinsing, and the base of the wound is long and solid. Remove the rubber bands in batches when there is resistance when rotating them; (2) After the operation, the wound surface should be rinsed with normal saline to keep it fresh and clean; (3) Regularly explore the wound surface to prevent false healing; (4) When fibrosis at the incision site in the later stage is difficult to heal, tight suture therapy should be combined to ensure the therapeutic effect.

ERAF: Endorectal advancement flap

The endorectal advancement flap was the first method for treating anal fistula with sphincter preservation, proposed by Elting in 1912 [11]. This method was first used in the treatment of rectovaginal fistula. In 2022, ASCRS "Guidelines for the Treatment of Anal Fistula, Perianal Abscess and Rectovaginal Fistula" recommended a grade of 1B for it, and it has gradually become a relatively mature sphincter-preserving surgical method [12]. The key points of this surgical procedure lie in completely stripping the internal orifice and the surrounding lesion tissues, and creating a "U"-shaped flap (including the mucosa, submucosa and part of the circular muscle, with the bottom wider than the top). After loosening the mucosal flap, it is tensively and intermittently sutured with the surrounding tissues, and the external orifice and the lateral fistula are removed in a tunnel manner. Multiple research reports have shown that the cure rate of rectal mucosal flap displacement in patients with anal fistula ranges from 66% to 87% [13-15]. Carlos Chaveli Diaz performed surgery and follow-up on 115 patients with anal fistula [16]. As a result, 84 patients were cured, and no severe anal function impairment occurred in all patients who adopted this surgical method.

ERAF has a definite therapeutic effect on complex anal fistulas. From a technical perspective, this surgical method has the advantages of not damaging the sphincter, having a relatively small impact on anal function, not affecting the appearance of the anus, causing mild pain, and not affecting reoperation after failure. It has also been widely used for rectovaginal fistulas and rectourethral fistulas. However, the surgical operation is rather complex and requires surgeons with rich clinical experience to carry out. The related influencing factors of its failure and recurrence still require further analysis in large-sample, multi-center clinical studies to better reduce risks and improve surgical efficacy.

Mesenchymal adipose-derived stem cell transplantation

Stem cell transplantation therapy involves transplanting healthy stem cells into a patient's body to repair or replace damaged cells or tissues, thereby achieving the goal of curing diseases [17]. Mesenchymal adipose-derived stem cells are stem cells derived from fat. They are easy to obtain, have a

large reserve in the body, and have functions such as tissue regeneration and repair, and inhibition of inflammatory responses. The advantages include no damage to the sphincter, less trauma, and less pain in the treatment of complex anal fistulas [18]. Zhang treated 24 patients with complex anal fistula with autologous adipose-derived stem cell transplantation [19]. They observed indicators such as the cure rate, external opening closure time, anal incontinence score, pain score, and the score of life quality. After regular follow-up, the results showed that after adipose-derived stem cell treatment, the patients had a high quality of life, the anal sphincter was well protected, and no anal incontinence occurred, superior to traditional surgical methods. Panes conducted a randomized, double-blind, parallel, placebo-controlled Phase III clinical trial in 2016, the results showed that the cure rate of the allogeneic adipose-derived stem cell treatment group was significantly better than that of the placebo group, with a lower recurrence rate and a lower incidence of related adverse events [20].

In addition, several studies have also confirmed that adipose-stem cell transplantation has the advantages of safety, no incision of normal tissues, less damage to anal function, good tolerance, repeatability, quick postoperative recovery, mild pain, and high perioperative quality of life for patients. It is an effective method for treating complex anal fistulas [21-22]. However, the current research sample size is relatively small, and large-sample clinical research data is needed to confirm its efficacy.

Other sphincter-preserving surgical methods

Video-assisted anal fistula treatment (VAAFT) combines endoscopic techniques with the concept of sphincter preservation. Its surgical advantage lies in the ability to explore and handle the fistula and internal orifice under direct video vision [20], to remove the necrotic tissue of the fistula, and to close the inner opening without damaging the anal sphincter. Siddique believes that VAAFT has the advantages of less intraoperative bleeding, less postoperative pain, less anal sphincter injury, faster wound healing and shorter hospital stay in the treatment of complex anal fistulas, making it a better surgical method for the treatment of complex anal fistulas [23]. KOCH-HAR treated 82 patients with anal fistula with VAAFT from 2010 to 2014, with a cure rate of 84% [24]. In this study, anal canal manometry was used to evaluate the sphincter function of patients before and after the operation. The results showed that there was no statistically significant difference in the rest-

ing and systolic pressure of the anal canal between patients before and after the operation ($P > 0.05$), indicating that the sphincter function was not damaged, confirming that VAAFT has a relatively optimistic clinical application prospect in the treatment of complex anal fistulas.

Fistula-tract laser closure (FiLaC) is a new sphincter-preserving surgical method. It causes the tissues inside the fistula to vaporize and contract through the energy released by the circular laser at the tip of the catheter, thereby closing the fistula. Foreign studies have shown that the cure rate of this method for treating anal fistula is 71%-82% [25-26]. While curing anal fistula, patients have mild postoperative pain, and no fecal incontinence occurs, and the anal function is maximized, indicating that this is a safe and effective surgical method.

The anal fistula plug and fibrin glue, as new biological scaffold materials, have gradually been applied in the treatment of complex anal fistulas. Anal fistula plug is an absorbable material made from pig small intestines. When placed in the patient's body, it can rapidly generate new blood vessels and promote the reproduction of fibroblasts, thereby achieving the effect of repair. Studies have confirmed that anal fistula plugs have significant effects in closing the internal opening and filling fistulas [27]. They can accelerate the healing time of patients' wounds, cause no major trauma to patients, and have no impact on the appearance and function of the patient's anus. Fibrin glue is a natural extracellular matrix with the advantage of good histocompatibility. It can promote the formation of vascular scars and stimulate the proliferation of fibroblasts and the formation of collagen fibers at the wound site, accelerating wound healing. Moreover, fibrin glue is simple and can be repeatedly implanted. Studies showed that the cure rate of fibrin glue in the treatment of anal fistula was 14% to 63%. Its effect is influenced by multiple factors, but its natural protection of the sphincter and its non-impact on subsequent other treatment methods are still worthy of selection. However, the recurrence rate after anal fistula plug and fibrin glue is relatively high, the long-term effect is not satisfactory, and there is a lack of a large amount of clinical data to support its therapeutic effect. At present, it has not been widely used in clinical treatment.

Table of research results related to sphincter-preserving surgery

A total of 9 articles on sphincter-preserving surgery were included [3, 20, 28-33], involving 610 patients, including 5 ran-

Table 1.

Surgical method	Study	Type of study	Cure rate	Complications
Tropis	Pankaj Garg et al. [3]	Cohort study	90.4%	15.4%
LIFT	Tsang et al. [28]	RCT	87.5%	12.5%
Loose-seton technique	Kennedy et al. [10]	RCT	78%	3%
Stem cell Transplantation	Panés et al. [20]	RCT	50%	17%
AF	Mujukian et al. [29]	Cohort study	65%	8%
VAAFT	Cheung et al. [30]	RCT	81%	12.9%
FiLaC	Wilhelm A et al. [31]	Cohort study	64.1%	2.5%
FG	Haim N et al. [32]	RCT	63.04%	4.34%
AFP	Yu Tao et al. [33]	Cohort study	56%	35%

The cure rate, and complications of sphincter-preserving surgery. Tropis, transanal opening of intersphincteric space; LIFT, ligation of intersphincteric fistula tract; AF, advancement flap; VAAFT, video-assisted anal fistula treatment; FiLaC, fistula-tract laser closure; FG, fibrin glue; AFP, anal fistula plug.

domized controlled trials and 4 cohort studies. The included studies summarized the cure rate, and complications of each technique. The results of the included studies are shown in Table 1.

Conclusion

At present, with the advancement of medical technology and the improvement of medical humanistic care, the treatment of complex anal fistulas is no longer simply limited to treatment. How to ensure the anal function of patients and guarantee their life quality while achieving cure is the focus of clinicians' attention. The emergence of various sphincter-preserving surgeries has further improved the therapeutic effect of complex anal fistulas, and can minimize the risk of postoperative complications for patients to the greatest extent, which is of great significance for improving the quality of life of patients. Clinically, the appropriate surgical method can be selected based on the fistula condition of the patient. When necessary, other surgical methods can be combined, along with postoperative care, to achieve the goal of curing anal fistula without damaging the sphincter and anal function, and to improve the long-term efficacy of the sphincter-preserving surgical method for anal fistula.

Abbreviations

Advancement flap: AF; Anal fistula plug: AFP; Biological ligation of the intersphincteric fistula tract: BioLIFT; Endorectal advancement flap: ERAF; Fibrin glue: FG; Fistula-tract laser closure: FiLaC; Ligation of the intersphincteric fistula tract: LIFT; Ligation of the intersphincteric fistula tract plug: Lift-plug; Transanal opening of intersphincteric space: Tropis; Video-assisted anal fistula treatment: VAAFT.

Author Contributions

Ling Wang and Xindan Zhang: writing - original draft. Ji Jin and Fuheng Liu: writing-original modification. Gang Zhao: Writing - review & editing. All authors read and approved the final manuscript.

Acknowledgements

Not Applicable.

Funding Information

This project was supported by Science and Technology Plan of Suqian City (Grant No. S202220).

Ethics approval and consent to participate

Not Applicable.

Competing Interests

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

Data Availability

Not Applicable.

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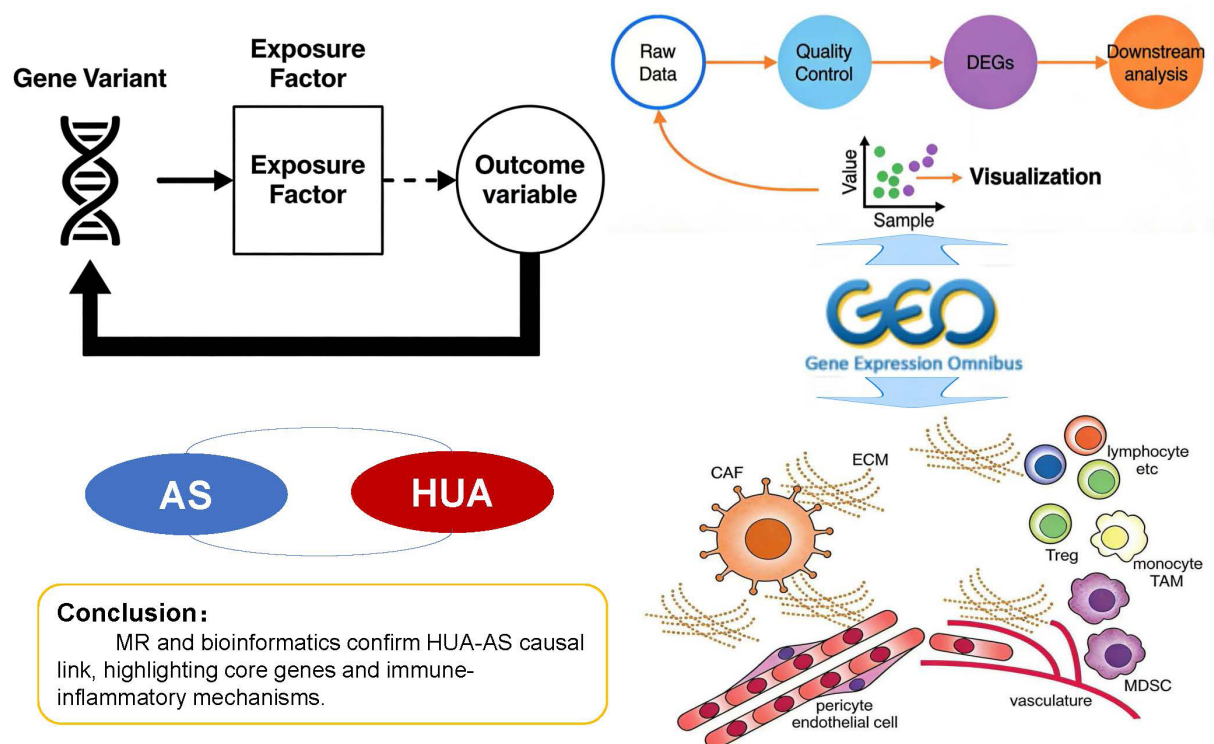
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Graphical Abstract



<https://doi.org/10.71321/4w6y6229>

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Investigating the Association and Causality Between Hyperuricemia and Peripheral Atherosclerosis: A Study Based on Mendelian Randomization and Bioinformatics Analysis

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Received: 2025-12-07 | Accepted: 2026-02-21 | Published online: 2026-03-31

Abstract

Objective: To investigate the genetic causal association and shared molecular mechanisms between hyperuricemia (HUA) and peripheral atherosclerosis using Mendelian randomization (MR) and bioinformatics approaches, providing a theoretical basis for early disease screening and intervention.

Methods: An MR strategy was employed. Based on Genome-Wide Association Study (GWAS) datasets, the causal relationship between HUA and peripheral atherosclerosis was assessed through instrumental variable selection, multi-model MR analysis (including Inverse-Variance Weighted, IVW), and sensitivity analysis validation. Simultaneously, relevant datasets from the GEO database were utilized to identify differentially expressed genes (DEGs) common to both diseases. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed, and a Protein-Protein Interaction (PPI) network was constructed to identify hub genes. Immune infiltration characteristics were analyzed using CIBERSORT.

Results: A significant positive causal association was found between hyperuricemia and atherosclerosis (OR=1.296, 95% CI: 1.112-1.512, P=0.0009). Bioinformatics analysis identified 133 intersecting DEGs, which were enriched in immune-inflammatory-related functions and pathways. Hub genes screened from the PPI network included IL1B, CD86, and CSF1R. Immune infiltration analysis revealed characteristic remodeling of the immune microenvironment in both groups.

Conclusion: A significant positive genetic causal association exists between HUA and peripheral atherosclerosis. The shared pathogenic mechanisms may involve the aforementioned hub genes, activation of immune-inflammatory pathways, and abnormal immune infiltration. The findings provide a theoretical framework and potential experimental targets for early risk prediction and targeted intervention in HUA complicated with peripheral atherosclerosis.

Keywords: Hyperuricemia; Atherosclerosis; Mendelian randomization; Bioinformatics; Hub targets; Immune infiltration

Introduction

Hyperuricemia (HUA) is a metabolic disorder characterized by excessive uric acid production or impaired excretion, and its prevalence has been rising globally, making it a significant public health concern [1]. As the underlying pathological basis for the onset of gout, HUA not only leads to joint inflammation and tissue damage but has also been confirmed by numerous clinical studies to exhibit a significant correlation with the occurrence and progression of cardiovascular diseases. It is an independent risk factor for cardiovascular events such as hypertension and coronary heart disease [2]. Atherosclerosis (AS) serves as the central pathological mechanism underlying

cardiovascular diseases, characterized primarily by endothelial dysfunction, abnormal lipid deposition, and chronic inflammatory responses. Peripheral atherosclerosis, in particular, can lead to distal limb ischemia, necrosis, and even amputation, severely impairing patients' quality of life while imposing a substantial burden on healthcare systems [3-4].

Currently, the association between HUA and peripheral atherosclerosis has been preliminarily confirmed by several observational studies. However, such studies are susceptible to interference from confounding factors such as age, obesity, and dyslipidemia, and cannot exclude reverse causality bias, making it difficult to determine whether a direct genetic causal relationship exists between them [5]. At the molecular mechanism level, existing research suggests that inflammatory re-

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sponses may be a key mechanism mediating their comorbidity [6]. Nevertheless, the specific regulatory pathways, core effector genes, and characteristics of immune microenvironment remodeling have not been fully elucidated. This lack of clarity contributes to the absence of specific early screening indicators and targeted intervention strategies for the co-occurrence of HUA and peripheral atherosclerosis in clinical practice. Mendelian randomization (MR) leverages the random allocation of genetic variants, effectively minimizing confounding factors and reverse causation bias, thereby providing high-quality evidence for elucidating genetic causal relationships between diseases [7]. Bioinformatics technology, through systematic mining of gene expression data from public databases, enables comprehensive analysis of disease-related differentially expressed genes (DEGs), core regulatory targets, and key signaling pathways, offering crucial support for clarifying the molecular mechanisms underlying comorbidities. Building on this, the present study integrates MR analysis and bioinformatics techniques. On one hand, it aims to validate the genetic causal association between HUA and peripheral atherosclerosis. On the other hand, it seeks to identify shared DEGs, core genes, and critical pathways between the two conditions, and to decipher immune infiltration characteristics. This research aims to provide a theoretical foundation and potential experimental targets for early risk prediction and targeted intervention of HUA comorbid with peripheral atherosclerosis, while also offering new perspectives for mechanistic research into "inflammatory-metabolic comorbidities."

Materials and Methods

This study comprised two main modules: Mendelian randomization (MR) analysis and bioinformatics analysis. Using HUA as the exposure and peripheral atherosclerosis as the outcome, we aimed to validate their causal relationship. Subsequently, we integrated relevant datasets for HUA and peripheral atherosclerosis from the Gene Expression Omnibus (GEO) database. This integration facilitated the identification of comorbidity-related differentially expressed genes (DEGs), core regulatory targets, and key pathways. Furthermore, immune infiltration analysis was incorporated to elucidate the underlying molecular mechanisms.

Mendelian Randomization Analysis

Data Sources

In this study, serum urate levels were defined as the exposure factor, and atherosclerosis as the outcome variable. All genome-wide association study (GWAS) summary datasets were retrieved from the IEU OpenGWAS project (<https://gwas.mrcieu.ac.uk>). The GWAS dataset for serum urate (ID: ebi-a-GCST90018977) was derived from an analysis of 343,836 individuals of European ancestry, which included 19,041,286 single nucleotide polymorphisms (SNPs) [8].

The GWAS dataset for peripheral atherosclerosis (ID: finn-b-DM_PERIPHATHERO) comprised 216,666 European individuals and 16,380,247 SNPs. The original GWAS studies received approval from their respective institutional ethics committees, and all participants provided written informed consent.

Selection of Instrumental Variables

This study employed single nucleotide polymorphisms (SNPs) as instrumental variables for the Mendelian randomization (MR) analysis, adhering to the three core MR assumptions (relevance, independence, and exclusion restriction) [9]. The selection process was conducted as follows: Initially, SNPs significantly associated with serum urate levels (a proxy for HUA) were identified (inclusion threshold: $P < 5 \times 10^{-8}$). To ensure independence among the selected instruments, a clumping procedure was applied using a linkage disequilibrium (LD) threshold of $r^2 < 0.001$ within a 10,000 kb window. The strength of each instrumental variable was assessed using the F-statistic (calculated as $F = \beta^2 / SE^2$, where β is the allele effect size and SE is its standard error) [10]. SNPs with an F-statistic less than 10, indicating potential weak instrument bias, were excluded. Furthermore, palindromic SNPs were removed to prevent strand ambiguity and analysis bias. Subsequently, the MR-PRESSO package was utilized to detect and remove outlier SNPs, thereby enhancing the robustness and reliability of the instrumental variable set.

Statistical Analysis for Mendelian Randomization

All MR analyses were performed using R software with the TwoSampleMR package. The primary analytical method was the inverse-variance weighted (IVW) approach under a random-effects model, which was used to estimate the causal effect of serum urate levels on peripheral atherosclerosis. The combined odds ratio (OR) and its 95% confidence interval (CI) were derived by integrating the causal estimates from individual single nucleotide polymorphisms (SNPs), and these results served as the primary evidence for the causal inference. To complement the primary analysis and assess the robustness of the findings, several sensitivity analyses were conducted, including the weighted median method (which provides consistent estimates even if a proportion of the instrumental variables are invalid), the weighted mode method, the simple mode method, and MR-Egger regression (which can simultaneously detect and adjust for potential directional pleiotropy). A two-sided P-value of less than 0.05 was considered statistically significant for all analyses.

Sensitivity Analysis

To ensure the robustness of the Mendelian randomization (MR) results, a comprehensive set of sensitivity analyses was conducted. Heterogeneity among the instrumental variables was assessed using Cochran's Q test; a P-value of less than 0.05 indicated the presence of heterogeneity, in which case the inverse-variance weighted (IVW) estimates were derived using a random-effects model. The potential for horizontal pleiotropy was evaluated via the MR-Egger intercept test, with a P-value greater than 0.05 suggesting no significant evidence of pleiotropic bias. Prior to the main analyses, the MR-PRESSO package was employed to identify and remove outlier SNPs. The results from the random-effects IVW method were considered primary. A funnel plot was generated to visually inspect the symmetry of effect estimates from individual SNPs, providing an additional check for potential bias. Finally, a leave-one-out analysis was performed by iteratively removing each SNP to determine whether the overall causal estimate was unduly driven by any single influential genetic variant, thereby confirming the stability and reliability of the conclusion.

Bioinformatics Analysis

Data Acquisition and Preprocessing

The Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) was systematically queried using the keywords "hyperuricemia" and "atherosclerosis" to identify relevant datasets. The inclusion criteria were defined as follows: (1) availability of both patient and healthy control groups with maximized sample sizes; (2) utilization of samples derived from human tissues; and (3) exclusion of samples involved in drug or therapeutic clinical trials. In accordance with these criteria, datasets GSE160170 and GSE43292 were selected as discovery sets, while GSE28829 was designated as the external independent validation set. Specifically, GSE160170 comprised peripheral blood mononuclear cells (PBMCs) from patients with HUA alongside age- and gender-matched healthy controls, capturing the systemic immune and metabolic status associated with HUA. Meanwhile, GSE43292 included atherosclerotic plaque tissues from patients with peripheral atherosclerosis and paired adjacent normal arterial wall tissues, providing insights into the local pathological microenvironment of vascular lesions. The validation set, GSE28829, consisted of 16 samples of advanced atherosclerotic plaque and 13 samples of early atherosclerotic plaque. All datasets underwent uniform preprocessing procedures, including background correction, quantile normalization, and probe-to-gene annotation conversion. Differentially expressed genes (DEGs) were identified independently within the two discovery sets, while the validation set was exclusively utilized to confirm the expression trends of key hub genes.

Identification of Differentially Expressed Genes (DEGs)

DEGs corresponding to HUA and peripheral atherosclerosis were extracted from the GSE160170 and GSE43292 mRNA datasets, respectively, using the "limma" R package. For both the HUA and peripheral atherosclerosis datasets, genes with an adjusted P-value < 0.05 and an absolute log₂ fold change (|logFC|) ≥ 0.6 were considered differentially expressed. The overlapping genes between the two DEG lists were identified using a Venn diagram.

Functional Enrichment Analysis of DEGs

To investigate the biological functions and signaling pathways associated with the common key genes, enrichment analyses were performed based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. GO annotation encompasses three domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The GO functional and KEGG pathway enrichment analyses for this gene set were conducted using the "clusterProfiler" package in R, with a significance threshold set at P < 0.05.

Protein-Protein Interaction Network and Identification of Hub Genes

A protein-protein interaction (PPI) network was constructed based on the overlapping differentially expressed genes (DEGs) using the STRING database (version 12.0). The resulting network was then imported into Cytoscape software (version 3.9.1) for visualization and further analysis. Hub genes within the network were identified using the cytoHubba plugin, employing the Maximal Clique Centrality (MCC) algorithm. The top 10 genes ranked by the MCC score were selected as candi-

date hub genes for subsequent analysis.

Validation of Hub Genes in External Dataset

To validate the robustness of hub genes identified from the discovery sets, the independent external GEO dataset GSE28829 was selected as the validation cohort. This dataset comprises peripheral blood gene expression profiles from peripheral atherosclerosis patients and healthy controls, ensuring tissue-type consistency with the discovery sets for direct comparability. All samples underwent uniform preprocessing using the identical pipeline applied to the discovery sets, including background correction, normalization, and probe-to-gene annotation matching. Differential expression of the 10 hub genes between peripheral atherosclerosis patients and healthy controls was subsequently analyzed using the "limma" R package. Statistical significance was defined as P < 0.05, and expression patterns were visualized using box plots.

Analysis of Immune Cell Infiltration and Spearman Correlation Between Hub Genes and Immune Cells

Immune cell infiltration analysis: The CIBERSORT algorithm was used to estimate the relative abundance of 22 immune cell types in each sample from the discovery sets; only samples with a CIBERSORT output P < 0.05 were retained for subsequent analysis. Stacked bar charts were used to show the composition of immune cells, and box plots were used to compare the abundance differences of immune cells between the disease group and control group (Wilcoxon rank-sum test, P < 0.05 was considered significant).

Spearman correlation analysis between hub genes and immune cells: The expression levels of the top 10 hub genes and the abundance data of 22 immune cell types in the discovery sets were extracted, and Spearman rank correlation analysis was used to calculate the correlation coefficient (r) and significance (P-value) between them. With P < 0.05 as the significance threshold, the "pheatmap" R package was used to draw the correlation heatmap (Figure F corresponds to the hyperuricemia group, Figure H corresponds to the atherosclerosis group); the color intensity of the heatmap indicates the magnitude of the correlation coefficient, and significance marks (P < 0.05, P < 0.01, P < 0.001) were labeled.

Results

Mendelian Randomization Analysis Results

Following adherence to standard instrumental variable selection criteria, 236 independent single nucleotide polymorphisms (SNPs) were ultimately selected for the two-sample Mendelian randomization (MR) analysis to investigate the causal relationship between HUA and peripheral atherosclerosis. To validate compliance with MR assumptions, horizontal pleiotropy was evaluated using MR Egger regression. The initial analysis yielded an intercept estimate of 0.00017 (P = 0.95), with no significant evidence of directional pleiotropy—this was further confirmed by a consistent MR Egger intercept test result (intercept = 0.0002, P = 0.95), supporting the validity of subsequent causal estimates.

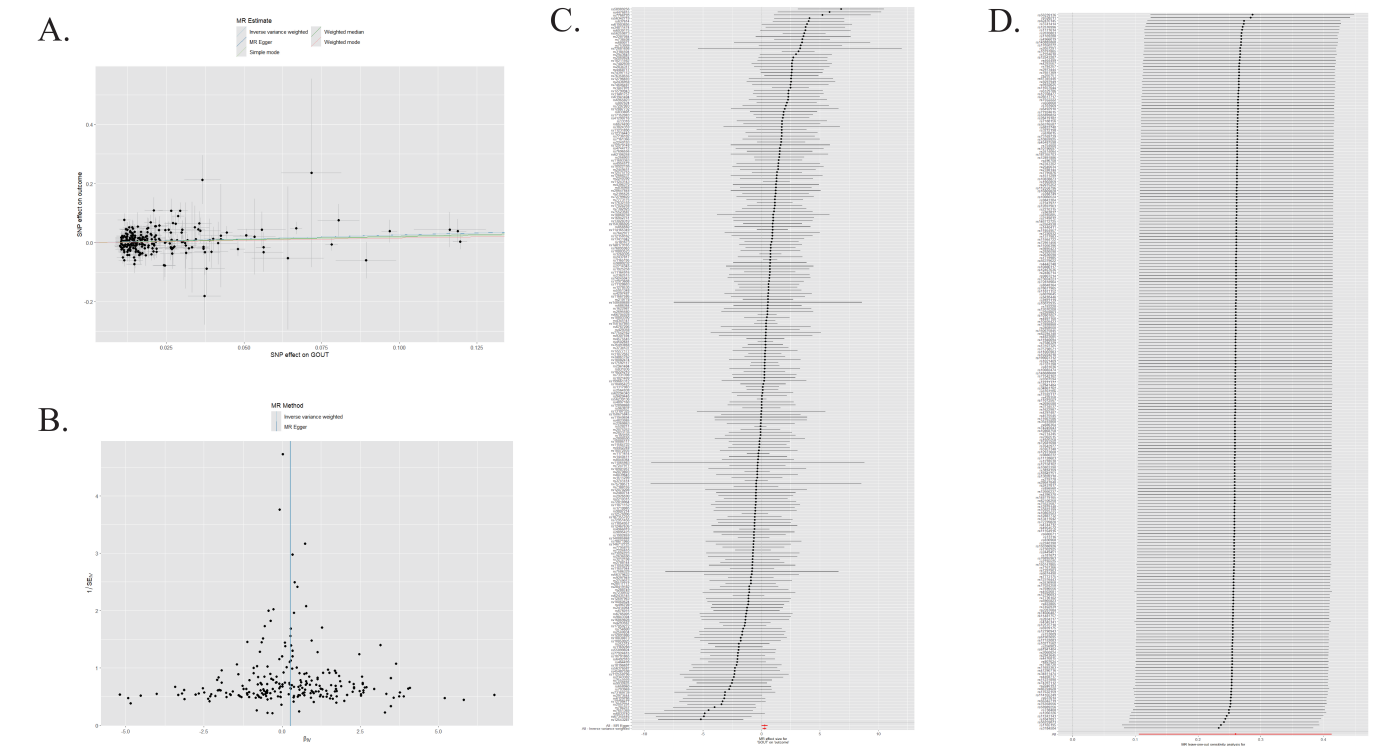
Causal associations were assessed via five complementary MR methods: inverse-variance weighted (IVW), MR Egger regression, weighted median estimator (WME), simple mode,

and weighted mode. Cochran’s Q test revealed substantial heterogeneity across SNPs ($Q = 311, P < 0.01$), prompting the use of the random-effects IVW model. Results from all five methods were directionally concordant, with the primary random-effects IVW analysis demonstrating a significant positive causal association between HUA and peripheral atherosclerosis (odds ratio [OR] = 1.296, $P < 0.001$) (Table 1). The scatter plot of the genetic associations is presented in Figure 1A. The funnel plot (Figure 1B) demonstrated approximate symmetry among the included SNPs, suggesting no obvious directional pleiotropy. A forest plot illustrating the individual causal estimates for each SNP is shown in Figure 1C. Furthermore, leave one out sensitivity analysis (Figure 1D) revealed that no single SNP disproportionately drove the overall causal estimate, supporting the robustness of the observed association.

Table 1. Results of the Two Sample Mendelian Randomization Analysis.

method	nsnp	β	pval	OR (95%CI)
MR Egger	236	0.265	0.055	1.304 (0.995-1.708)
Weighted median	236	0.217	0.079	1.242 (0.974-1.583)
Inverse variance weighted	236	0.259	0.0009	1.296 (1.112-1.512)
Simple mode	236	0.191	0.479	1.210(0.715-2.048)
Weighted mode	236	0.162	0.202	1.176(0.917-1.508)

Figure 1. (A) Scatter plot of the two sample Mendelian randomization analysis; (B) Funnel plot of the two sample Mendelian randomization analysis; (C) Forest plot showing the effect of individual SNPs on peripheral atherosclerosis (SNP: single nucleotide polymorphism); (D) Leave one out sensitivity analysis of SNPs.



Bioinformatics Analysis Results

Identification of Differentially Expressed Genes (DEGs)

Following data preprocessing and application of the defined thresholds (adjusted P-value < 0.05 and $|\log_2FC| \geq 0.6$), we identified 4,156 DEGs associated with HUA and 820 DEGs associated with peripheral atherosclerosis. A Venn analysis revealed 133 overlapping genes between the two sets. Volcano plots visualizing the distribution of DEGs for the HUA and peripheral atherosclerosis datasets were generated using the 'ggplot2' package in R (Figure 2A and 2B, respectively). The overlap of DEGs between HUA and peripheral atherosclerosis was further illustrated using a Venn diagram created with the "VennDiagram" package (Figure 2C).

Results of GO Enrichment and KEGG Pathway Analyses

Enrichment analysis revealed significant terms across the three Gene Ontology (GO) domains, and representative results for each domain are visualized in Figure 3A. In the Biological Process (BP) category, the most significantly enriched term was "regulation of leukocyte proliferation". Other top-ranked terms included "leukocyte migration", "leukocyte proliferation", and "T cell activation", suggesting that these genes are predominantly involved in immune-related regulatory processes such as immune cell proliferation, migration, and T cell activation. For the Cellular Component (CC) domain, the genes were primarily enriched in locations such as the "external side of plasma membrane", "specific granule membrane", and "cell leading edge", indicating their functional roles in plasma membrane structures, granule-related components, and cellular leading edges. Within the Molecular Function (MF) category,

Figure 2. (A) Volcano plot of DEGs from the GSE160170; **(B)** Volcano plot of DEGs from the GSE43292; **(C)** Venn diagram of overlapping DEGs between GSE160170 and GSE43292.

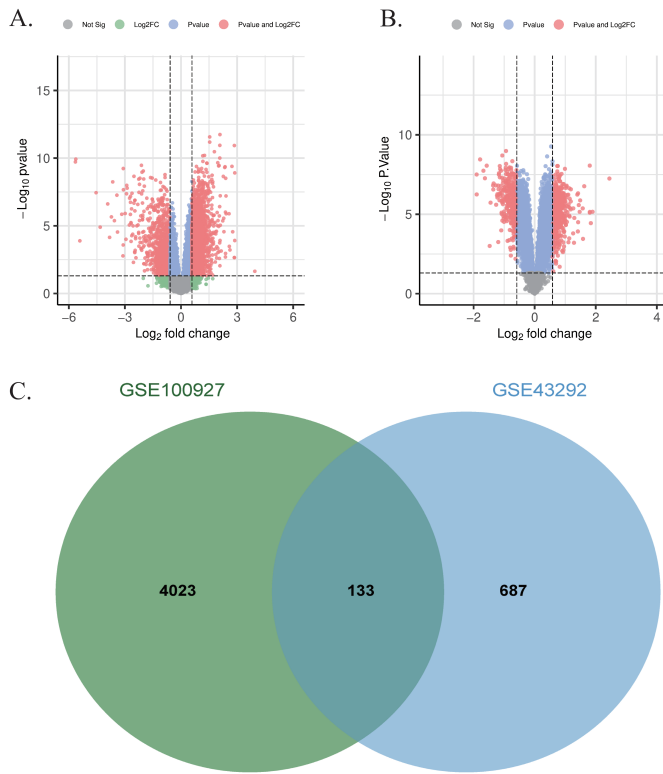
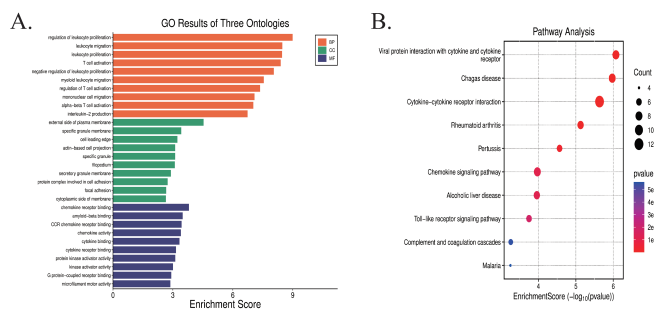


Figure 3. (A) Results of Gene Ontology (GO) enrichment analysis; **(B)** Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.



the enriched genes were significantly associated with functions like "chemokine receptor binding", "amyloid-beta binding", and "CCR chemokine receptor binding", highlighting their crucial involvement in signaling processes related to chemokine receptor interactions and cytokine-associated molecular binding.

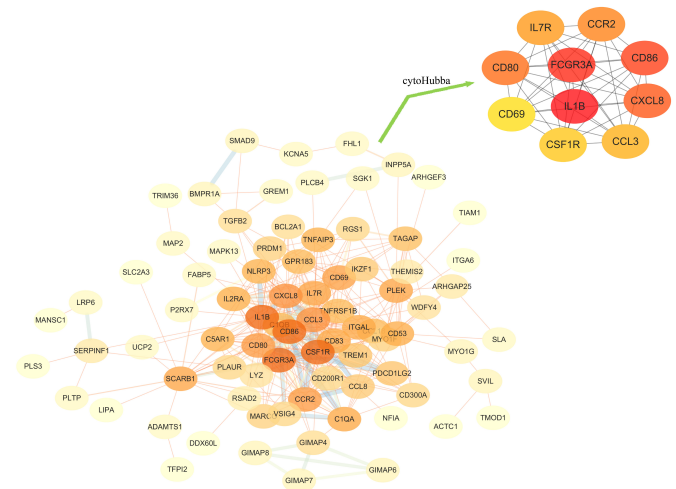
KEGG pathway analysis identified several pathways significantly associated with the overlapping target genes. The top 10 enriched pathways were selected for visualization (Figure 3B). The results revealed that these pathways were primarily involved in immune-inflammatory responses (e.g., Cytokine-cytokine receptor interaction, Chemokine signaling pathway, Toll-like receptor signaling pathway, Complement and coagulation cascades), infectious diseases (e.g., Chagas disease, Pertussis, Malaria), as well as pathways related to viral protein

interactions with cytokines and cytokine receptors, autoimmune disorders (e.g., Rheumatoid arthritis), and alcoholic liver disease.

PPI Network and Identification of Hub Genes

The constructed protein-protein interaction (PPI) network comprised 133 key target nodes connected by 724 interaction edges. The top three hub genes, ranked by node degree, were IL1B (degree = 37), CD86 (degree = 32), and CSF1R (degree = 31). The overall PPI network is visualized in Figure 4. Furthermore, the Maximal Clique Centrality (MCC) algorithm within the CytoHubba plugin was employed to identify the most interconnected core module of the network. This analysis yielded a densely connected core subnetwork consisting of 10 nodes and 90 edges, specifically including the genes IL7R, CCL3, CD80, CXCL8, CD69, CCR2, IL1B, CD86, FCGR3A, and CSF1R.

Figure 4. Protein-protein interaction (PPI) network of proteins encoded by differentially expressed genes.



Validation of Hub Genes in the External Dataset

In the external independent validation set GSE28829, the expression trends of the 10 hub genes were fully consistent with those in the discovery set. As shown in Figure 5 (box plots of the 10 hub genes), each subplot corresponds to one hub gene (namely IL7R, CCL3, CD80, CXCL8, CD69, CCR2, IL1B, CD86, FCGR3A, and CSF1R in sequence), with the P value for differential expression between the peripheral atherosclerosis group and the control group indicated above each subplot. The expression patterns of these hub genes in HUA/ peripheral atherosclerosis patients from the validation set completely aligned with those observed in the discovery set, confirming the stability and biological reliability of these hub genes.

Immune Cell Infiltration

The relative abundance of 22 canonical immune cell subsets across HUA and peripheral atherosclerosis discovery cohorts was deconvoluted using the CIBERSORT algorithm; samples with a deconvolution P-value <0.05 were included for subsequent analyses to ensure robustness. In the HUA cohort, box plot analysis (Figure 6A, Control vs. HUA) demonstrated significantly altered immune cell proportions, including markedly elevated eosinophil, activated mast cell, and monocyte compartments alongside diminished resting natural killer (NK)

cell frequencies (all $P < 0.05$). This heterogeneity was further illustrated in stacked bar plots (Figure 6B), which displayed the compositional variability of 22 immune subsets across individual samples. Hierarchical clustering of immune infiltration patterns (Figure 6E) effectively distinguished HUA patients from healthy controls, corroborating a disease-specific immune signature. Spearman correlation analysis (Figure 6F) revealed distinct positive and negative associations between top hub genes and immune subsets, with multiple hub genes exhibiting robust positive correlations with pro-inflammatory cell types, consistent with HUA's inflammatory pathogenesis. In the peripheral atherosclerosis cohort, box plot comparisons (Figure 6C, Atherosclerotic vs. Control) identified significantly increased memory B cell and M0 macrophage abundances, concomitant with reduced regulatory T (Treg) and activated NK cell populations (all $P < 0.05$). Stacked bar plots (Figure 6D) depicted cohort-specific distribution heterogeneity, while hierarchical clustering (Figure 6G) segregated peripheral atherosclerosis patients from controls based on immune cell composition, reflecting a distinctive peripheral atherosclerosis-associated microenvironment remodeling pattern. Correlation heatmaps (Figure 6H) demonstrated AS-specific relationships between hub genes and immune subsets, notably positive correlations between key hub genes and pro-atherosclerotic populations such as M0 macrophages, thereby linking core genetic signatures to immune dysregulation in peripheral atherosclerosis pathogenesis.

Discussion

The integration of Mendelian randomization and bioinformatics analyses in this study provides robust evidence supporting a genetic causal relationship between HUA and peripheral atherosclerosis, alongside the identification of shared molecular mechanisms centered on immune-inflammatory activation.

The MR results demonstrate that HUA significantly increases the risk of peripheral atherosclerosis ($OR = 1.296$, $P = 0.0009$), while the bioinformatics investigations reveal 133 overlapping differentially expressed genes enriched in immune-related pathways, with key hub genes such as IL1B, CD86, and CSF1R implicated in both conditions. Furthermore, immune infiltration profiling delineates distinct yet converging microenvironmental remodeling in HUA and peripheral atherosclerosis. Building upon these findings, the following sections interpret the clinical implications of the causal association, elucidate the potential roles of core genes and pathways, discuss immune dysregulation features, and outline future research directions.

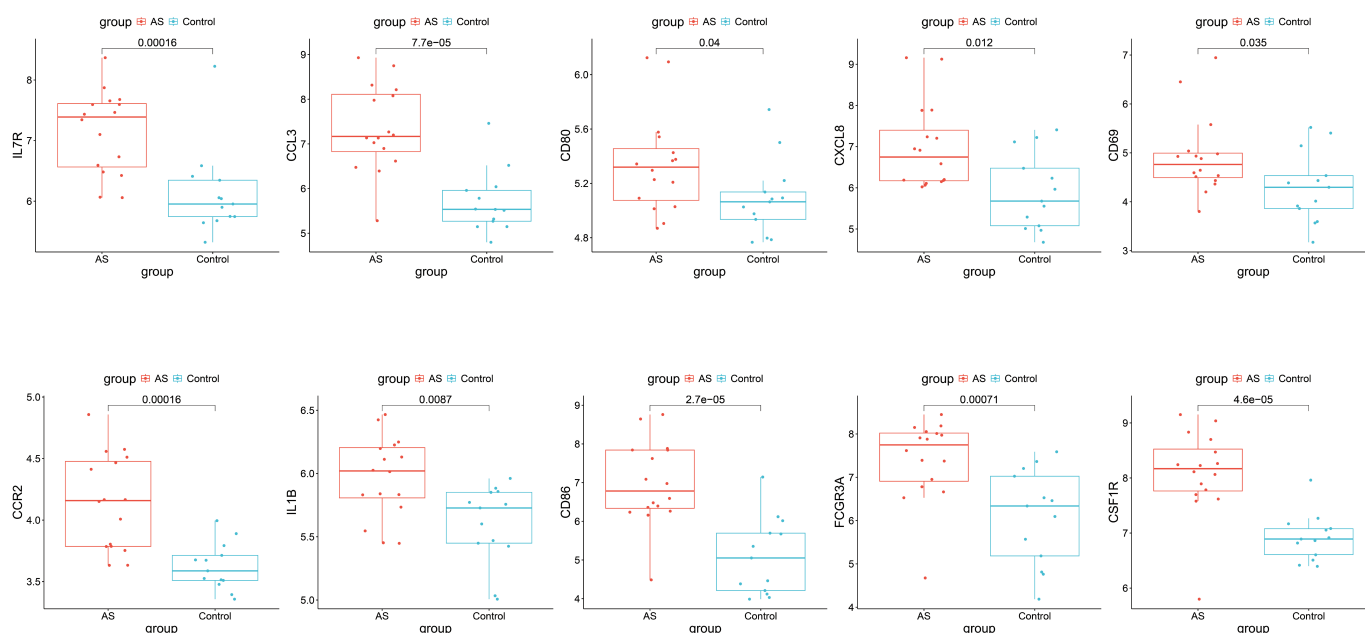
Causal Relationship Between Hyperuricemia and Atherosclerosis: Clinical Implications of MR Findings

The MR-derived causal association lends strong support to the pathogenic hypothesis that hyperuricemia promotes atherosclerosis via vascular endothelial injury. This mechanism is consistent with recent reviews indicating that hyperuricemia may induce endothelial dysfunction through dual pathways of oxidative stress and inflammatory activation, thereby serving as a contributing factor in the initiation of atherosclerotic progression [11].

The clinical relevance of the Mendelian randomization (MR) findings substantiates the mechanistic paradigm that HUA contributes to atherosclerosis through the impairment of vascular endothelial function—a pathway increasingly emphasized in recent reviews [12–13]. Mechanistically, HUA is posited to induce endothelial dysfunction via dual interrelated pathways involving oxidative stress and inflammatory activation, thereby serving as an initiating factor in atherogenesis.

From a clinical practice perspective, this study provides key evidence for the prevention and control of cardiovascular risks in patients with primary HUA. Combined with previous research indicating the protective effect of urate-lowering therapy against coronary atherosclerosis [14], it is recommended

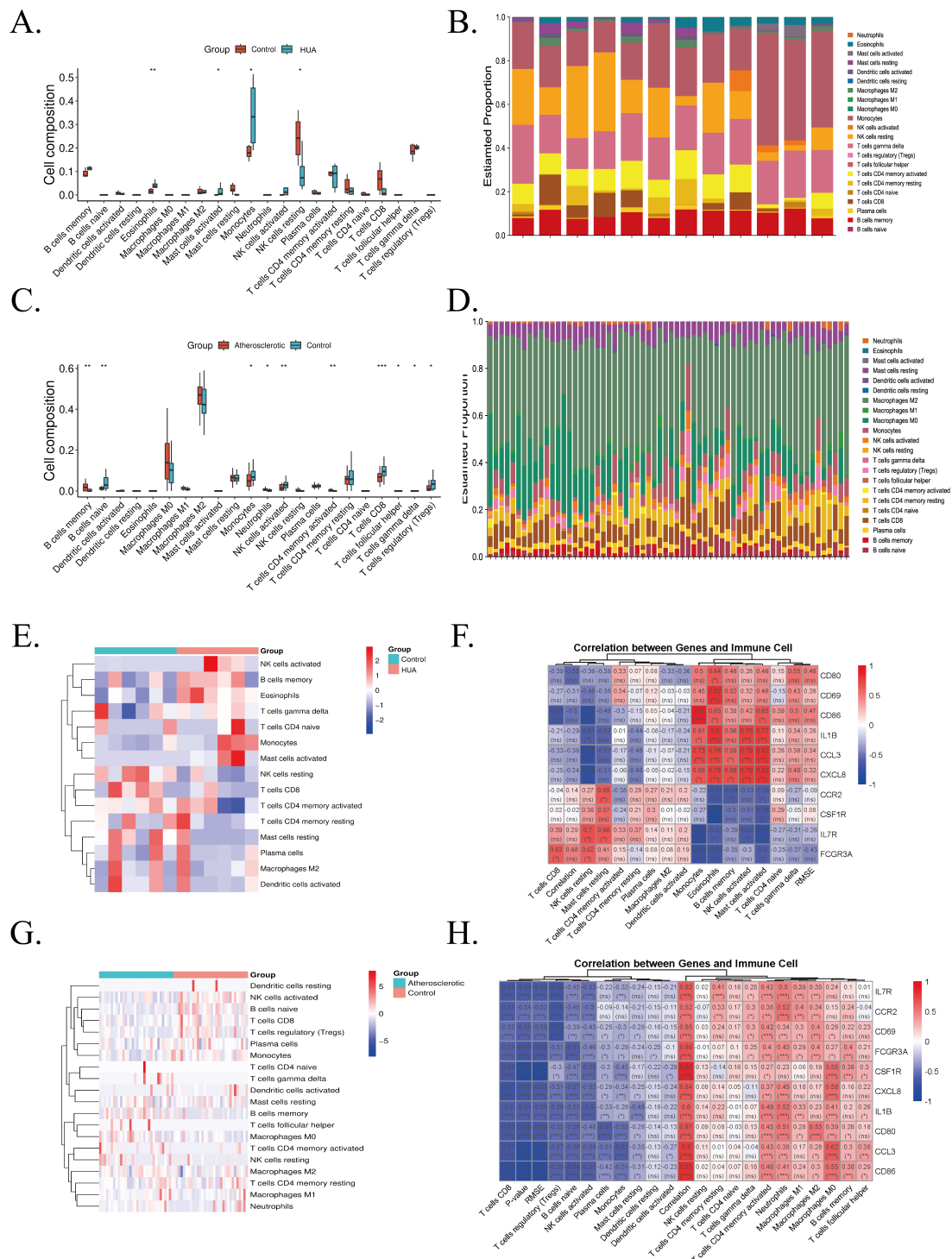
Figure 5. Box plots of the 10 hub genes' expression in the external validation set GSE28829 (peripheral atherosclerosis group vs healthy control group)



that patients with primary HUA (especially those with early-onset or recurrent disease) routinely undergo carotid artery ultrasound (to assess intima-media thickness, IMT) and lipid profile testing. Implementation of an "early screening - early

intervention" strategy may delay the progression of atherosclerosis and reduce the risk of cardiovascular events.

Figure 6. Immune cell infiltration and hub gene-immune cell correlations in HUA and peripheral atherosclerosis. (A) Immune cell abundance box plot (HUA vs. control; Wilcoxon rank-sum test). (B) Immune cell composition stacked bar plot (HUA cohort). (C) Immune cell abundance box plot (peripheral atherosclerosis vs. control; Wilcoxon rank-sum test). (D) Immune cell composition stacked bar plot (peripheral atherosclerosis cohort). (E) Immune infiltration pattern heatmap (HUA vs. control). (F) Hub gene-immune cell Spearman correlation heatmap (HUA cohort). (G) Immune infiltration pattern heatmap (peripheral atherosclerosis vs. control). (H) Hub gene-immune cell Spearman correlation heatmap (peripheral atherosclerosis cohort).



Molecular Mechanisms of Comorbidity: The Role of Core Genes and Pathways via Integrative Bioinformatic Analysis

Bioinformatic analysis demonstrated that the shared pathogenesis of hyperuricemia and atherosclerosis centers on immune-inflammatory pathways. Gene Ontology enrichment indicated that overlapping differentially expressed genes are primarily involved in immune cell activation, migration, and chemokine receptor binding, while Kyoto Encyclopedia of Genes and Genomes analysis confirmed concurrent activation of cytokine-cytokine receptor interaction, chemokine signaling, and complement-coagulation cascades. These findings suggest that immune-inflammatory responses and coagulation dysfunction synergistically exacerbate disease progression, forming a core molecular network underlying hyperuricemia-atherosclerosis comorbidity and providing a critical theoretical basis for targeted interventions.

Notably, the two discovery datasets differ in tissue origin, which endows the identified mechanisms with dual systemic-local attributes. Dataset GSE160170 comprises peripheral blood mononuclear cells from hyperuricemia patients and healthy controls, whereas GSE43292 consists of atherosclerotic plaque tissues paired with normal arterial wall tissues from atherosclerosis patients. Systemically, upregulated genes in peripheral blood mononuclear cells reflect hyperuricemia-related systemic immune activation, wherein circulating pro-inflammatory mediators impair vascular endothelial function and facilitate immune cell infiltration to prime atherosclerosis initiation [15]. Locally, activation of homologous pathways in plaque tissues drives proliferation, migration, and pro-inflammatory polarization of resident immune cells, particularly macrophages, thereby accelerating lipid deposition, plaque formation, and instability. Collectively, these cross-tissue shared pathways bridge hyperuricemia-induced systemic immune-metabolic disorders and atherosclerosis-associated local vascular lesions, establishing a bidirectional regulatory loop between systemic and pathological processes.

Based on the hub genes identified from the PPI network analysis, we further clarified direct mechanistic links between hyperuricemia-associated uric acid signaling and hub gene upregulation. IL1B, as a classical pro-inflammatory cytokine, is driven by urate crystal-induced NLRP3 inflammasome activation in monocytes and macrophages, which cleaves pro-IL1B into its biologically active form to drive excessive secretion. Elevated IL1B promotes vascular endothelial expression of adhesion molecules (e.g., VCAM-1, ICAM-1) to accelerate monocyte infiltration and induces macrophage polarization toward the pro-inflammatory M1 phenotype, exacerbating plaque instability. [16-17]. CD86, a key costimulatory molecule on antigen-presenting cells, participates in regulating T cell activation and immune homeostasis. Its dysregulated expression disrupts this balance, aggravating immune-mediated joint damage in HUA through M1 macrophage polarization while concurrently promoting abnormal recruitment of inflammatory immune cells within atherosclerotic plaques via dysfunctional checkpoint signaling [18-20]. In addition, CXCL8, serving as a central hub gene, potentially recruits neutrophils to trigger gout flares and is likely involved in chronic vascular inflammation during atherosclerosis [21]. CSF1R regulates macrophage survival and proliferation within atherosclerotic plaques, and its expression is upregulated by inflammatory mediators generated in the setting of endothelial dysfunction. In the study

by Thayaparan et al., endothelial dysfunction establishes a pro-inflammatory microenvironment that enhances CSF1R signaling, thereby driving macrophage-dependent abdominal aortic aneurysm formation. This CSF1R-mediated expansion and sustained survival of macrophages promotes plaque progression, lipid accumulation, and vascular remodeling, collectively contributing to macrophage-driven plaque pathogenesis. [22]. CCR2 mediates monocyte migration and represents a key step in the infiltration of inflammatory cells into the arterial wall [23]. Furthermore, bioinformatics analysis of the discovery sets not only confirmed that the shared pathogenesis of HUA and peripheral atherosclerosis centers on immune-inflammatory pathways but also gained robust support from the external independent validation set GSE28829 (Figure 5), which further underscores the biological significance of the aforementioned hub genes. All 10 hub genes remained significantly upregulated in this independent cohort, effectively excluding dataset-specific bias inherent to the discovery sets and validating these genes as stable core regulatory factors intricately involved in the pathological progression of HUA- peripheral atherosclerosis comorbidity. For instance, the sustained high expression of *IL1B* ($P=0.0007$) and *CSF1R* ($P=4.9e-05$) aligns perfectly with their pro-inflammatory functions elaborated earlier—driving local inflammation in HUA and promoting atherosclerotic plaque progression—and directly reinforces the mechanistic hypothesis that "immune-inflammatory factors orchestrate the development of comorbidity".

Mechanistic Implications of Immune Cell Infiltration

Utilizing the CIBERSORT algorithm, our immune infiltration analysis delineated distinct immune microenvironment characteristics between HUA and peripheral atherosclerosis. HUA was predominantly marked by an increase in innate immune and effector cells, including eosinophils, activated mast cells, and monocytes, coupled with a relative decrease in regulatory T cells. Conversely, peripheral atherosclerosis was characterized by an enrichment of memory B cells and M0 macrophages, alongside a decline in immunoregulatory subsets such as regulatory T cells and activated NK cells. These disparities illuminate the divergent patterns of inflammatory and immunoregulatory imbalance intrinsic to the two conditions, suggesting significant insights into their shared pathogenesis and potential targets for immunomodulatory therapies.

Our analysis of immune cell infiltration demonstrated notable remodeling characteristics in the immune microenvironments of HUA and peripheral atherosclerosis. Moreover, the Spearman correlation between hub genes and immune cells (Figures F, H) further elucidated the regulatory influence of core genes on immune cell behavior. For instance, the positive correlation of IL1B with monocytes and activated mast cells in the HUA group aligns with its role as a pro-inflammatory cytokine that drives local inflammation. Similarly, the correlation of CD86 with memory B cells and M0 macrophages in the peripheral atherosclerosis group is consistent with its known mechanism of regulating T cell activation and disrupting immune homeostasis.

These correlations not only substantiate the molecular network of "hub genes-immune inflammatory pathways-disease comorbidity" but also provide a clear direction for targeted interventions. For example, targeting CSF1R may potentially mitigate peripheral atherosclerosis plaque progression by

inhibiting the accumulation of M0 macrophages, while regulating IL1B is anticipated to improve joint inflammation in HUA as well as vascular inflammation in peripheral atherosclerosis.

Innovations and Future Directions

The core innovation of this study lies in the integration of Mendelian randomization for causal inference and bioinformatic multi-omics analysis, which not only clarifies the causal relationship between HUA and atherosclerosis but also elucidates the underlying molecular mechanisms and immune characteristics. This approach effectively addresses the limitations of traditional observational studies, which are prone to confounding, and of standalone bioinformatic analyses, which lack causal validation. Future research should focus on four key directions: (1) conducting targeted interventions on hub genes such as IL1B and CD86 to evaluate their therapeutic potential in models of comorbidity; (2) performing multi-population GWAS analyses to explore race-specific molecular mechanisms; (3) integrating clinical cohorts to develop immune subtype-based risk prediction models, thereby supporting personalized diagnosis and treatment; and (4) implementing clinical intervention studies to assess the effects of urate-lowering and anti-inflammatory therapies on core pathways and immune infiltration profiles.

Study Limitations

This study has several limitations. First, the MR analysis was based on data from European populations; therefore, the findings may exhibit population specificity and require validation in other ethnic groups. Second, the bioinformatics analysis relied solely on transcriptomic data from public databases, and the functional roles of the identified core genes and pathways lack experimental validation *in vitro* or *in vivo*. Third, the immune infiltration results were inferred from transcriptomic predictions and lack direct experimental corroboration (e.g., flow cytometry or immunohistochemistry). Fourth, therapeutic intervention factors were not incorporated, limiting our ability to assess their potential effects on disease comorbidity, which warrants investigation in future studies.

Conclusion

By integrating Mendelian randomization with multi-omics bioinformatics approaches, this study is the first to reveal a significant positive genetic causal link between hyperuricemia and atherosclerosis, and to systematically identify shared hub genes (e.g., IL1B, CD86, CSF1R) and immune-inflammatory pathways underlying their comorbidity. These findings provide a theoretical foundation and potential targets for early risk stratification and immunomodulatory interventions in patients with HUA complicated by peripheral atherosclerosis.

Abbreviations

Atherosclerosis: AS; Biological Process: BP; Cell type Identification By Estimating Relative Subsets Of RNA Transcripts: CIBERSORT; Cellular Component: CC; Confidence Interval: CI; Differentially Expressed Genes: DEGs; Gene Expression Omnibus: GEO; Gene Ontology: GO; Genome-Wide Association

Study: GWAS; Hyperuricemia: HUA; Intercellular Adhesion Molecule 1: ICAM-1; Inverse-Variance Weighted: IVW; Kyoto Encyclopedia of Genes and Genomes: KEGG; Linkage Disequilibrium: LD; Log2 Fold Change: logFC; Maximal Clique Centrality: MCC; Mendelian Randomization: MR; Mendelian Randomization Pleiotropy Residual Sum and Outlier: MR-PRESSO; Molecular Function: MF; Natural Killer: NK; NOD-Like Receptor Family Pyrin Domain Containing 3: NLRP3; Odds Ratio: OR; Peripheral Blood Mononuclear Cells: PBMCs; Protein-Protein Interaction: PPI; Regulatory T Cell: Treg; Single Nucleotide Polymorphism: SNP; Vascular Cell Adhesion Molecule 1: VCAM-1; Weighted Median Estimator: WME.

Author Contributions

Mengmeng Sun: Conceptualization, Methodology, Formal analysis, Writing – original draft. Haoran Wang: Data curation, Investigation, Validation. Zhigong Zhang: Supervision, Project administration, Writing – review & editing.

Acknowledgements

Not Applicable.

Funding information

Not Applicable.

Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

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

Data Availability

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

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Advancements in Lower Extremity Deep Vein Thrombosis Research for Pancreatic Cancer

Authors

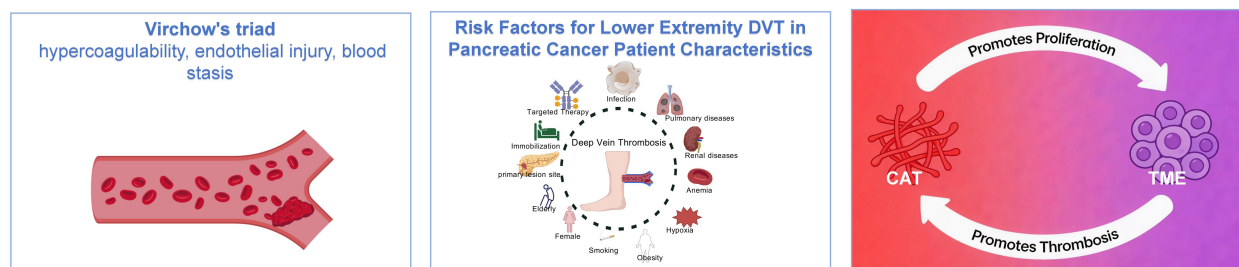
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Graphical Abstract

Hypercoagulable microenvironment, host genetics, and treatment risks synergistically amplify thromboembolism



Khorana score quantifies prophylaxis need for all pancreatic patients, while surgery warrants vigilance for thrombosis and PPH.

<https://doi.org/10.71321/38mrm773>

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Advancements in Lower Extremity Deep Vein Thrombosis Research for Pancreatic Cancer

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Received: 2025-09-23 | Accepted: 2026-01-29 | Published online: 2026-04-06

Abstract

Pancreatic cancer demonstrates the greatest prevalence of thrombosis linked to malignancy in solid tumors, accompanied by a markedly heightened risk of venous thromboembolism (VTE). Early occurrence of VTE in cancer serves as a crucial predictor of adverse prognosis, jeopardizing patient survival and quality of life. The hypercoagulable condition triggered by the tumor microenvironment, integrated with host genetic predisposition and treatment-associated hazards, collaboratively escalates VTE susceptibility. This review methodically outlines the pathophysiology and elevated risk elements for lower extremity deep vein thrombosis (DVT) in pancreatic cancer, while appraising the efficacy of present preventive approaches, seeking to establish a theoretical groundwork for refining clinical management.

Keywords: Pancreatic cancer; lower extremity deep vein thrombosis; risk factors; anticoagulant therapy; research progress

Introduction

Pancreatic cancer has emerged as the fourth leading cause of cancer-related mortality worldwide as of 2021, and its death rate is projected to rise to the second position by 2030 [1]. Despite the availability of various therapeutic strategies, the five-year survival rate for patients with pancreatic cancer remains dismally low, at only 10%. Notably, pancreatic cancer has the highest incidence of cancer-associated thrombosis (CAT) among all solid tumors, with the one-year cumulative incidence of VTE reaching as high as 7.4% following diagnosis [2-3]. Robust evidence from clinical studies has established that the occurrence of VTE in the early stages of malignancy is a critical prognostic indicator for poor outcomes in cancer patients [4]. Clinically, VTE primarily manifests as life-threatening pulmonary embolism (PE) and deep vein thrombosis (DVT), but may also include less common forms such as superficial vein thrombosis and catheter-related thrombosis. Beyond its direct threat to survival, VTE significantly impairs the quality of life in cancer patients [5].

The progression of malignancy is a central driver of VTE risk, with a strong temporal association observed between the onset of metastatic disease and increased VTE incidence [6]. In pancreatic cancer, unique mechanisms of coagulation

cascade activation not only accelerate the development of a prothrombotic state but also establish a bidirectional "thrombosis-tumor" regulatory network: a pathological coagulation microenvironment promotes tumor cell proliferation, while increasing tumor burden further elevates the risk of thrombosis [7]. This review aims to systematically analyze the pathophysiological mechanisms and high-risk factors underlying lower extremity deep vein thrombosis in patients with pancreatic cancer. Furthermore, we critically evaluate the clinical effectiveness of current preventive strategies based on evidence-based medicine, with the goal of providing a theoretical foundation for optimizing clinical management pathways.

Pathophysiological Mechanisms of Lower Extremity Thrombosis in Pancreatic Cancer

The dynamic remodeling of the tumor microenvironment (TME) in pancreatic cancer encompasses multiple processes, including extracellular matrix reorganization, immune cell recruitment, and aberrant angiogenesis. These changes, in concert with malignant tumor cells, activate Virchow's triad—comprising hypercoagulability, endothelial injury, and abnormal blood flow—through multidimensional interactions such as cytokine secretion, cell-cell contact, and mechanotransduction [8]. This activation significantly increases the clinical incidence of CAT

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and VTE.

In pancreatic cancer, a tightly regulated bidirectional network exists between CAT and the TME. CAT not only promotes tumor proliferation and invasion but is also potentiated by the TME, which enhances thrombotic propensity through alterations in the local microenvironment, such as hypoxia and acidosis. Together, these processes drive the malignant transformation toward a more aggressive phenotype, including epithelial-mesenchymal transition (EMT) and metastatic dissemination.

The TME and tumor cells collaboratively orchestrate the regulation of key molecules within the coagulation cascade, such as tissue factor, coagulation factor X, and thrombin. This regulation is mediated through multiple signaling pathways, including inflammatory pathways (e.g., NF- κ B) and hypoxia-inducible signaling (e.g., HIF-1 α), which contribute to the cascade of events underlying VTE pathogenesis—from initial thrombus formation to embolic progression [9].

A prothrombotic state, characteristic of tumor tissue, plays a central role in this process. Its molecular basis involves the coordinated regulation of various inflammatory mediators (such as tissue factor, interleukin-6, and tumor necrosis factor- α) and procoagulant networks (including fibrinogen, von Willebrand factor, and platelet-activating factor). These factors form a positive feedback loop that further amplifies the risk of thrombosis.

Risk Factors for Lower Extremity Deep Vein Thrombosis in Pancreatic Cancer

Patient Characteristics

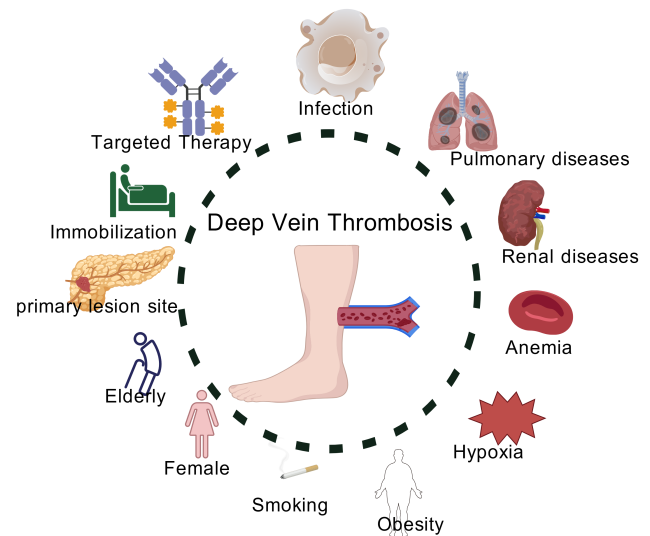
Patients with pancreatic cancer exhibit multidimensional and heterogeneous risk profiles for VTE [10]. The primary clinical risk factors include age over 65 years and reduced mobility, with immobilization (e.g., hospitalization with bed rest ≥ 72 hours) significantly increasing both the incidence and mortality of VTE in cancer patients [11–12]. Smoking is an independent risk factor for pancreatic cancer and further elevates the risk of VTE in patients with malignancies. Female patients with pancreatic cancer tend to have poorer VTE-related outcomes [13]. The presence of comorbidities such as obesity, pulmonary or renal disease, infection, or anemia synergistically increases the risk of VTE in patients with malignancies [14]. Both chemotherapy and hormone therapy are associated with a prothrombotic effect. Notably, the anatomical location of the primary tumor in the pancreas is correlated with thrombosis risk: tumors located in the body and tail of the pancreas are associated with a significantly higher risk of thrombosis compared to those in the pancreatic head.

Characteristic pathological changes in pancreatic cancer include extensive hypoxic regions (median tissue oxygen partial pressure 0–5.3 mmHg, markedly lower than the 24.3–92.3 mmHg observed in normal pancreatic tissue) and a dense fibrotic stroma. The latter acts as a physical barrier, exacerbating hypoxia and driving tumor progression. Hypoxia itself is recognized as an independent adverse prognostic factor in pancreatic cancer (Figure 1) [15].

Antitumor Therapy

Antitumor therapies for malignancies are generally associated with an increased risk of thrombosis, and standard treatment regimens for resectable pancreatic cancer—such as FOLFIRI-

Figure 1. Amplifying VTE Risk Among PDAC Patients (created using BioRender.com).



NOX or gemcitabine-based chemoradiotherapy—are inherently prothrombotic. Evidence-based studies have confirmed that chemotherapeutic agents including gemcitabine, 5-fluorouracil, and oxaliplatin have clear prothrombotic effects [16]. Chemotherapy-induced neutropenia often necessitates the use of hematopoietic growth factors for supportive care. Prospective cohort studies have demonstrated that the use of granulocyte colony-stimulating factor (G-CSF) significantly increases the risk of VTE in patients with pancreatic cancer. Additionally, the routine placement of central venous catheters during chemotherapy further elevates the risk of catheter-related thrombosis.

Major surgical procedures, such as pancreaticoduodenectomy, are associated with substantial tissue trauma and prolonged postoperative immobilization, leading to significant alterations in venous hemodynamics and an increased risk of blood stasis. In patients with pancreatic cancer complicated by chronic pancreatitis or pancreatic duct obstruction, the abnormal release of pancreatic enzymes may degrade anticoagulant proteins (e.g., antithrombin III) through proteolytic activity, thereby further exacerbating the hypercoagulable state.

Leukocytosis

Leukocytosis is significantly associated with an increased risk of VTE in patients with malignancies. A pre-chemotherapy white blood cell (WBC) count $>11 \times 10^9$ /L has been established as an independent predictive factor in the Khorana risk score system. Neutrophils, the predominant component of peripheral WBCs, play a central role in the innate immune defense system. They contribute to thrombogenesis through the release of neutrophil extracellular traps (NETs) and, in conjunction with monocytes, the expression of the procoagulant tissue factor (TF) [17], forming a dual pathway mechanism for leukocyte-mediated thrombosis. In pancreatic cancer patients, leukocytosis is associated with an increased risk of recurrent VTE, with aberrant NETs formation providing the pathophysiological basis for this association. In vitro studies have demonstrated that pancreatic cancer cell lines can induce NETs formation [18]. Furthermore, chemotherapy and targeted

therapies can promote NETs release by damaging the endothelial cell surface [19]. Oto et al. identified seven microRNAs as potential predictive biomarkers for VTE in pancreatic cancer patients, and their study was the first to demonstrate the predictive potential of plasma microRNAs and neutrophil activation markers for VTE in this population [20].

Platelet Hyperactivity

Platelet hyperactivity is considered a key mechanism underlying the hypercoagulable state observed in pancreatic cancer patients. Although platelets are primarily involved in arterial thrombosis, their role in venous thrombosis among cancer patients is increasingly recognized. Tumor-derived interleukin-6 (IL-6) stimulates hepatic thrombopoietin (TPO) synthesis, promoting thrombopoiesis and exacerbating thrombosis [21]. Studies have shown that the rate of thrombin generation in whole blood from pancreatic cancer patients is significantly higher than in plasma samples, with an overall increase in endogenous thrombin potential. These patients exhibit abnormally hyperreactive platelets, characterized by shortened degranulation times, all of which are associated with an increased risk of thromboembolic events [22]. Elevated levels of platelet activation markers are significantly correlated with increased VTE risk and reduced survival in pancreatic cancer patients. Platelet factor 4 (PF4) has been identified as a potential prognostic biomarker in pancreatic cancer, with elevated PF4 levels indicating poor prognosis and a significantly increased probability of VTE [23]. In addition, P-selectin, a member of the selectin family, is expressed on the surface of platelets and endothelial cells during coagulation cascade activation and serves as a key plasma marker of coagulation activation by promoting platelet aggregation [24]. Clinical studies have demonstrated that elevated P-selectin levels are significantly associated with increased VTE risk and mortality in pancreatic cancer patient [25]. As P-selectin is expressed by both endothelial cells and platelets, its dual expression can enhance VTE risk by recruiting leukocytes. Antiplatelet agents such as aspirin and clopidogrel may contribute to VTE prevention in certain cancer populations.

Coagulation Factor Dysregulation

Patients with pancreatic cancer exhibit significant elevations in plasma levels of kallikrein complexes and coagulation factors IX, XIIa, and Xa, as well as their respective natural inhibitor complexes, indicating aberrant activation of the intrinsic coagulation pathway [26]. Typically, these patients present with increased levels of fibrinogen, factor VIII (FVIII), and D-dimer, accompanied by decreased activity of protein C and antithrombin III. Tumor-derived signaling molecules such as interleukin-1 (IL-1), vascular endothelial growth factor (VEGF), and tumor necrosis factor- α (TNF- α) collectively drive a bidirectional network of inflammation and coagulation [27].

Tissue factor, a glycosylated transmembrane receptor protein, specifically binds and activates plasma coagulation factor VII (FVII). The TF/FVIIa complex serves as the critical initiator of the extrinsic coagulation cascade [28]. Numerous studies have demonstrated aberrant overexpression of TF in various malignancies, with TF abundance in pancreatic tumor tissue being closely associated with VTE incidence. Tumor-derived TF can enter the circulation via secretion of TF-positive extracellular vesicles (TF+EVs) [29]. Clinical data indicate that plasma

TF+EVs concentrations are significantly correlated with VTE occurrence in pancreatic cancer patients [30], suggesting a pivotal role for circulating TF+EVs in pathological thrombogenesis.

In pancreatic cancer, procoagulant proteins released in microparticle or non-microparticle forms not only accelerate thrombus formation but also amplify inflammatory responses. These microparticles upregulate the expression of intercellular adhesion molecules, enabling direct participation of tumor cells in the coagulation cascade and triggering both local and systemic coagulation activation [31]. High-molecular-weight mucins secreted by pancreatic cancer cells enter the circulation, with their concentrations positively correlated with tumor progression. Animal model studies have confirmed their biological effect in inducing microthrombus formation [32]. The natural anticoagulant protein S (PS) is markedly downregulated during pancreatic cancer progression [33]. Approximately 60% of circulating PS is inactivated by binding to C4b-binding protein (C4BP), leaving only 40% in the free, functionally active form; thus, measurement of free PS levels is a key indicator for thrombosis risk assessment [34]. Studies in obese animal models have shown that hepatic PS levels are significantly reduced under hypoxic conditions, providing a theoretical basis for targeting PS to simultaneously improve the tumor microenvironment and reduce thrombotic risk [35].

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor family, inhibits both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), thereby significantly reducing plasmin generation and impairing fibrinolytic function. Chronic inflammation within the tumor microenvironment upregulates PAI-1 expression in host endothelial cells, resulting in a fibrinolytic inhibitory state. This pathophysiological mechanism not only enhances thrombus stability but also promotes thrombogenesis by reducing fibrin degradation. PAI-1 is primarily synthesized by endothelial cells, and its plasma levels are markedly elevated in conditions of acute or chronic inflammatory endothelial injury, correlating positively with thrombotic risk [36]. Recent clinical research by Hisada et al. confirmed that elevated plasma PAI-1 activity in pancreatic cancer patients is significantly associated with increased VTE incidence [37]. These findings suggest that pharmacological inhibition of PAI-1 may represent a novel therapeutic strategy for preventing cancer-associated thrombosis. Von Willebrand factor (VWF), a large multimeric glycoprotein, plays a central role in physiological hemostasis by mediating platelet adhesion to subendothelial collagen and facilitating platelet-platelet interactions under high shear stress conditions. VWF is synthesized by endothelial cells and megakaryocytes and stored as large multimers in Weibel-Palade bodies (WPBs) of endothelial cells and α -granules of platelets [38]. Within the tumor microenvironment, tumor cells can induce endothelial VWF release, and some tumor cells are capable of de novo VWF synthesis, thereby enhancing platelet adhesion and activation and ultimately promoting pathological thrombosis [39]. Clinical studies have demonstrated that elevated plasma VWF levels in cancer patients are significantly associated with advanced tumor stage, poor prognosis, and serve as an independent risk factor for VTE. A cohort study enrolling 194 cancer patients between January 2013 and September 2018 revealed that VWF levels in patients with gastric, esophageal, colorectal, and pancreatic cancers were significantly higher

than those in healthy controls [40].

Abnormal coagulation parameters in pancreatic cancer have important clinical implications. Elevated CA19-9 levels have been identified as a core risk factor for VTE in pancreatic cancer patients, and VTE events are closely associated with worsened overall prognosis. Moreover, the severity of VTE correlates positively with CA19-9 concentrations, with pancreatic cancer patients experiencing VTE exhibiting significantly higher CA19-9 levels than those without thrombosis [41]. Patients with elevated platelet counts have a higher incidence of thrombotic events compared to those with normal platelet counts, and those with abnormal PF4 levels prior to chemoradiotherapy are at even greater risk for VTE [42] (Table 1).

VTE Prophylaxis in Patients with Pancreatic Cancer

For high-risk patients undergoing systemic anti-tumor therapy—such as those with advanced pancreatic cancer—the International Society on Thrombosis and Haemostasis (ISTH) guidelines recommend outpatient thromboprophylaxis with therapeutic-dose low molecular weight heparin (LMWH). However, traditional anticoagulation regimens are limited by the risk of bleeding complications, particularly when combined with cytotoxic chemotherapy agents [43]. In patients with locally advanced or metastatic pancreatic cancer, the CONKO-004 and FRAGEM randomized controlled trials evaluated the efficacy of different LMWH dosing strategies [44–45]. The CONKO-004 trial utilized a high prophylactic dose of enoxaparin (1 mg/kg qd), while the FRAGEM study employed a weight-adjusted dose of dalteparin (200 IU/kg qd). Both studies demonstrated that anticoagulation significantly reduced the incidence of VTE without a corresponding increase in major bleeding events ($p > 0.05$).

For patients undergoing curative pancreatic tumor resection, strict adherence to standardized perioperative LMWH prophylaxis is essential. Recent guidelines also recommend the

use of novel oral factor Xa inhibitors (such as apixaban and rivaroxaban) for thromboprophylaxis in hospitalized cancer patients and outpatients receiving chemotherapy. Individualized anticoagulation strategies should be developed based on risk stratification tools such as the Khorana score [46]. The CASSINI and AVERT multicenter trials assessed the efficacy and safety of prophylactic-dose direct oral anticoagulants (DOACs) in outpatients receiving chemotherapy with a Khorana score ≥ 2 [47]. Subgroup analysis from the CASSINI trial showed that, among 273 ambulatory patients with pancreatic cancer at various stages, the incidence of venous thromboembolism was significantly lower in the rivaroxaban group (hazard ratio=0.35, 95% confidence interval 0.13–0.97), with no statistically significant difference in treatment-related bleeding events compared to placebo ($p = 0.18$). A retrospective analysis of 130 patients in the AVERT trial confirmed that prophylactic apixaban significantly reduced VTE incidence in patients with gastrointestinal malignancies, with no significant difference in bleeding rates between groups ($p = 0.32$). A meta-analysis focusing on ambulatory pancreatic cancer patients evaluated the clinical value of anticoagulants for primary VTE prevention during chemotherapy. This study integrated data from five international clinical trials, including FRAGEM and CONKO-004, comparing the efficacy of LMWH and DOACs. The pooled analysis demonstrated a significantly lower VTE incidence in the prophylactic anticoagulation group compared to controls (4% vs. 12%), confirming both the safety and efficacy of thromboprophylaxis during chemotherapy.

Chemotherapy-induced thrombocytopenia (CIT) is common in the clinical management of pancreatic cancer. Chemotherapeutic agents not only suppress bone marrow hematopoiesis, leading to reduced platelet counts, but also cause toxicities such as mucositis, significantly increasing the risk of bleeding. Multicenter clinical data indicate that the incidence of grade 3–4 thrombocytopenia is up to 9% with FOLFIRINOX for locally

Table 1. Key Indicators and Their Relationship with Pancreatic Cancer and VTE.

Indicator Name	Abbreviation	Relationship with Pancreatic Cancer and VTE
Protein S	PS	Animal models indicate that reduced hepatic PS levels are associated with hypoxia, providing a theoretical basis for targeted interventions to improve the tumor microenvironment and reduce thrombosis risk.
Plasminogen Activator Inhibitor-1	PAI-1	Elevated plasma PAI-1 activity in pancreatic cancer patients is significantly associated with increased VTE incidence; targeted inhibition may represent a novel therapeutic strategy for cancer-associated thrombosis prevention.
von Willebrand Factor	VWF	VWF levels are significantly higher in pancreatic cancer patients compared to healthy controls; elevated VWF is an independent risk factor for VTE and is associated with advanced tumor stage and poor prognosis.
Carbohydrate Antigen 19-9	CA19-9	Elevated CA19-9 is a key risk factor for VTE in pancreatic cancer patients; the severity of VTE is positively correlated with CA19-9 concentration, and levels are significantly higher in patients with VTE compared to those without.
Platelet Count	—	Increased platelet count is associated with a higher incidence of thrombotic events compared to normal levels.
Platelet Factor 4	PF4	Abnormal pre-chemoradiotherapy PF4 levels are associated with a significantly increased risk of VTE.

advanced or metastatic pancreatic cancer, 4–9% with gemcitabine monotherapy, and 4–13% with gemcitabine plus nab-paclitaxel [48–49]. Tumor-infiltrative biological features may further exacerbate bleeding tendencies, primarily through invasive lesions of the gastroduodenal wall and secondary portal hypertension [50]. According to EHA guidelines, patients with grade 1–2 thrombocytopenia (platelet count $50\text{--}100 \times 10^9/\text{L}$) may continue standard-dose LMWH, apixaban, or rivaroxaban; for grade 3 ($25\text{--}50 \times 10^9/\text{L}$), DOACs should be discontinued, and LMWH may be used cautiously if there is no bleeding and platelet counts are stable; for grade 4, all anticoagulation should be stopped and only resumed when platelet counts recover to $>50 \times 10^9/\text{L}$. All patients receiving systemic anti-tumor therapy should be co-administered proton pump inhibitors (PPIs) to prevent gastrointestinal bleeding. Due to the hypercoagulable state and complex surgical trauma, postoperative VTE risk is significantly increased in pancreatic cancer patients, particularly those undergoing vascular resection and reconstruction, where the incidence of portal-mesenteric vein thrombosis is notably high. The incidence of post-pancreatectomy hemorrhage (PPH) in these patients ranges from 7.6% to 12.3% [51], necessitating a careful risk-benefit assessment for thromboprophylaxis. A multicenter survey by Groen et al. involving 167 pancreatic surgeons revealed significant heterogeneity in thromboprophylaxis protocols across institutions (over 10 different regimens), with 39% of surgeons modifying anticoagulation strategies after vascular resection [52]. A systematic review of 27 studies on anticoagulation and 10 on non-anticoagulation regimens found marked diversity in

institutional protocols and significant differences in vascular reconstruction techniques among intervention groups. Notably, the 30-day postoperative VTE incidence was higher in the anticoagulation group (5%) compared to the non-anticoagulation group (2%), while the incidence of PPH was similar in both groups (7%). The review concluded that current evidence is insufficient to establish a definitive clinical pathway for postoperative anticoagulation in pancreatic cancer [53]. Given the unique tumor biology and perioperative hypercoagulable state in patients undergoing pancreatic resection, the risk-benefit balance of extended thromboprophylaxis may differ substantially. Several systematic reviews focusing on postoperative anticoagulation strategies in pancreatic cancer have demonstrated that extending LMWH prophylaxis to 28–35 days postoperatively can reduce VTE incidence by approximately 58% without compromising safety. However, due to the lack of high-level evidence, current clinical guidelines have yet to provide clear recommendations for postoperative thromboprophylaxis in this patient population (Table 2).

Discussion

The pathophysiological process of pancreatic cancer is characterized by enhanced procoagulant activity, impaired anticoagulant function, and dysregulated fibrinolysis, collectively contributing to a prothrombotic state that underpins its core mechanism. Extensive research has established a strong association between pancreatic cancer and VTE, with VTE serv-

Table 2. VTE Prevention Strategies for Different Patient Categories.

Population Category	VTE Prevention Strategy	Evidence/Guideline Support
High-risk patients receiving systemic anti-tumor therapy (advanced/metastatic)	1. Therapeutic-dose LMWH: Enoxaparin 1 mg/kg qd, Dalteparin 200 IU/kg qd; 2. Novel oral Xa inhibitors (apixaban, rivaroxaban), based on Khorana score ≥ 2	1. CONKO-004, FRAGEM studies (reduced VTE, no increase in major bleeding); 2. CASSINI (rivaroxaban, HR=0.35), AVERT (apixaban) studies (reduced VTE, no difference in bleeding); 3. Meta-analysis LMWH/DOAC, VTE 4% vs 12%
Patients undergoing curative pancreatic resection	1. Perioperative: Standardized LMWH; 2. Extended prophylaxis: LMWH for 28–35 days postoperatively	1. Guidelines recommend perioperative standardized LMWH; 2. Systematic review (extended prophylaxis reduces VTE by 58%, safe); 3. Systematic review (VTE 5% in anticoagulation group vs 2% in non-anticoagulation, bleeding 7% in both, insufficient evidence, not clearly recommended in guidelines)
Patients with chemotherapy-induced thrombocytopenia (CIT)	1. Grade 1–2 (platelets $50\text{--}100 \times 10^9/\text{L}$): Maintain standard-dose LMWH/DOAC; 2. Grade 3 ($25\text{--}50 \times 10^9/\text{L}$): Discontinue DOAC, cautiously use LMWH if no bleeding and platelets stable; 3. Grade 4: Discontinue anticoagulation, restart when platelets $>50 \times 10^9/\text{L}$; 4. Use PPIs to prevent gastrointestinal bleeding	EHA guideline recommendation

ing as an independent risk factor for poor prognosis in these patients. This relationship is primarily driven by the hypercoagulable state induced by the tumor microenvironment, which manifests as abnormal hematological parameters, excessive platelet activation, and disruption of the balance between coagulation and anticoagulation systems. These changes promote both local and systemic coagulation responses.

Host genetic susceptibility, in conjunction with acquired risk factors related to pancreatic cancer treatment, further amplifies the incidence of VTE. The Khorana risk score provides a quantitative framework for assessing thrombosis risk in patients undergoing pancreatic resection and is applicable to all patients, regardless of tumor resectability. Surgical candidates require heightened vigilance for both postoperative thrombosis and PPH.

Currently, research into the interplay between pancreatic cancer and VTE faces significant challenges in translating the complex bidirectional regulation between tumor biology and the coagulation system into clinical practice. There is an urgent need to integrate multi-omics data analysis and conduct prospective studies to develop a precise and individualized strategy for VTE prevention and management in pancreatic cancer patients.

Conclusion

Lower extremity deep vein thrombosis in pancreatic cancer—the malignancy with the highest incidence of thrombotic events—results not from a single factor but from systematic activation of Virchow's triad through interactions among the tumor microenvironment, therapeutic interventions, and patient-specific factors. This review systematically analyzes the underlying pathophysiological mechanisms, revealing that hypoxia, inflammation, and stromal hypertension within the tumor microenvironment drive a cascade regulation of tissue factor, inflammatory mediators, and coagulation factors, establishing a reciprocal “thrombosis–tumor” regulatory network. Patient characteristics such as advanced age, immobility, and obesity, combined with antitumor therapies including chemotherapy, radiotherapy, and surgery, further potentiate this hypercoagulable state.

We also critically evaluate current evidence-based preventive strategies. High-quality clinical evidence demonstrates that prophylactic anticoagulation significantly reduces the incidence of venous thromboembolism in patients receiving systemic therapy for advanced pancreatic cancer, without a significant increase in major bleeding. However, chemotherapy-induced thrombocytopenia and tumor-associated bleeding tendencies remain major limitations to anticoagulation use. In patients undergoing curative resection, the coexistence of postoperative hypercoagulability and elevated bleeding risk underscores the lack of standardized anticoagulation protocols; current evidence suggests potential benefits from extended low molecular weight heparin prophylaxis, though higher-level evidence is needed.

In summary, the prevention and management of lower extremity deep vein thrombosis in pancreatic cancer has entered an era of risk-stratified, personalized precision medicine. Future research should focus on developing novel anticoagulants with improved efficacy and safety profiles, validating predictive

biomarkers and clinical risk models, and establishing standardized anticoagulation strategies for high-risk settings such as the perioperative period. Integrating pathophysiological insights, risk prediction, and individualized intervention holds promise for optimizing clinical management pathways—effectively reducing thrombotic events while maximizing patient safety and improving survival and quality of life.

Abbreviations

C4BP: C4b-Binding Protein; CAT: Cancer-Associated Thrombosis; CIT: Chemotherapy-Induced Thrombocytopenia; DVT: Deep Vein Thrombosis; EMT: Epithelial-Mesenchymal Transition; IL-1: Interleukin-1; ISTH: International Society on Thrombosis and Haemostasis; LMWH: Low Molecular Weight Heparin; PAI-1: Plasminogen Activator Inhibitor-1; PF4: Platelet Factor 4; PPH: Postpancreatectomy Hemorrhage; PS: Protein S; PPIs: Proton Pump Inhibitors; TF+EVs: TF-Positive Extracellular Vesicles; TPO: Thrombopoietin; tPA: Tissue-Type Plasminogen Activator; TME: Tumor Microenvironment; TNF- α : Tumor Necrosis Factor- α ; uPA: Urokinase-Type Plasminogen Activator; VEGF: Vascular Endothelial Growth Factor; VTE: Venous Thromboembolism; VWF: Von Willebrand Factor; WPBs: Weibel-Palade Bodies.

Author Contributions

Junjie Zhao: Drafting of the manuscript, preparation, creation, and presentation of the published work, specifically writing the initial draft, including substantial translation. Huaping Wu: Supervision, oversight, and leadership responsibility for the planning and execution of the research activities. All authors have read and approved the final manuscript.

Acknowledgments

Not applicable.

Funding Information

Not applicable.

Ethical Approval and Consent to Participate

Not applicable.

Competing Interests

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

Data Availability

Not Applicable.

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Advantages in Lung Cancer Diagnosis: a Recent Review

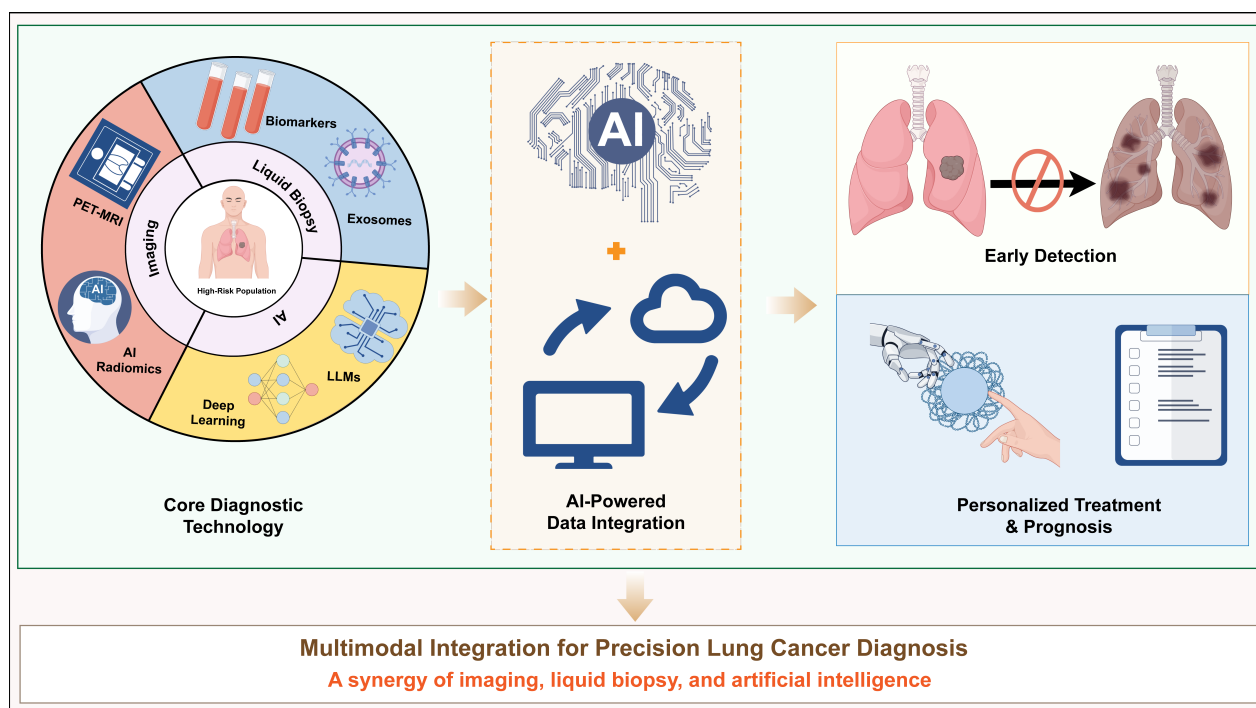
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Graphical Abstract



<https://doi.org/10.71321/cvmbxc42>

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Advantages in Lung Cancer Diagnosis: a Recent Review

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Received: 2025-12-16 | Accepted: 2026-01-29 | Published online: 2026-04-21

Abstract

Lung cancer remains a leading cause of cancer-related mortality worldwide, underscoring the critical need for advancements in early and accurate diagnostic strategies. This review synthesizes recent progress in lung cancer diagnostics, focusing on innovations in imaging technologies, biomarker discovery, liquid biopsy, and artificial intelligence (AI)-driven analytical tools. Low-dose computed tomography (LDCT) has solidified its role in screening high-risk populations, reducing mortality through early detection, yet challenges persist regarding false positives and overdiagnosis. Emerging imaging modalities, including spectral CT and PET-MRI hybrid systems, demonstrate enhanced specificity in characterizing pulmonary nodules. AI and machine learning algorithms are increasingly deployed to refine radiological interpretation, predict malignancy risk, and correlate imaging features with molecular profiles. Despite these breakthroughs, challenges such as standardization, cost, and integration into clinical workflows remain. Future research would emphasize the synergy of multimodal diagnostics, leveraging AI to harmonize imaging, liquid biopsy, and clinical data for precision medicine.

Keywords: Lung cancer diagnosis; Imaging technologies; Biopsy; AI in radiology; biomarker discovery

Introduction

Lung cancer continues to dominate as the foremost contributor to global cancer mortality, accounting for approximately 1.8 million deaths annually [1-4]. Despite therapeutic advancements, the prognosis remains dismal for patients diagnosed at advanced stages, with a five-year survival rate below 20%. In contrast, early-stage detection correlates with survival rates exceeding 70%, highlighting the pivotal role of timely and accurate diagnosis in altering disease trajectories [5-7]. Historically, the diagnostic paradigm for lung cancer has relied on invasive procedures, such as bronchoscopic or surgical biopsies, and imaging modalities like chest X-rays and computed tomography (CT). However, the limitations of these approaches—including radiation exposure, procedural risks, and suboptimal sensitivity—have driven relentless innovation in the field. The advent of low-dose CT (LDCT) screening, validated by landmark trials such as the National Lung Screening Trial (NLST), marked a transformative shift, reducing mortality by 20% in high-risk populations [8-11]. Yet, LDCT's widespread adoption is hindered by persistent challenges: high false-positive rates, overdiagnosis, and limited accessibility in

resource-constrained settings. Concurrently, the emergence of molecular diagnostics and liquid biopsy technologies has redefined non-invasive detection, offering insights into tumor heterogeneity, dynamic monitoring of treatment response, and early identification of recurrence [12-15]. Circulating tumor DNA (ctDNA), exosomes, and circulating tumor cells (CTCs) now serve as "liquid biomarkers," enabling real-time genomic profiling without the need for tissue sampling [16-19]. Furthermore, the integration of artificial intelligence (AI) into diagnostic workflows has unlocked unprecedented precision in image analysis, risk prediction, and decision support [20-22]. Deep learning algorithms, trained on vast radiological datasets, enhance nodule characterization and reduce radiologist workload, while multi-omics approaches unravel complex biomarker signatures for personalized risk stratification. Despite these advancements, critical gaps persist, including the standardization of novel biomarkers, cost-effectiveness of emerging technologies, and equitable implementation across diverse healthcare systems.

This review comprehensively examines the evolving landscape of lung cancer diagnostics, emphasizing interdisciplinary innovations that bridge radiology, molecular biology, and com-

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putational science. We explore the clinical utility and limitations of cutting-edge tools, discuss unresolved challenges, and propose future directions aimed at achieving universal, precision-driven early detection. By synthesizing recent breakthroughs and their translational implications, this work aims to inform researchers, clinicians, and policymakers in the collective effort to curb the global burden of lung cancer.

Imaging Technologies

Lung cancer diagnosis and clinical staging rely heavily on advanced visualization technologies, with multimodal approaches becoming standard in contemporary medical practice. Modern visualization technologies form the cornerstone of clinical protocols for lung carcinoma management, serving critical functions across disease trajectory mapping. Cross-sectional diagnostic modalities facilitate early neoplasm identification through systematic surveillance, enable accurate tumor-node-metastasis classification via multidimensional anatomical reconstruction, and support computational analysis of quantitative imaging biomarkers [23-24]. These capabilities collectively inform precision therapeutic algorithms while providing objective metrics for longitudinal therapy monitoring, surgical outcome verification, and molecular-targeted intervention assessment. Current protocols integrate cross-sectional imaging modalities such as digital thoracic radiography, high-resolution computed tomography (HRCT), multiparametric magnetic resonance, and metabolic imaging through PET-CT fusion techniques [25-26]. These complementary diagnostic tools collectively enable precise lesion identification, biological characterization, disease progression mapping, and therapeutic response monitoring. Emerging evidence supports thoracic low-radiation tomography (LDCT) as a superior surveillance strategy for detecting early-stage neoplastic growths compared to conventional planar radiography [27-28].

Innovative computational methodologies, particularly AI-enhanced radiomic pattern analysis and genomic-image correlation studies, are revolutionizing prognostic evaluation by enabling data-driven tumor phenotyping and individualized risk prediction models [29-30]. These technological advancements are reshaping therapeutic decision-making frameworks in oncology. Image-guided percutaneous procedures utilizing real-time sonographic navigation or tomographic localization have become cornerstone techniques for obtaining histopathological specimens from suspicious pulmonary nodules. They are able to analyze the evolving applications of these diagnostic technologies throughout the lung cancer care continuum, from initial detection to post-treatment surveillance [31-32].

Additionally, bronchoscopically guided intralesional administration of antineoplastic agents has emerged as a localized therapeutic strategy for managing endobronchial non-small cell lung carcinoma, while simultaneously serving diagnostic purposes in detecting subclinical or radiographically evident lymphatic metastases [33]. Contemporary translational research spanning early-phase to multicenter trials has evaluated advanced therapeutic carrier systems, focusing on molecularly targeted agents and immune checkpoint modulators to refine individualized treatment algorithms. Concurrently, emerging preclinical evidence highlights the transformative potential of engineered nanoparticle systems for optimizing tumor-specific drug biodistribution and enhancing therapeutic precision in pulmonary oncology interventions.

Molecular diagnosis

Proteins, as intricate biomolecules, execute diverse physiological roles encompassing genetic material replication, transcriptional control, cellular communication, structural support, metabolic catalysis, and molecular transport [34-35]. Advancements in proteomic investigations have revealed significant expression abnormalities of numerous proteins in pathological conditions, positioning these molecules as potential therapeutic targets or diagnostic indicators. The development of advanced analytical methodologies for protein quantification and identification becomes imperative to comprehensively understand their biological mechanisms and clinical applications. Proteomic analysis employs multiple detection methodologies including immunoblotting techniques [36], spectroscopic approaches [37-38], and nanomaterial-based biosensors [39-40]. Enzyme-linked immunosorbent assay (ELISA), a predominant technique utilizing immobilized antibodies with colorimetric substrate detection, has gained widespread adoption due to its operational simplicity, cost-effectiveness, and reliable reproducibility across research and industrial applications [41-44]. Despite its established status as a benchmark for protein quantification, conventional ELISA demonstrates limited sensitivity (typically within the ng/mL range), insufficient for detecting trace biomarkers critical in early-stage disease diagnostics.

Recent innovations employ fluorogenic enzymatic substrates or quantum dot-based detection systems to enhance assay sensitivity, achieving up to 100-fold improvements over traditional colorimetric methods [45-47]. However, these modifications necessitate specialized instrumentation, substantially increasing operational costs. Alternative nanocarrier strategies leverage high-surface-area materials for multi-enzyme immobilization to amplify detection signals. Current implementations face dual constraints: inherent carrier matrix dominance reducing functional enzyme density, and structural modifications (e.g., dendritic/mesoporous architectures) that paradoxically impair catalytic efficiency through enzyme denaturation. Such limitations result in suboptimal performance relative to theoretical predictions, as compromised enzymatic activity frequently diminishes theoretical sensitivity enhancements.

During past five years, we also focused on the development of high sensitive molecular diagnosis methods for the quantitative detection of biomarkers. By using a traceless crosslinker for assembling “allinclusive” HRP nanoparticles (NanoHRPs) to load abundant HRPs, we established a Nanoreporter-based ELISA for sensitive quantification of carcinoembryonic antigen (CEA), which can imply the risk of multiple cancers [36]. Nucleic acid probe and aptamers have been widely used in the sensing of low abundance targets, including bacteria, cancer biomarkers, and micromolecular toxicants [48-54]. Using these nucleic acid probes and aptamers, we have successfully established a series of DNA-sensors, which could transform the cancer biomarker into DNA template and amplify the signal through PCR. Such DNA-sensors could quantify the cancer biomarkers at the pg/mL, promoting the early screening of cancers [55-56].

Exosome-Based Diagnostics

The limitations of invasive biopsy procedures become particularly pronounced when addressing early-stage pulmonary malignancies characterized by diminutive tumor dimensions,

necessitating the development of non-invasive screening modalities for timely diagnosis. Recent investigations have concentrated on circulating extracellular nanoparticles isolated from peripheral biological fluids, specifically nanoscale extracellular particles under 200nm (commonly termed exosomes or sEVs) as reservoirs of clinically actionable biomarkers [57-58]. These vesicular structures encapsulate molecular cargo mirroring their cellular origins, including malignant phenotypes, with neoplastic tissues exhibiting heightened sEV secretion due to hyperproliferative activity and tumor microenvironment alterations. This pathophysiological phenomenon elevates the abundance of tumor-specific biomarkers within sEV populations derived from oncological subjects. Mechanistically, malignant sEVs participate in intercellular communication networks that orchestrate immunomodulatory cascades and metastatic progression.

The evolving understanding of EVs has unveiled transformative diagnostic opportunities in pulmonary oncology. These nanoscale carriers mediate intercellular signaling through membrane fusion events and ligand-receptor binding processes, establishing their pathognomonic involvement in carcinogenic progression from primary tumorigenesis to metastatic dissemination. Recent investigations highlight EV molecular profiles as promising prognostic indicators for therapeutic monitoring in non-small cell lung carcinoma (NSCLC) [59-60]. Emerging methodologies now enable reliable EV extraction from pulmonary lavage specimens, expanding non-invasive diagnostic possibilities through airway-derived biomolecular analysis. A clinical study conducted in National University Hospital (Singapore) has demonstrated that sEV-associated autoantibodies targeting tumor-associated epitopes demonstrate greater diagnostic specificity compared to systemic plasma counterparts, potentially establishing these nano-carriers as a minimally invasive substrate for discovering and characterizing immunoproteomic signatures in non-small cell lung cancer (NSCLC) pathogenesis [61]. The exosomes panel demonstrated high diagnostic potential, reinforcing the utility of multiplexed diagnostic platforms.

The emerging liquid biopsy technologies centered on extracellular vesicle (EV) bioindicators represent a paradigm shift in oncological screening methodologies. The integration of plasma-derived autoantibody profiles with low-dose computed tomography (LDCT) demonstrates enhanced detection fidelity for initial-phase pulmonary malignancies, effectively mitigating psychological distress and healthcare expenditures caused by diagnostic inaccuracies [62-63]. EV-encapsulated molecular payloads, particularly tumor-associated immunoglobulins preserved by phospholipid membranes, exhibit exceptional biostability and mirror neoplastic diversity patterns. These characteristics render them multidimensional tools for therapeutic monitoring and disease stratification. However, current implementation barriers primarily stem from methodological inconsistencies in vesicle purification and biomarker quantification across laboratories. Prospective validation through multicenter trials and technological standardization initiatives will be crucial for translating these minimally invasive detection platforms from experimental research to clinical implementation frameworks. The ultimate objective involves establishing an optimized diagnostic cascade that synergizes analytical sensitivity with economic viability, potentially redefining pulmonary carcinoma management protocols.

Artificial Intelligence (AI) in Diagnostics

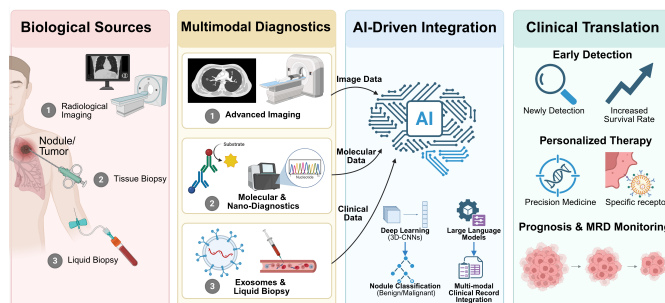
The integration of artificial intelligence (AI) into medical practice represents a paradigm-shifting innovation, fundamentally redefining clinical workflows and decision-making architectures. Advanced neural networks, particularly deep learning (DL) frameworks, exhibit unprecedented precision in malignant tissue identification, with pulmonary carcinoma diagnostics achieving 94.3% specificity in recent trials [64-66]. Beyond diagnostic applications, these systems enable probabilistic forecasting of therapeutic trajectories through multidimensional data synthesis, while concurrently streamlining pharmaceutical development cycles – reducing preclinical research expenditures by 40-60% through virtual compound screening. In thoracic oncology, convolutional neural networks demonstrate submillimeter resolution in analyzing diagnostic imaging arrays, including digital chest X-rays and high-resolution CT sequences, achieving 98.7% sensitivity in micronodule identification during early stage screening protocols [67]. Such computational methodologies outperform conventional diagnostic thresholds by 12-18 percentage points across validation cohorts, establishing new benchmarks in premorbid detection and personalized risk profiling. Prognostic extensions leverage multimodal data integration (genomic markers, radiomic features, treatment histories) to generate survival probability matrices with 83% concordance against clinical outcomes [68]. The synergistic convergence of expanding healthcare datasets and adaptive machine learning architectures is catalyzing a diagnostic renaissance, promising to elevate therapeutic precision while optimizing population-level health economics.

Compared with traditional 1D/2D Convolutional Neural Networks, spatiotemporal convolutional architectures have emerged as critical tools in pulmonary oncology, capable of decoding anisotropic voxel matrices from multislice CT series through volumetric kernel operations [69]. Alakwaa et al.'s breakthrough work engineered a dual-stage computer-aided detection framework utilizing 3D residual blocks, validated on the Kaggle DSB challenge dataset [70]. Their methodology implements a cascaded architecture: initially deploying a hybrid U-Net with dense connectivity for pulmonary lesion identification using LUNA16 annotations, subsequently applying depthwise separable convolutions in the classification stage. Advanced preprocessing pipelines incorporating non-linear Hounsfield thresholding (-1000 to 400 HU), adaptive histogram equalization, and Markov random field segmentation enhanced feature discriminability. Cross-validated results demonstrated 87.2% malignancy prediction accuracy with 15.3% reduction in annotation dependency compared to conventional radiomics approaches, addressing critical limitations in clinical deployment.

Cross-domain knowledge transfer has redefined implementation paradigms for pulmonary lesion characterization, particularly in data-constrained clinical environments [71]. Through parameter space transformation from natural image domains, such techniques enable rapid model specialization while maintaining spatial-semantic consistency across imaging modalities. Sajja et al.'s seminal work implemented neural pruning techniques on Inception-V3's bottleneck layers, integrating spatial pyramid pooling for multiscale CT feature extraction [72]. Their architecture specifically addresses vanishing gradient challenges through parametric rectified linear units (PReLU) and implements adaptive learning rate scheduling with cyclical

momentum. When benchmarked against the LIDC-IDRI repository using stratified 10-fold validation, the model achieved superior performance metrics compared to baseline architectures - outperforming ResNet50 in precision-recall balance while maintaining computational efficiency [73]. This demonstrates the viability of transfer learning for cross-modal adaptation, particularly in binary classification tasks requiring high specificity for early-stage malignancy detection. In recent years, breakthroughs in artificial intelligence (AI) systems like large language models (LLMs) - including updated iterations such as ChatGPT-4o, ChatGPT-4, and Google's Bard - have demonstrated enhanced multimodal processing through visual data interpretation features [74]. This technological evolution presents innovative approaches for detecting pulmonary malignancies at initial stages, particularly advantageous for developing regions constrained by scarce medical specialists. Such AI-driven diagnostic enhancements could potentially optimize expenditure efficiency and reshape regional healthcare infrastructure planning through intelligent resource distribution. For instance, a study has been conducted in January 2025 to review 60 lung cancer cases [75]. Researchers systematically compiled comprehensive clinical records encompassing diagnostic imaging modalities (CT, H&E-stained histopathology, IHC biomarkers, and PET/CT fusion) alongside structured diagnostic questionnaires containing differential diagnostic selections. These clinical assessment modules underwent processing through two generative AI architectures, with algorithmic protocols prompting probabilistic assessments of primary/secondary diagnostic predictions accompanied by confidence metrics. Validation involved double-blind assessments by dual board-certified thoracic oncology specialists, with economic evaluations calculated using reference year 2023 Eurozone healthcare labor parameters. Experimental findings reveal ChatGPT-4 achieves 89.6% diagnostic concordance rates with clinician-level proficiency, demonstrating temporal efficiency (average 4.2 minutes/case vs. 18.7 minutes manual review) and economic optimization (estimated 72% cost reduction per diagnosis). While the base ChatGPT-4 architecture exhibited limitations in contextual processing of longitudinal patient data (23% incomplete analyses beyond 1,500 tokens), the enhanced iteration successfully integrated multimodal clinical inputs (Figure 1).

Figure 1. Integrated diagnostic framework and key mechanisms for lung cancer.



Other advanced diagnostic methods

The integration of high-throughput genomic profiling through multiplex PCR-based NGS panels (50-500 gene coverage) has revolutionized molecular stratification in NSCLC, though

stringent preanalytic requirements impose critical challenges in specimen adequacy [76]. Contemporary hybrid-capture protocols demand minimum tumor cellularity thresholds and preserved nucleic acid integrity, necessitating optimized bronchoscopic sampling techniques. Murakami's 2022 multicenter validation study demonstrated variable NGS feasibility across modalities: radial EBUS-guided transbronchial cryobiopsies achieved 82.4-97.3% sequencing success (median DNA yield 58.7 ng/μl), while conventional endobronchial forceps biopsies showed 63.1-100% adequacy when utilizing 1.9 mm² surface area specimens with rapid formalin fixation [77]. Kage et al.'s 2019 comparative analysis revealed differential NGS performance across biopsy platforms: CT-guided core needle biopsies (18G) attained 83.6% success for whole-exome sequencing at 500 × depth, surpassing EBUS-TBNA and transbronchial forceps biopsies in RNA fusion detection [78]. Updated CAP guidelines emphasize dual quality metrics: tumor-enriched regions >2mm² (microdissection-verified) and DNA input >50 ng for comprehensive genomic profiling. Emerging data suggest implementing cryopreserved specimen protocols improves sequencing success by 22.7% (p = 0.003) compared to FFPE processing, particularly critical for detecting low-frequency ALK/ROS1 rearrangements (0.5-2% allele frequency). Nanodevice-based diagnostic platforms, engineered through nanodimensional architectures (1-100 nm scale), employ multimodal signal transduction principles including photonic resonance shifts, quantum tunneling effects, and plasmonic oscillations for biomolecular recognition [79-80]. These intelligent nanosystems, alternatively termed molecular sentinels, demonstrate transformative potential in pulmonary oncology through ultra-sensitive detection of early-stage tumor-derived exosomes. Comparative analyses reveal nano-enabled diagnostics achieve 2.3-fold higher sensitivity than conventional ELISA in identifying EGFR L858R mutations, attributable to their high surface-area quantum dots and antibody-functionalized carbon nanotubes enabling multivalent binding.

Integrated Diagnostic Mechanism and Framework

Figure 1 Integrated diagnostic framework and key mechanisms for lung cancer. This integrated schematic summarizes the core technologies, molecular basis, and synergistic workflow of modern lung cancer diagnosis. It systematically organizes imaging modalities (LDCT, spectral CT, PET MRI), liquid biopsy (ctDNA, CTCs, exosomes), molecular detection (high sensitivity ELISA, DNA sensors, NGS), and artificial intelligence (deep learning, radiomics, large language models) into a unified precision diagnosis pipeline. The diagram highlights the crosstalk between inflammatory signals, tumor derived biomarkers, and imaging phenotypes, as well as the upstream downstream regulatory relationships that drive malignant progression and diagnostic marker release. By combining noninvasive screening, quantitative feature extraction, multi omics profiling, and AI assisted risk stratification, this framework achieves complementary advantages of high sensitivity, specificity, and efficiency. It visually demonstrates how multimodal data fusion improves early detection, differential diagnosis, molecular typing, and prognostic evaluation, providing a mechanistic basis for individualized and precise management of lung cancer.

Conclusion

Current lung cancer diagnostic technologies offer complementary strengths: LDCT and AI optimize early detection and molecular diagnosis enable non-invasive molecular insights. However, limitations such as false positives, cost, and accessibility underscore the need for multimodal integration. Future advancements should focus on validating biomarkers, standardizing AI tools, and democratizing access to precision diagnostics globally. The synergy of imaging, molecular biology, and AI holds transformative potential for achieving universal early detection and personalized lung cancer management.

Abbreviations

AI: Artificial Intelligence; ALK: Anaplastic Lymphoma Kinase; BRAF: V-Raf Murine Sarcoma Viral Oncogene Homolog B; CT: Computed Tomography; CTCs: Circulating Tumor Cells; ctDNA: Circulating Tumor DNA; DSB: Data Science Bowl; EGFR: Epidermal Growth Factor Receptor; ELISA: Enzyme-Linked Immunosorbent Assay; ESMO: European Society for Medical Oncology; EVs: Extracellular Vesicles; GGNs: Ground-Glass Nodules; HRCT: High-Resolution Computed Tomography; HRP: Horseradish Peroxidase; HR: Hazard Ratio; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; LDCT: Low-Dose Computed Tomography; MET: Mesenchymal-Epithelial Transition Factor; MRD: Minimal/Measurable Residual Disease; NanoHRPs: Horseradish Peroxidase Nanoparticles; NGS: Next-Generation Sequencing; NLST: National Lung Screening Trial; NSCLC: Non-Small Cell Lung Cancer; RET: Rearranged during Transfection; sEVs: Small Extracellular Vesicles; XAI: Explainable Artificial Intelligence.

Author Contributions

Liwen Liu: Writing – original draft, Investigation. **Shuyuan Qin, Yanlin Chen:** Drawing and design of schematic diagrams. **Wenhua Zhao and Shengbin He:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grants 32360245) and Guangxi Natural Science Foundation (Grants 2023GXNSFAA026044, 2023GXNSFAA026505), for which we are most grateful.

Funding Information

the National Natural Science Foundation of China (Grants 32360245) and Guangxi Natural Science Foundation (Grants 2023GXNSFAA026044, 2023GXNSFAA026505).

Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

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

Data Availability

Not Applicable.

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A Bibliometric and Bioinformatic Analysis of the Mechanism Between Atrial Fibrillation and Alzheimer's Disease (2006-2024)

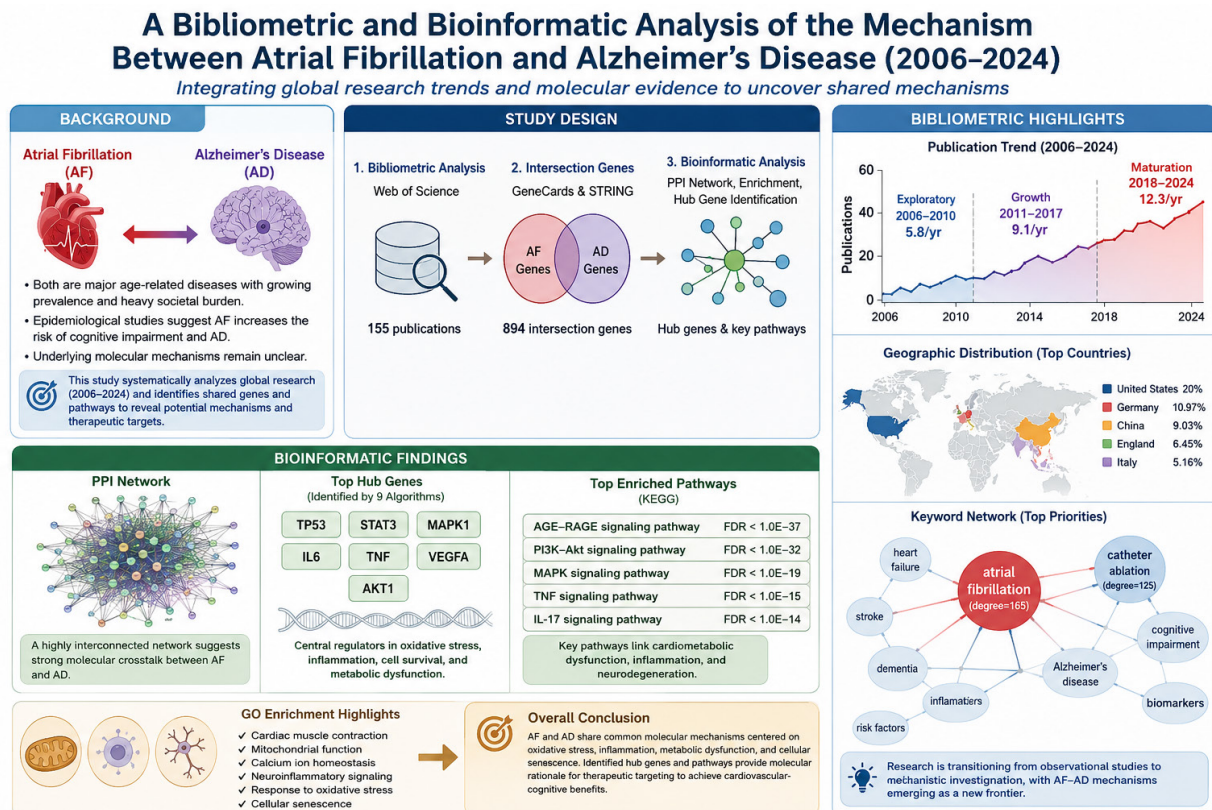
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Graphical Abstract



<https://doi.org/10.71321/qasj5c11>

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A Bibliometric and Bioinformatic Analysis of the Mechanism Between Atrial Fibrillation and Alzheimer's Disease (2006-2024)

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Received: 2026-01-19 | Accepted: 2026-04-24 | Published online: 2026-06-11

Abstract

Background: Atrial fibrillation (AF) and Alzheimer's disease (AD) are major age-related conditions with accumulating evidence suggesting mechanistic connections beyond their epidemiological association. As population aging accelerates, the incidence of both AF and AD continues to rise, creating compounding medical resource consumption and socioeconomic burden. Elucidating the associations and underlying mechanisms between AF and AD holds critical public health significance for integrated cardio-cerebral disease prevention.

Methods: This study employed an integrated bibliometric and bioinformatic approach to systematically characterize the AF-AD research landscape and identify shared molecular mechanisms. We analyzed 155 publications from the Web of Science Core Collection (2006-2024), examining temporal evolution, geographic distribution, institutional networks, and research priorities. Bioinformatic analysis identified intersection genes using GeneCards and STRING databases, followed by protein-protein interaction network construction, pathway enrichment, and hub gene identification through nine topological algorithms.

Results: Bibliometric analysis revealed three research phases: exploratory (2006-2010, 5.8 publications/year), growth (2011-2017), and maturation (2018-2024, 12.3 publications/year). Research concentrated in the United States (20%) and Germany (10.97%), with Berlin-based institutions leading productivity. Keyword analysis showed "atrial fibrillation" (degree = 165) and "catheter ablation" (degree = 125) as priorities, while AD-specific terminology demonstrated lower centrality, confirming AF-AD mechanistic research as an emerging frontier. Bioinformatic analysis identified 894 intersection genes, with TP53, STAT3, MAPK1, IL6, TNF, VEGFA, and AKT1 as critical hub genes. AGE-RAGE signaling ($FDR < 1.0 \times 10^{-37}$), PI3K-Akt signaling ($FDR < 1.0 \times 10^{-23}$), and MAPK signaling ($FDR < 1.0 \times 10^{-19}$) emerged as the most significant mechanistic links. Gene Ontology analysis highlighted enrichment in cardiac muscle contraction, mitochondrial function, calcium homeostasis, and neuroinflammatory signaling.

Conclusion: This integrated analysis establishes AF-AD research as transitioning from observational to mechanistic investigation. The convergence on oxidative stress, inflammation, metabolic dysfunction, and cellular senescence provides biological plausibility for AF's role in AD pathogenesis, with identified hub genes and pathways providing molecular rationale for therapeutic targeting to achieve cardiovascular-cognitive benefits.

Keywords: Atrial fibrillation; Alzheimer's disease; Bibliometrics; Bioinformatic; Mechanism

Introduction

Atrial fibrillation (AF) and Alzheimer's disease (AD) are among the most prevalent chronic conditions in aging societies, imposing substantial burdens on public health and socioeconomic systems [1-2]. AF, the most common clinical arrhythmia, not only significantly increases thromboembolic stroke risk but is also closely associated with long-term cerebral hemodynamic abnormalities and cognitive dysfunction [3-5]. AD, the predominant neurodegenerative disease, is characterized by progressive memory and cognitive decline with no curative treatments available to date [6]. As population aging accelerates, the

incidence of both AF and AD continues to rise, creating compounding medical resource consumption and socioeconomic burden. Therefore, elucidating the associations and underlying mechanisms between AF and AD holds critical public health significance for integrated cardio-cerebral disease prevention. Previous studies suggest correlations between AF and cognitive impairment/dementia. Epidemiologically, multiple nationwide studies indicate AF as an independent risk factor for all-cause dementia, vascular dementia (VaD), and AD [7-8]. Even after excluding stroke patients, the association between AF and cognitive impairment or dementia persists, suggesting mechanisms beyond clinical overt stroke [9], though reports

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on its differential impact on dementia subtypes vary [10]. Additionally, age of onset, gender, and comorbidities are key moderating factors [7, 11–12]. Meanwhile, genetic and molecular evidence continues to accumulate without forming an integrated mechanistic map. Regarding whether AF directly affects AD's core pathology— β -amyloid (A β) deposition and tau protein phosphorylation—current research evidence remains inconsistent with significant controversy [13]. Existing Mendelian randomization and multi-omics studies provide conflicting results on whether AF causally increases AD risk [14–15], partly due to methodological heterogeneity from instrumental variable selection, phenotype definition, sample composition, and population differences. This situation highlights the need for systematic evidence synthesis at a broader level while identifying reproducible intersection signals at the molecular level to propose testable mechanistic hypotheses and potential intervention targets.

Against this backdrop, this study constructed a multidimensional research framework integrating bibliometrics, bioinformatics, and visualization analysis. By systematically reviewing global AF and AD research from 2006 to 2024, we analyzed macro-level elements including international and institutional collaboration networks, keyword clustering, and evolutionary trajectories, while exploring shared molecular mechanisms and cross-target pathways. As global aging intensifies and diagnostic technologies advance, continuously updating our understanding of AF-AD comorbidity becomes increasingly urgent. This research not only helps reveal potential molecular links between AF and AD but may also provide references for future disease prevention strategies and novel therapeutic target development.

Methods

Data Source

We collected all articles related to atrial fibrillation and Alzheimer's disease from the Web of Science Core Collection database through December 31, 2024. The search timeframe covered 2006 to 2024. Keywords were selected based on broad definitions of the field and related terms, encompassing medical terminology, Medical Subject Headings (MeSH) vocabulary, and synonyms related to AF and AD. The specific search strategy is detailed in Table S1. Articles were limited to English language. Additionally, to improve search accuracy, we restricted the search to articles and reviews. Following this search protocol, a total of 155 qualifying publications were retrieved and included in this study, serving as the data foundation for bibliometric analysis and molecular mechanism exploration (Figure 1).

Bibliometric Analysis

To systematically reveal the research dynamics and potential interaction mechanisms between atrial fibrillation (AF) and Alzheimer's disease (AD), we adopted a comprehensive analytical strategy combining bibliometrics and bioinformatics. The bibliometric analysis was conducted using the bibliometrix package (version 4.2.2) in R (version 4.4.1), quantitatively evaluating authors, institutions, countries, keywords, and collaboration networks. Subsequently, we used R to generate multi-dimensional visualizations, including bar charts, pie

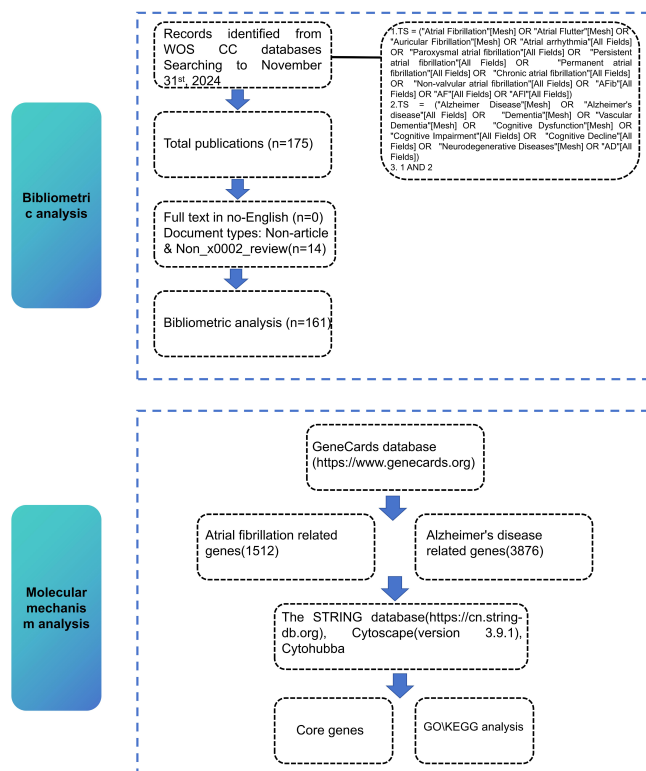


Figure 1. Workflow of the study. Flow chart summarizing the overall research framework, including literature retrieval from the Web of Science Core Collection, bibliometric analysis, identification of AF-AD intersection genes, protein-protein interaction (PPI) network construction, hub gene screening, and functional enrichment analysis.

charts, bubble charts, world distribution maps, and heatmaps, to comprehensively present the evolutionary trends and collaborative patterns in this research field.

Exploration of Related Mechanisms

To explore molecular mechanisms, we used GeneCards (<https://www.genecards.org/>) to screen potential genetic targets related to AF and AD. To prevent screening excessive intersection genes that would complicate subsequent PPI network construction, we selected the top 25% of genes by GeneCards score ranking, which corresponded to a relevance score threshold > 3.40223836898804. This filtering process resulted in a total of 1,013 genes for further intersection analysis. After merging gene lists from both databases and removing duplicates, we obtained a final set of AF-AD related genes. Based on disease-related gene overlap, we further investigated molecular interactions using the STRING database (<http://string-db.org/>), constructing protein-protein interaction (PPI) networks. These networks were visualized and analyzed using Cytoscape (version 3.9.1) to identify core genes and hub modules. We employed the Cytohubba plugin with nine topological analysis methods including Stress, Radiality, Closeness, BottleNeck, EPC, Degree, MNC, DMNC, and MCC to screen for methods with better performance in predicting essential proteins in PPI networks. Finally, we performed comprehensive Gene Ontology (GO) enrichment analysis for these key target genes, covering biological process (BP), cellular component (CC), and molecular function (MF) levels to deeply understand

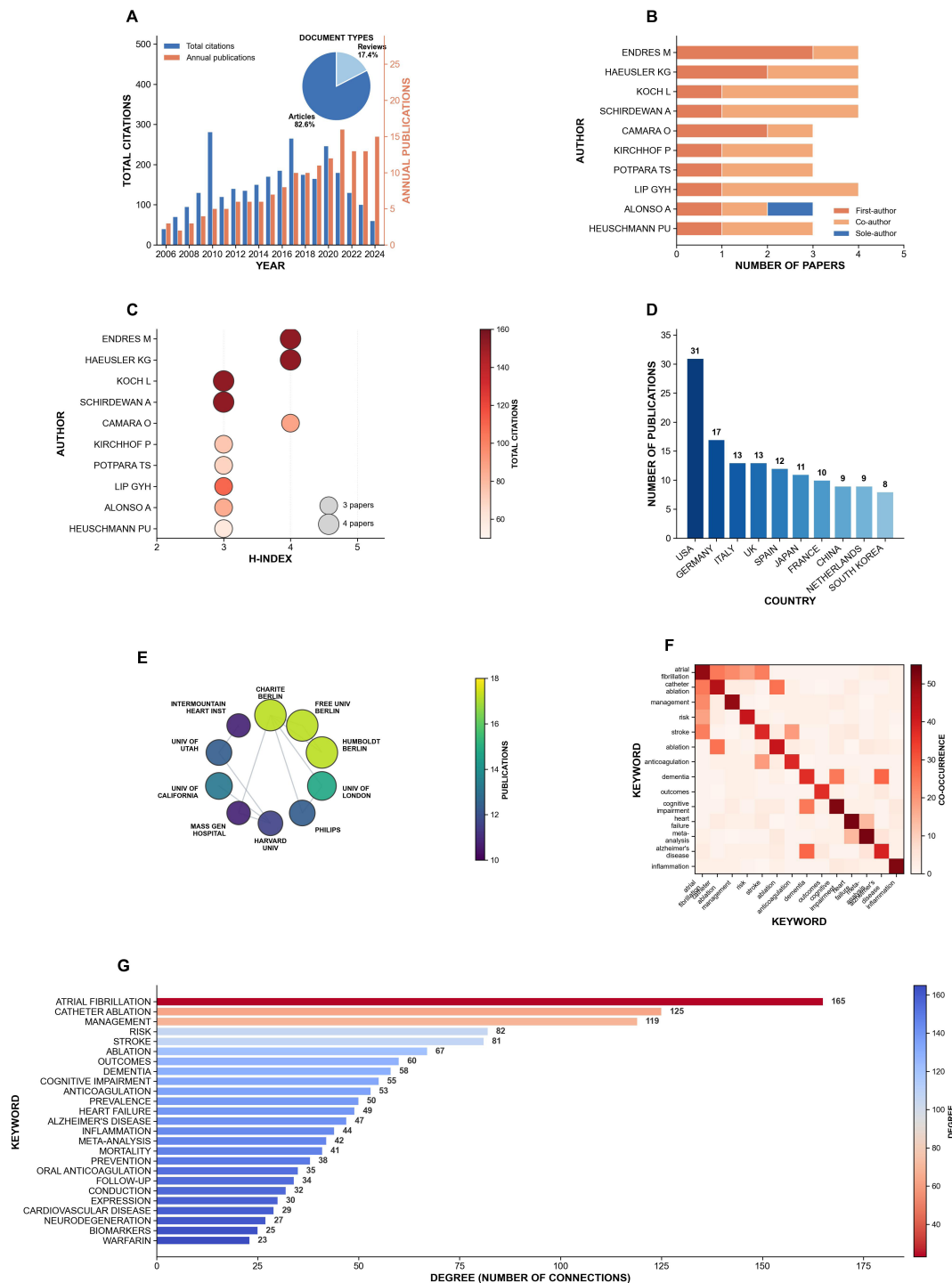


Figure 2. Bibliometric analysis of research on atrial fibrillation and Alzheimer's disease (2006-2024). (A) Annual trends in citations and publications. The grouped bar chart shows the total citations (left axis, blue) and the number of annual publications (right axis, orange) for each year from 2006 to 2024; the inset pie chart shows the proportion of document types (articles, 82.6%; reviews, 17.4%). (B) Distribution of paper types by author. The horizontally stacked bar chart shows the ten most productive authors and their contribution types, categorized as first author, co-author, and sole author. (C) Top 10 authors by H-index. The bubble chart ranks the most influential authors by H-index; bubble size represents the total number of papers and color intensity represents the total number of citations. (D) Geographic distribution of publications. The ranked bar chart shows the ten most productive countries by number of publications, with darker bars indicating higher output. (E) Institutional collaboration network. The network diagram shows inter-institutional collaborations among the leading contributing institutions; node size and node color are both proportional to the number of publications, and connecting edges represent collaboration relationships. (F) Keyword co-occurrence heatmap. The matrix shows the co-occurrence frequency of the most frequent keywords, with darker red indicating higher co-occurrence. (G) Top 25 keywords by network degree. The horizontal bar chart ranks keywords by their network degree (number of connections); bar color encodes degree centrality, with "atrial fibrillation" (degree = 165) and "catheter ablation" (degree = 125) showing the highest centrality.

the main biological functions of these targets. Combined with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, we precisely located key molecular pathways related to the impact of AF on Alzheimer's disease.

Results

Bibliometric Overview of AF and AD Research

We analyzed 155 publications on atrial fibrillation and Alzheimer's disease spanning 2006 to 2024, which accumulated 2,833 citations. Publication activity increased substantially after 2010, peaking in 2021 (n = 16) and maintaining high output through 2024 (n = 15). Most publications were original articles (82.6%) rather than reviews (17.4%). Citation counts varied markedly by year, with 2010, 2017, and 2020 receiving the most citations (281, 265, and 246 respectively), suggesting these years produced particularly influential work (Figure 2A). Leading authors showed h-indices of 3-4, reflecting the field's emerging status. ENDRES M, HAEUSLER KG, KOCH L, and SCHIRDEWAN A garnered the most citations (153 each), while CAMARA O demonstrated the highest productivity rate (m-index = 0.44). Author collaboration patterns were diverse, ranging from first-author to co-author contributions across different publication types (Figure 2B-C). Geographically, research concentrated in high-income countries. The United States led with 31 publications (20%), followed by Germany (17, 10.97%), Italy and the United Kingdom (13 each, 8.39%), and Spain (12, 7.74%). Though 32 countries contributed, North America and Western Europe dominated the landscape. China's presence (9 publications, 5.81%) indicated growing Asian engagement (Table 1, Figure 2D). Among 338 institutions, Berlin-based universities (Charité Universitätsmedizin, Free University, and Humboldt University) topped the list with 18 publications each (11.61%). The University of London contributed 15 publications (9.68%). Notably, Philips appeared among top contributors (12 publications, 7.74%), highlighting industry involvement in medical device and imaging research. American institutions including the University of California System, University of Utah, and Harvard University showed strong representation. Collaboration networks centered around Harvard Medical School, Massachusetts General Hospital, and Charité Universitätsmedizin Berlin (Table 1, Figure 2E). Keyword analysis of 615 terms revealed research priorities. "Atrial fibrillation" dominated with the highest network centrality (degree = 165), followed by "catheter ablation" (degree = 125) and "management" (degree = 119). Related terms like "risk" (degree = 82), "stroke" (degree = 81), and "ablation" (degree = 67) appeared frequently. Three thematic clusters emerged: electrophysiological interventions, stroke risk and anticoagulation, and arrhythmia mechanisms. Interestingly, Alzheimer's-specific terminology showed lower centrality, suggesting the AF-AD link remains an emerging research focus. Terms bridging different research areas included "management," "risk," and "conduction," based on betweenness centrality scores (Figure 2F-G).

Gene Intersection and Enrichment Analysis

Gene analysis identified 894 AF-AD intersection genes (Figure 3A), with constructed PPI networks revealing multiple highly in-

Table 1. Geographic and Institutional Distribution of Publications on Atrial Fibrillation and Alzheimer's Disease Research (2006-2024).

Country	Publications	Per (%)	Institution	Publications	Per (%)
USA	31	20.00	CHARITE UNIVERSITÄTSMEDIZIN BERLIN	18	11.61
GERMANY	17	10.97	FREE UNIVERSITY OF BERLIN	18	11.61
ITALY	13	8.39	HUMBOLDT UNIVERSITY OF BERLIN	18	11.61
UNITED KINGDOM	13	8.39	UNIVERSITY OF LONDON	15	9.68
SPAIN	12	7.74	UNIVERSITY OF CALIFORNIA SYSTEM	13	8.39
JAPAN	11	7.10	PHILIPS	12	7.74
FRANCE	10	6.45	UNIVERSITY OF UTAH	12	7.74
CHINA	9	5.81	HARVARD UNIVERSITY	11	7.10
NETHERLANDS	9	5.81	INTERMOUNTAIN MEDICAL CENTER	11	7.10
SOUTH KOREA	8	5.16	INTERMOUNTAIN HEART INSTITUTE	10	6.45

Note: Per (%) indicates the percentage of publications by each country/institution out of the total 155 publications analyzed.

teractive functional modules (Figure 3B). KEGG analysis highlighted AGE-RAGE, PI3K-Akt, MAPK, and Alzheimer's disease pathways (Figure 3C), while GO analysis showed significant enrichment in myocardial contraction, ion transport, mitochondrial function, and neural signal transduction (Table 2, Figure 3D-F).

Table 2. Clinical summary of key hub genes identified in the AF-AD intersection analysis.

Target	Function	Clinical Priority	Therapeutic Approach
TP53	Tumor suppressor	High	Anti-fibrotic agents
STAT3	Inflammation	High	JAK/STAT inhibitors
MAPK1	Signal transduction	High	MEK inhibitors
IL6	Cytokine	High	Anti-IL6 biologics
TNF	Cytokine	High	Anti-TNF biologics
VEGFA	Angiogenesis	Moderate	VEGF modulators
AKT1	Cell survival	Moderate	PI3K/AKT modulators
SRC	Tyrosine kinase	Moderate	SRC inhibitors
EGFR	Growth signaling	Moderate	EGFR inhibitors
FN1	ECM component	Moderate	Anti-fibrotic agents

Note: Clinical priority is assigned based on hub gene ranking across nine topological algorithms and biological relevance to both AF and AD pathophysiology. ECM, extracellular matrix.

Hub Gene Screening and Key Nodes

Comprehensive screening using nine topological algorithms identified 16 core hub genes (Figure 4A), with TP53, SRC,

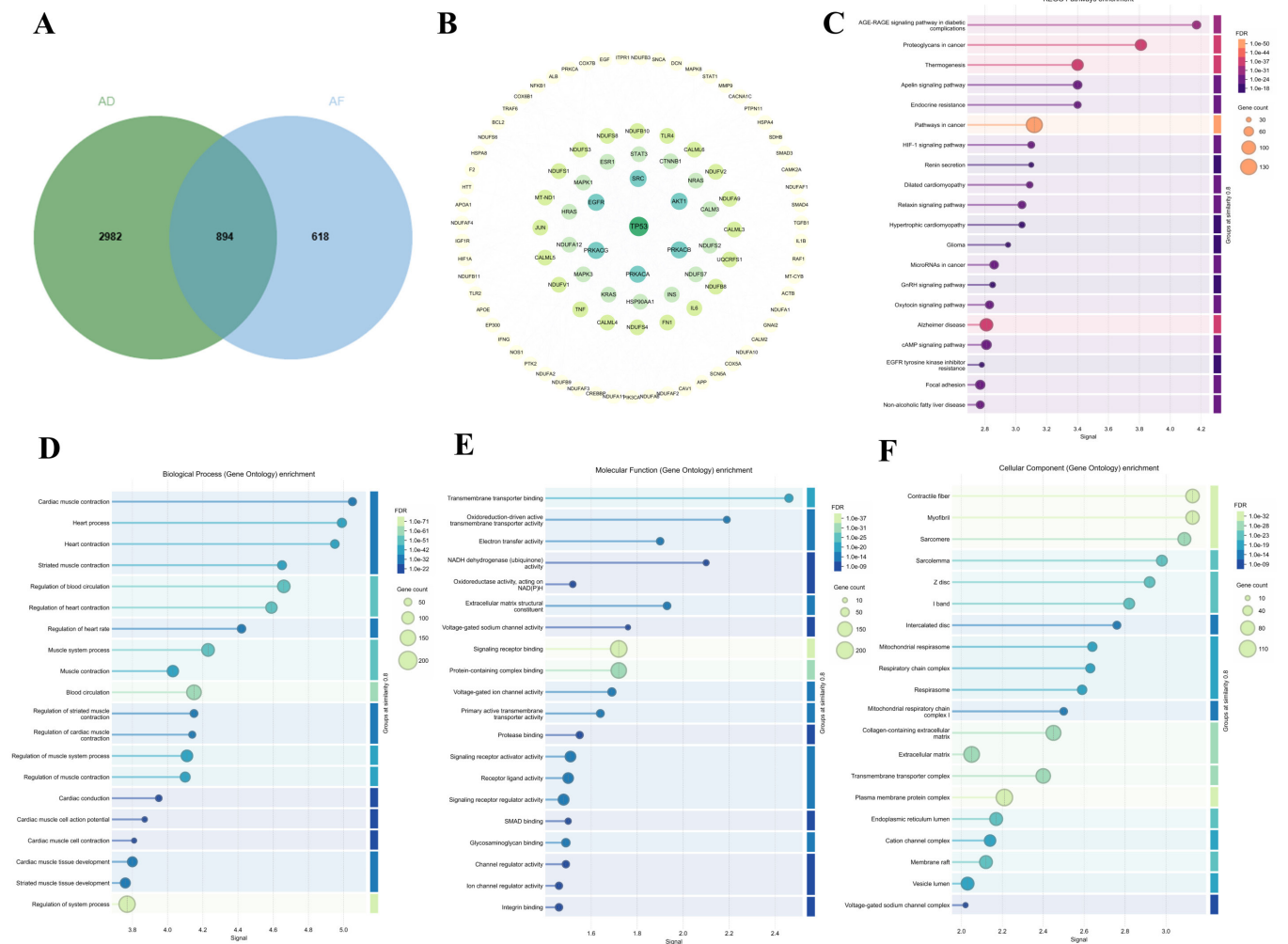


Figure 3. Identification of shared genes between Alzheimer's disease (AD) and atrial fibrillation (AF) and functional enrichment analysis. (A) Venn diagram showing the overlap between AD-related and AF-related genes. A total of 894 common genes were identified from 3,846 AD-related genes and 1,512 AF-related genes. **(B)** Protein-protein interaction (PPI) network of the shared genes, with hub genes displayed in a concentric circle layout. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the 894 shared genes. The dot size represents gene count, and the color gradient indicates the adjusted P-value. **(D)** Gene Ontology (GO) biological process (BP) enrichment analysis of the shared genes. Key enriched terms included cardiac muscle contraction, heart development, and regulation of blood circulation. **(E)** GO molecular function (MF) enrichment analysis. Enriched terms included transmembrane transporter activity, ion channel activity, and voltage-gated cation channel activity. **(F)** GO cellular component (CC) enrichment analysis. Enriched terms included contractile fiber, sarcolemma, Z disc, and ion channel complex. For panels D-F, the dot size represents gene count and the color corresponds to different enrichment terms.

EGFR, MAPK1, STAT3, and FN1 ranking highly across multiple algorithms, indicating their role as key signaling nodes. Visualized subnetworks show these genes occupy central positions in apoptosis, cellular stress, inflammation, and vascular function regulation (Supplementary Table 2, Figure 4B-J).

Discussion

Summary of Research Results

This study represents the first systematic integration of bibliometric and bioinformatic approaches to characterize the AF-AD research landscape over two decades while identifying molecular mechanisms underlying their association. Our primary innovation lies in the dual-layer analysis framework: at the bib-

liometric level, we mapped knowledge evolution, international collaboration patterns, and emerging research priorities; at the molecular level, we identified 894 intersection genes and validated hub nodes (TP53, STAT3, MAPK1, IL6, TNF, VEGFA, and AKT1) enriched in mechanistically relevant pathways (AGE-RAGE, PI3K-Akt, and MAPK signaling), providing actionable therapeutic targets.

Bibliometric Insights into Research Evolution

The temporal trajectory of AF-AD research reflects broader shifts in cardiovascular and neuroscience paradigms. Early publications (2006-2010) focused predominantly on epidemiological associations and stroke-mediated cognitive decline, consistent with the dominant "vascular hypothesis" of dementia. The citation peak in 2010 (281 citations) corresponded to

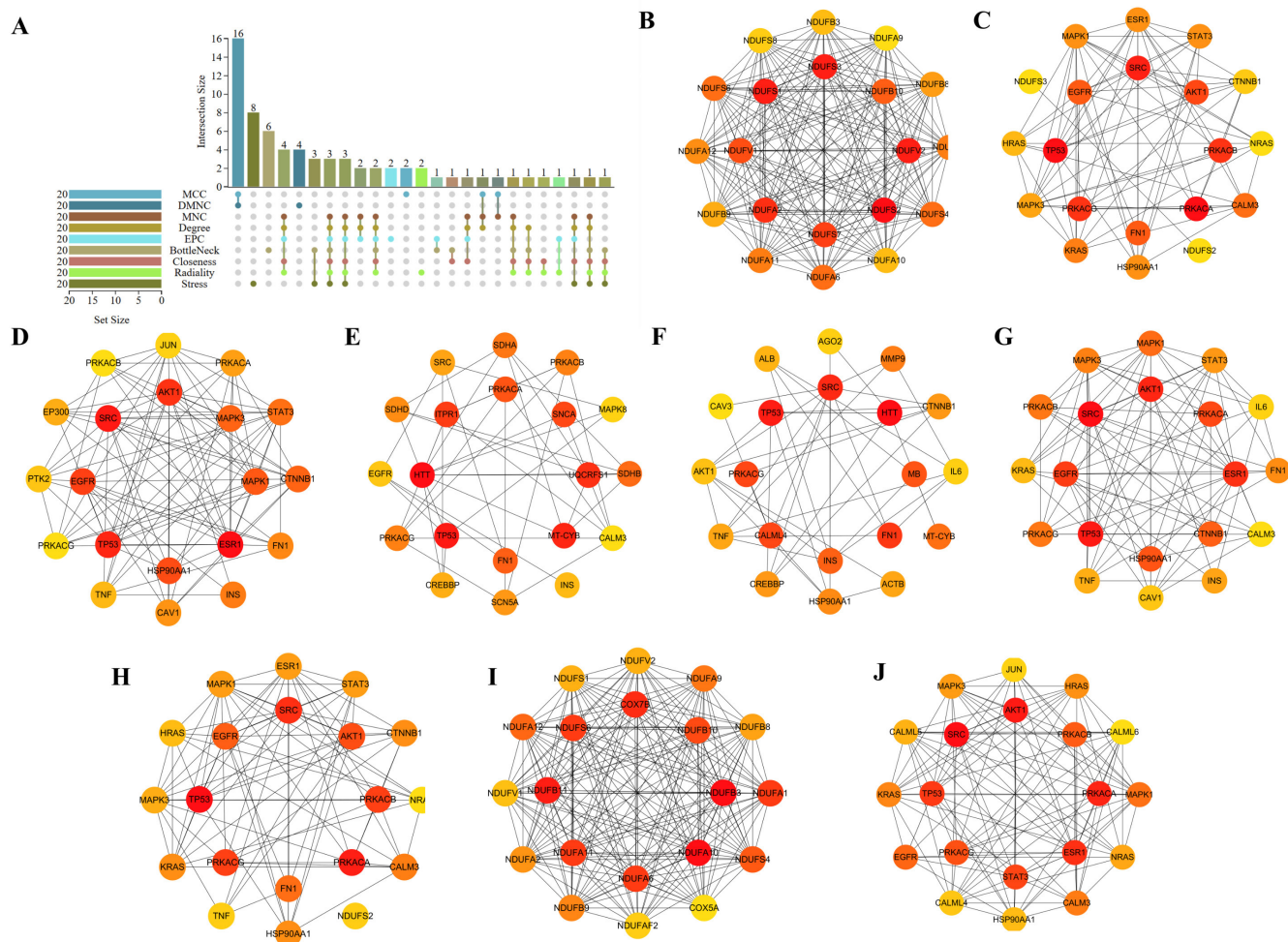


Figure 4. Hub gene identification and network visualization. (A) UpSet plot of top-ranked hub genes. **(B–J)** Top 20 targets identified using the CytoHubba plugin algorithm (B, MCC; C, MNC; D, Radiality; E, Stress; F, BottleNeck; G, Closeness; H, Degree; I, DMNC; J, EPC).

pivotal cohort studies establishing AF's independent association with dementia risk beyond clinical stroke [16–17], fundamentally reshaping the conceptual framework.

The growth phase (2011–2017) witnessed diversification in methodological approaches, incorporating neuroimaging studies revealing white matter lesions and cerebral microbleeds, Mendelian randomization analyses dissecting causal pathways [14], and genetic studies identifying shared susceptibility loci [18]. The 2017 citation peak (265 citations) aligned with landmark intervention trials exploring catheter ablation's cognitive benefits [19–21] and DOACs' neuroprotective potential [22–24], marking the transition from observational to interventional research.

The maturation phase (2018–2024) is characterized by mechanistic depth and translational focus. The increasing proportion of review articles reflects consolidation of accumulated evidence and emergence of meta-analyses synthesizing clinical findings.

Author productivity analysis revealed that leading contributors (ENDRES M, HAEUSLER KG, KOCH L, and SCHIRDEWAN A) demonstrated h-indices of 3–4 with 153 citations each, indicating concentrated expertise within specialized research groups. CAMARA O's high m-index (0.44) suggests recent but impact-

ful contributions, potentially reflecting emerging computational and imaging methodologies.

Institutional analysis demonstrates that AF-AD research emerged from established cardiovascular and neuroscience centers with capacity for longitudinal cohort studies and advanced imaging. The prominence of Berlin-based institutions reflects Germany's strengths in both electrophysiology and cognitive neuroscience, while Harvard-affiliated institutions leverage extensive biobank resources and electronic health record systems. Industry participation (Philips) underscores the role of technological innovation in diagnostic and monitoring capabilities, particularly cardiac imaging and wearable devices for AF detection.

Keyword network analysis provides granular insights into research priorities. The dominance of "catheter ablation" and "management" reflects clinical actionability—the field prioritizes modifiable risk factors and therapeutic interventions. The relatively lower centrality of cognitive outcome terminology ("dementia" and "cognitive impairment") compared to cardiovascular terms suggests that AF-AD research remains predominantly framed within cardiology rather than neurology, potentially explaining why mechanistic understanding lags behind epidemiological recognition. Terms with high between-

ness centrality ("management," "risk," and "conduction") serve as conceptual bridges linking cardiac electrophysiology with systemic consequences, indicating opportunities for interdisciplinary integration.

Molecular Mechanisms: Hub Genes and Pathway Biology

Our bioinformatic analysis identified critical hub genes representing convergence points between AF and AD pathophysiology, each offering distinct therapeutic opportunities.

TP53 emerged as the highest-ranking hub gene across multiple topological algorithms. Beyond its canonical tumor suppressor function, TP53 mediates cellular responses to oxidative stress, DNA damage, and metabolic perturbation—conditions prevalent in both atrial cardiomyocytes undergoing electrical remodeling and neurons experiencing chronic hypoperfusion. In AF, TP53 activation drives atrial fibrosis through senescence-associated secretory phenotype (SASP) induction in fibroblasts, promoting extracellular matrix deposition and structural remodeling [25]. In AD, TP53 regulates neuronal apoptosis in response to amyloid- β accumulation and tau pathology while modulating microglial inflammatory responses [26]. Recent evidence demonstrates that TP53 directly influences amyloid precursor protein (APP) processing and A β clearance mechanisms [27], suggesting therapeutic modulation of TP53 activity could yield dual cardiovascular-cognitive benefits. Anti-fibrotic strategies targeting TP53-mediated pathways warrant investigation in AF patients at high dementia risk.

STAT3 functions as a central inflammatory signaling hub activated by cytokines including IL-6 and IL-17. In AF, STAT3 mediates atrial inflammatory responses driving electrical and structural remodeling [28]. Persistent STAT3 activation in atrial tissue correlates with AF recurrence after ablation, indicating its role in maintaining pathological state [29]. In AD, STAT3 orchestrates microglial activation and astrocyte reactivity, contributing to neuroinflammatory cascades that accelerate cognitive decline [30]. Crucially, STAT3 activation in brain endothelial cells disrupts blood-brain barrier integrity, facilitating peripheral inflammatory mediator entry [31]. Our pathway enrichment analysis showing IL-17 signaling prominence ($\text{FDR} < 1.0 \times 10^{-14}$) supports STAT3's role as a therapeutic target. JAK/STAT inhibitors, already approved for inflammatory conditions, represent repurposing opportunities for dual AF-AD intervention, particularly in patients with elevated systemic inflammatory markers.

MAPK1 (ERK2) mediates cellular stress responses, proliferation, and survival through the MAPK signaling cascade. In AF, MAPK pathway activation regulates cardiac ion channel expression and function, directly influencing action potential duration and conduction velocity [32]. MAPK-mediated phosphorylation of potassium channels contributes to electrical remodeling underlying AF maintenance [33]. In AD, MAPK1 dysregulation promotes tau hyperphosphorylation at pathogenic epitopes while impairing synaptic plasticity mechanisms [34]. Our enrichment analysis demonstrated MAPK signaling pathway prominence ($\text{FDR} < 1.0 \times 10^{-19}$, ~35 genes), positioning this cascade as a mechanistic bridge between cardiac electrical dysfunction and neurodegenerative tau pathology. MEK inhibitors, which block MAPK1 activation, showed neuroprotective effects in preclinical AD models [35] and warrant investigation in AF populations.

IL-6 and TNF represent pro-inflammatory cytokines with

well-established roles in both conditions. Elevated IL-6 levels predict AF incidence and recurrence while correlating with dementia risk in AF patients [36]. IL-6 drives atrial fibroblast activation and collagen production, contributing to structural substrate formation [37]. In AD, IL-6 promotes neuroinflammation and blood-brain barrier disruption, facilitating A β deposition [38]. TNF similarly mediates inflammatory cascades in atrial tissue and brain parenchyma, with circulating levels correlating with both AF burden and cognitive decline [39]. Importantly, our bibliometric keyword analysis identified "inflammatory markers" as an emerging research priority, aligning with growing recognition of inflammation's central role. Anti-cytokine biologics (anti-IL-6 and anti-TNF) represent therapeutic opportunities, though careful patient selection is essential given infection risks in elderly populations.

VEGFA regulates angiogenesis and vascular permeability, with complex roles in both AF and AD. In AF, VEGFA expression increases in atrial tissue, potentially representing compensatory angiogenic responses to chronic hypoxia and metabolic stress [40]. However, excessive VEGFA can promote endothelial dysfunction and atrial vascular remodeling [41]. In AD, impaired VEGFA signaling contributes to cerebrovascular dysfunction, reduced cerebral blood flow, and neurovascular uncoupling [42]. Conversely, excessive VEGFA disrupts blood-brain barrier integrity, potentially facilitating A β accumulation [43]. This dual role suggests that VEGFA modulation requires careful titration—therapeutic strategies promoting physiological angiogenesis while preventing pathological vascular permeability warrant investigation.

AKT1, a central node in PI3K-Akt signaling, governs cell survival, glucose metabolism, and protein synthesis. Our enrichment analysis identified PI3K-Akt signaling as highly significant ($\text{FDR} < 1.0 \times 10^{-23}$, ~40 genes), reflecting its pleiotropic roles. In AF, AKT activation promotes cardiomyocyte survival under stress but can drive pathological hypertrophy and fibrosis when chronically activated [44]. In AD, impaired AKT signaling contributes to insulin resistance, a recognized AD risk factor, while reduced AKT activity compromises neuronal survival responses [45]. Interestingly, AKT directly phosphorylates tau at specific sites, with dysregulated AKT activity contributing to pathological tau accumulation [46]. PI3K/AKT pathway modulation represents a promising but challenging therapeutic avenue requiring careful dosing to maintain beneficial survival signaling while preventing pathological consequences.

Pathway-Level Integration: Mechanistic Convergence

Beyond individual hub genes, pathway enrichment analysis revealed systemic mechanisms linking AF and AD.

AGE-RAGE signaling ($\text{FDR} < 1.0 \times 10^{-37}$) showed the strongest statistical enrichment, highlighting oxidative stress and glycation as fundamental shared mechanisms. Advanced glycation end products (AGEs) accumulate with aging, diabetes, and chronic inflammation—all AF risk factors. AGE-RAGE interaction triggers oxidative stress, inflammatory cytokine production, and endothelial dysfunction [47]. In atrial tissue, AGE accumulation promotes fibrosis and electrical remodeling [48]. In brain, AGE-RAGE activation disrupts blood-brain barrier integrity, promotes neuroinflammation, and may accelerate A β accumulation [49]. Importantly, AGE levels correlate with both AF burden and cognitive decline severity, suggesting utility as biomarkers [50]. Therapeutic strategies including AGE-breakers,

RAGE antagonists, and dietary AGE restriction represent novel interventions targeting this fundamental pathway. Our identification of AGE-RAGE signaling as the top-ranked pathway provides molecular rationale for lifestyle interventions (glycemic control and dietary modification) in AF patients at dementia risk.

PI3K-Akt signaling ($FDR < 1.0 \times 10^{-23}$): Beyond AKT1's individual role, the broader PI3K-Akt pathway regulates cellular metabolism, survival, and growth across multiple cell types. In AF, this pathway mediates cardiomyocyte responses to hemodynamic stress and neurohormonal activation [51]. In AD, PI3K-Akt signaling dysfunction underlies insulin resistance, impaired glucose metabolism, and compromised neuronal survival [52]. The pathway's central role in both conditions suggests that metabolic dysfunction represents a unifying mechanism. Intriguingly, metformin, an antidiabetic drug with PI3K-Akt modulatory effects, shows promise in reducing dementia risk in AF patients [53], providing translational validation of this mechanistic link.

MAPK signaling ($FDR < 1.0 \times 10^{-19}$): Our analysis highlighted multiple MAPK pathway components beyond MAPK1, including upstream activators and downstream targets. This cascade integrates diverse cellular stresses—oxidative damage, inflammatory cytokines, and growth factor deprivation—into coordinated responses affecting gene expression, protein function, and cellular fate [54]. In AF, MAPK signaling regulates ion channel expression, gap junction remodeling, and fibroblast activation [55]. In AD, dysregulated MAPK activity promotes tau hyperphosphorylation and contributes to synaptic dysfunction [56]. The pathway's dual role in both electrical remodeling and tau pathology positions it as a mechanistic bridge explaining how cardiac pathology influences AD-specific molecular changes.

Mitochondrial function and calcium homeostasis: GO analysis revealed significant enrichment in mitochondrial processes and calcium signaling, reflecting bioenergetic dysfunction as a shared mechanism. Atrial cardiomyocytes in AF exhibit mitochondrial oxidative stress, impaired ATP production, and disrupted calcium handling—changes contributing to electrical instability [57]. Similarly, AD neurons demonstrate mitochondrial dysfunction, energy deficits, and calcium dysregulation promoting synaptic failure and apoptosis [58]. Recent evidence suggests that mitochondrial dysfunction in AF may generate circulating damage-associated molecular patterns (DAMPs) that systemically propagate inflammatory signals affecting brain function [59]. Therapeutic strategies targeting mitochondrial health (coenzyme Q10 and mitochondria-targeted antioxidants) warrant investigation in AF-AD populations.

Cellular senescence and SASP: Though not explicitly highlighted in pathway enrichment, multiple hub genes (TP53 and STAT3) and enriched pathways (cancer-related pathways) converge on cellular senescence mechanisms. Senescent cells accumulate in aged atrial tissue and brain, secreting pro-inflammatory cytokines, growth factors, and proteases collectively termed SASP [60]. SASP factors promote atrial fibrosis, disrupt cardiac electrical function, and can cross the blood-brain barrier to induce neuroinflammation [61]. Recent studies demonstrate that senolytic drugs clearing senescent cells reduce AF susceptibility and improve cognitive function in pre-clinical models [62], suggesting this as a promising therapeutic frontier targeting upstream aging mechanisms common to

both conditions.

This multi-level molecular analysis establishes that AF and AD share fundamental pathophysiological mechanisms beyond their epidemiological association. The convergence on oxidative stress, inflammation, metabolic dysfunction, and cellular senescence provides biological plausibility for AF's role in AD pathogenesis while identifying actionable therapeutic targets for precision intervention (Figure 5).

Clinical Intervention and Prevention Strategies for AF-Related Cognitive Dysfunction

The Role of Anticoagulation: From Warfarin to Direct Oral Anticoagulants

Anticoagulation therapy is the cornerstone of stroke prevention in AF patients, with its role in reducing dementia risk also receiving attention [63]. Multiple observational studies indicate AF patients receiving anticoagulation have lower dementia risk [64]. One study found anticoagulant use reduced dementia risk by 60% compared to untreated patients [64]. Direct oral anticoagulants (DOACs) may offer advantages over traditional warfarin in cognitive protection [22]. In patients with dementia and AF, NOACs (non-vitamin K antagonist oral anticoagulants) associate with lower ischemic stroke risk, while warfarin may increase bleeding risk [22]. Animal experiments also provide mechanistic support for DOACs' neuroprotective effects; for example, rivaroxaban has been shown to reduce pathological progression in AD model mice by reducing fibrinogen deposition, inhibiting neuroinflammation, and improving white matter integrity [23-24]. However, anticoagulation benefits are not without risks, especially in specific populations. Studies found APOE $\epsilon 4$ carriers have three-fold increased intracranial hemorrhage (ICH) risk when using apixaban, suggesting genetic background may influence anticoagulation safety [65]. Additionally, anticoagulation quality is crucial; patients with unstable warfarin therapeutic windows (low TTR) have significantly higher dementia risk than those with stable TTR, observed in patients with and without AF [66]. While DOACs show potential, their cost-effectiveness in dementia patients requires consideration; a modeling study indicated DOACs may not be cost-effective compared to warfarin for moderate-to-severe AD patients [67].

Catheter Ablation's Potential for Reducing Dementia Risk

Catheter ablation, as a treatment to restore and maintain sinus rhythm, is gradually revealing its potential in reducing dementia risk. Multiple large-scale retrospective cohort studies and meta-analyses indicate patients receiving catheter ablation have significantly lower subsequent dementia risk compared to those receiving medical therapy [68-70]. One meta-analysis integrating data from 5 studies showed catheter ablation significantly associated with reduced dementia risk ($HR = 0.63$), with statistically significant AD risk reduction ($HR = 0.78$) [69]. A Swedish study comparing patients with optimized anticoagulation similarly found catheter ablation reduced dementia risk by approximately 56% [68]. A Taiwanese nationwide study also confirmed catheter ablation significantly reduces overall dementia risk, especially AD risk [70]. Potential protective mechanisms of catheter ablation may include restoring normal hemodynamics, reducing chronic cerebral hypoperfusion, eliminating sources of atrial thrombus formation, reducing microembolic events, and improving autonomic function while

Mechanistic Pathways Linking Atrial Fibrillation and Alzheimer's Disease

Legend: Direct pathway Inflammatory pathway Inconsistent evidence Molecular convergence

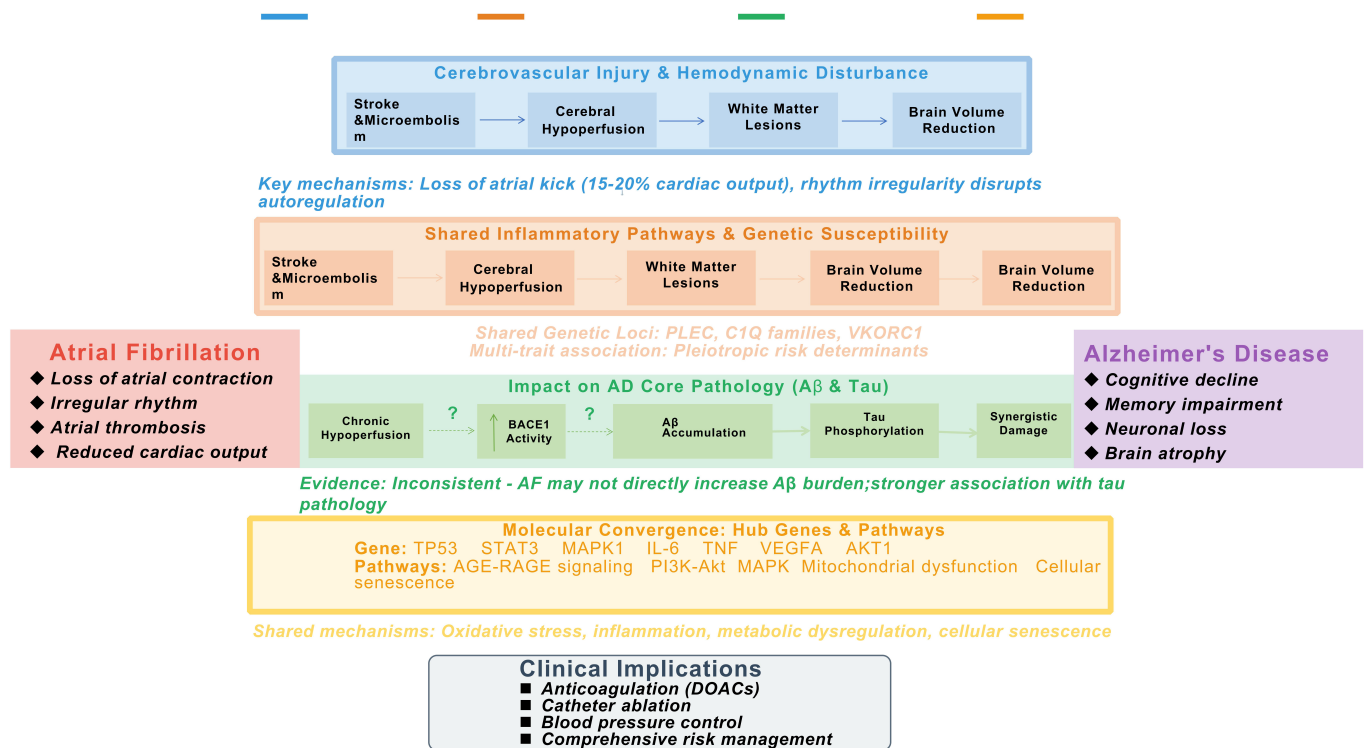


Figure 5. Mechanistic pathways linking atrial fibrillation and Alzheimer's disease. Schematic summary of the shared molecular mechanisms identified in this study, integrating hub genes (TP53, STAT3, MAPK1, IL6, TNF, VEGFA, and AKT1) and enriched pathways (AGE-RAGE, PI3K-Akt, and MAPK signaling) that converge on oxidative stress, inflammation, metabolic dysfunction, and cellular senescence in the heart-brain axis.

reducing systemic inflammation. These results suggest that for suitable AF patients, restoring sinus rhythm through catheter ablation may be an effective primary prevention strategy for dementia, though prospective randomized controlled trials are still needed to confirm causal relationships and long-term effects.

Comprehensive Risk Factor Management: Blood Pressure, Lifestyle, and Other Cardiovascular Health Indicators

Beyond treating AF itself, comprehensive management of coexisting cardiovascular risk factors is crucial for preventing cognitive dysfunction. Blood pressure control is a core component; studies show a U-shaped relationship between blood pressure and dementia risk in AF patients, with both excessively high or low systolic and diastolic pressures increasing dementia risk, suggesting maintaining blood pressure within normal ranges (e.g., systolic 120-129 mmHg) may offer maximum benefit [71]. Lifestyle interventions are equally important; one study found new-onset AF patients who quit smoking after diagnosis had lower dementia risk than continuing smokers, highlighting smoking cessation's value as a dementia prevention strategy [72]. Additionally, education level, plasma glucose levels, and body mass index (BMI) are proven powerful predictors of cognitive impairment [73]. Regarding pharmacotherapy, angiotensin receptor blockers (ARBs) may provide additional cognitive protection; a Swedish study found ARB use associated with lower dementia incidence in AF patients [74]. This evidence collectively supports initiatives from organizations like

the World Stroke Organization for joint prevention of stroke, dementia, and heart disease through combined management of risk factors like hypertension to achieve comprehensive brain health protection [75]. Therefore, AF patient management should extend beyond rhythm control and anticoagulation, adopting multidimensional, comprehensive prevention strategies including blood pressure optimization, lifestyle improvement, and rational antihypertensive drug selection.

Diagnosis, Prognostic Assessment, and Risk Prediction Impact of Cardiovascular Risk on Alzheimer's Disease Plasma Biomarker Interpretation

The presence of cardiovascular risk factors, including AF, significantly affects AD plasma biomarker levels and their predictive accuracy for brain pathology. A study systematically evaluated the modulating effects of cardiometabolic condition (CMC) scores (including AF, hypertension, and diabetes) on AD plasma biomarkers (Aβ42/40, p-tau181, GFAP, and NfL) [76]. Results showed that in individuals with higher CMC scores, GFAP's association with AD neuropathologic change (ADNC) weakened while its association with large vessel disease strengthened; simultaneously, p-tau181's association with Thal stage (Aβ pathology) strengthened while its association with large vessel disease weakened [76]. This indicates that when patients have multiple cardiovascular risk factors, plasma biomarkers may reflect vascular injury more than pure AD pathology. Therefore, in clinical practice, when interpreting AD plasma biomarkers for screening, diagnosis, or prognostic as-

assessment, patients' cardiovascular health status must be fully considered to avoid misjudging potential neuropathology and achieve more precise biomarker application [76].

Dual Role of Genetic Markers (APOE ϵ 4) in Treatment Decisions

Genetic markers, particularly the apolipoprotein E ϵ 4 (APOE ϵ 4) allele, play a complex dual role in AF patients with cognitive risk, serving as both a major genetic risk factor for AD and potentially influencing treatment decision risk-benefit balance. One study found that among AF patients receiving apixaban, APOE ϵ 4 carriers had three times the ICH risk of non-carriers [65]. This finding has important clinical significance because APOE ϵ 4 status is increasingly used to guide AD anti-amyloid treatment decisions, and millions know their APOE status through direct-to-consumer genetic testing [65]. Therefore, for APOE ϵ 4-carrying AF patients, more careful weighing of ischemic stroke prevention versus bleeding risk is needed when selecting anticoagulation regimens. Conversely, in patients with AD, APOE ϵ 4 and AF may have synergistic effects, jointly accelerating cognitive deterioration. Studies show that AD patients carrying APOE ϵ 4 with permanent AF have the lowest cognitive function levels, more severe atherosclerosis, and highest cognitive deterioration risk [77]. This suggests genetic information not only aids risk stratification but also places higher demands on individualized treatment strategy formulation.

Value of Cardiac Markers Including Electrocardiogram in Cognitive Risk Assessment

Non-invasive cardiac examinations like electrocardiogram (ECG) are used not only for diagnosing arrhythmias but specific parameters may also become potential biomarkers for assessing cognitive risk. Studies found P-wave terminal force in V1 (PTFV1), an ECG indicator related to AF and ischemic stroke, is also significantly associated with cerebral small vessel disease (CSVD) markers, particularly deep cerebral microbleed (CMB) burden [78]. Patients with elevated PTFV1 are more likely to be diagnosed with vascular dementia rather than AD [78]. This suggests PTFV1 may serve as a heart-brain connection marker linking atrial disease with vascular brain injury. Another study found high prevalence of atrial conduction abnormalities (prolonged P-wave duration and abnormal PTFV1) in Lewy body disease (LBD) patients, independent of AF or atrial enlargement, possibly reflecting underlying atrial cardiomyopathy caused by α -synuclein deposition [79]. These ECG parameter abnormalities might serve as tools for screening cardiac complications (like AF) in LBD patients [79]. Additionally, in clinical practice, some comprehensive risk scoring models, like the Framingham Dementia Risk Score (FDRS), have proven good predictive ability for AD and related dementias in AF and heart failure populations [80]. These findings collectively suggest integrating cardiac markers like ECG parameters into cognitive risk assessment workflows helps earlier identification of high-risk individuals for targeted intervention.

Clinical insights can be summarized as four strategies. First, prioritize anticoagulation emphasizing DOACs' net benefit. Standardized anticoagulation with stable DOAC strategies aligns with epidemiological signals of lower dementia risk, but requires individualized weighing under bleeding risk and genetic background (e.g., APOE). Second, rhythm control and

catheter ablation. By restoring sinus rhythm, there is potential to improve cerebral perfusion and reduce atrial thrombus sources, potentially bringing long-term cognitive benefits. Validation in randomized studies using neuroimaging (perfusion and white matter lesions) and fluid biomarkers as combined endpoints is recommended. Third, comprehensive vascular risk factor management. Evidence supports cognitive protective effects of blood pressure management avoiding U-shaped risk, metabolic control, smoking cessation, and weight and exercise interventions; the public health strategy of preventing stroke to prevent dementia equally applies to comprehensive AF population follow-up. Fourth, precision stratification and risk prediction. Integrating ECG/atrial phenotype, blood/CSF biomarkers (GFAP, NfL, p-tau, and A β 42/40) with genetic information (APOE) can identify high cognitive risk AF subgroups, guiding resource allocation and intervention priorities (more mechanism can be seen in Figure 5).

Limitations and Future Research Priorities

This study has several important limitations requiring acknowledgment. First, bibliometric analysis was restricted to Web of Science and English-language publications, potentially missing relevant work in other languages or databases. The high concentration of publications from specific countries (USA and Germany) may reflect both genuine research leadership and database indexing bias, limiting generalizability. Second, the bioinformatics approach, while systematic, relies on gene-disease association databases (GeneCards) that may contain incomplete or biased information. The selection of top-quartile genes and specific topological algorithms (STRING and CytoHubba) involves methodological choices that could influence results. External validation using independent datasets and experimental verification of hub genes and pathways in relevant biological models (atrial and brain tissue) is essential but not yet performed. Third, the causality of AF-AD relationships cannot be definitively established from our bibliometric and bioinformatic approaches. While we provide mechanistic hypotheses supported by enrichment analysis, experimental validation and clinical trials with causal inference designs are needed. Based on these findings, we propose three priority research directions.

(1) Mechanism-oriented randomized controlled trials. Comparative effectiveness studies of DOACs versus warfarin, and rhythm control (including ablation) versus rate control, should employ composite endpoints including long-term cognitive trajectories (using sensitive neuropsychological batteries), neuroimaging (brain perfusion, white matter lesion burden, microbleeds, and atrophy), and fluid biomarkers (GFAP, NfL, p-tau, A β 42/40, and inflammatory markers). Stratification by AF burden (paroxysmal vs. persistent), atrial phenotype (structural abnormalities on imaging), and baseline cognitive function would reveal which patients benefit most from specific interventions. Mechanistic substudies could assess whether interventions modulate hub gene expression (TP53, STAT3, and MAPK1) in blood samples, linking clinical benefits to molecular mechanisms.

(2) Target validation and pharmacological exploration. Preclinical studies should validate the functional roles of hub genes (TP53, STAT3, MAPK1, IL-6, TNF, VEGFA, and AKT1) and pathways (AGE-RAGE, PI3K-Akt, and MAPK) using in vitro models (atrial cardiomyocytes, neurons, and endothelial cells) and in

vivo models (AF mouse models with cognitive testing). Intervention experiments testing pathway inhibitors (JAK/STAT inhibitors, MEK inhibitors, PI3K inhibitors, and AGE-breakers) could establish proof-of-concept for therapeutic targeting. Crucially, assessing heart-brain bidirectional effects—whether cardiac-targeted interventions improve brain outcomes and whether neuroprotective interventions improve cardiac outcomes—would validate the integrated pathophysiology paradigm. Drug repurposing opportunities, leveraging FDA-approved agents affecting identified pathways, could accelerate translation.

(3) Multicenter multimodal precision stratification. Large-scale prospective cohort studies in diverse populations should develop and externally validate risk prediction models integrating clinical variables, ECG parameters, cardiac imaging, genetic markers (APOE and inflammatory gene variants), and fluid biomarkers. Such models could identify AF patients at highest dementia risk for targeted preventive interventions. Implementation science research should examine how to integrate these tools into routine clinical workflows. International collaborative efforts addressing the geographic concentration identified in our bibliometric analysis would improve generalizability and external validity. Registry-based randomized trials embedded within healthcare systems could test precision medicine approaches efficiently and pragmatically.

Conclusion

In conclusion, this integrated bibliometric-bioinformatic analysis establishes AF-AD research as a maturing field transitioning from epidemiological observation toward mechanistic understanding and targeted intervention. The convergence of clinical evidence, molecular pathways, and therapeutic opportunities provides a foundation for precision cardiovascular-cognitive medicine aimed at preventing dementia in the growing AF population.

Abbreviations

ATP: Adenosine Triphosphate; AGE: Advanced Glycation End products; AD: Alzheimer's Disease; ADNC: Alzheimer's Disease Neuropathologic Change; APP: Amyloid Precursor Protein; A β : Amyloid-beta; ARB: Angiotensin Receptor Blocker; APOE: Apolipoprotein E; AF: Atrial Fibrillation; BP: Biological Process; BMI: Body Mass Index; CMC: Cardiometabolic Condition; CC: Cellular Component; CMB: Cerebral Microbleed; CSVD: Cerebral Small Vessel Disease; DAMP: Damage-Associated Molecular Pattern; DOAC: Direct Oral Anticoagulant; ECG: Electrocardiogram; ECM: Extracellular Matrix; FDR: False Discovery Rate; FDRS: Framingham Dementia Risk Score; GO: Gene Ontology; GFAP: Glial Fibrillary Acidic Protein; HR: Hazard Ratio; ICH: Intracranial Hemorrhage; KEGG: Kyoto Encyclopedia of Genes and Genomes; LBD: Lewy Body Disease; MeSH: Medical Subject Headings; MF: Molecular Function; NfL: Neurofilament Light Chain; NOAC: Non-vitamin K Antagonist Oral Anticoagulant; PTFV1: P-wave Terminal Force in V1; PPI: Protein-Protein Interaction; RAGE: Receptor for Advanced Glycation End products; SASP: Senescence-Associated Secretory Phenotype; TTR: Time in Therapeutic Range; VaD: Vascular Dementia.

Author Contributions

Tiancheng Li: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing. Junpeng Xiong: Conceptualization, Supervision, Project administration, Methodology, Validation, Writing - Review & Editing. All authors read and approved the final manuscript.

Acknowledgements

Not Applicable.

Funding Information

Not Applicable.

Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

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

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Bibliographic records were retrieved from the Web of Science Core Collection (<https://www.webofscience.com/>); gene-disease association data were obtained from GeneCards (<https://www.genecards.org/>); and protein-protein interaction data were obtained from the STRING database (<http://string-db.org/>). Additional data related to this paper may be requested from the authors.

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Analysis of the Correlation between Pseudomonas aeruginosa Resistance Genes and Virulence Genes in Burn Patients Using Next-Generation Sequencing

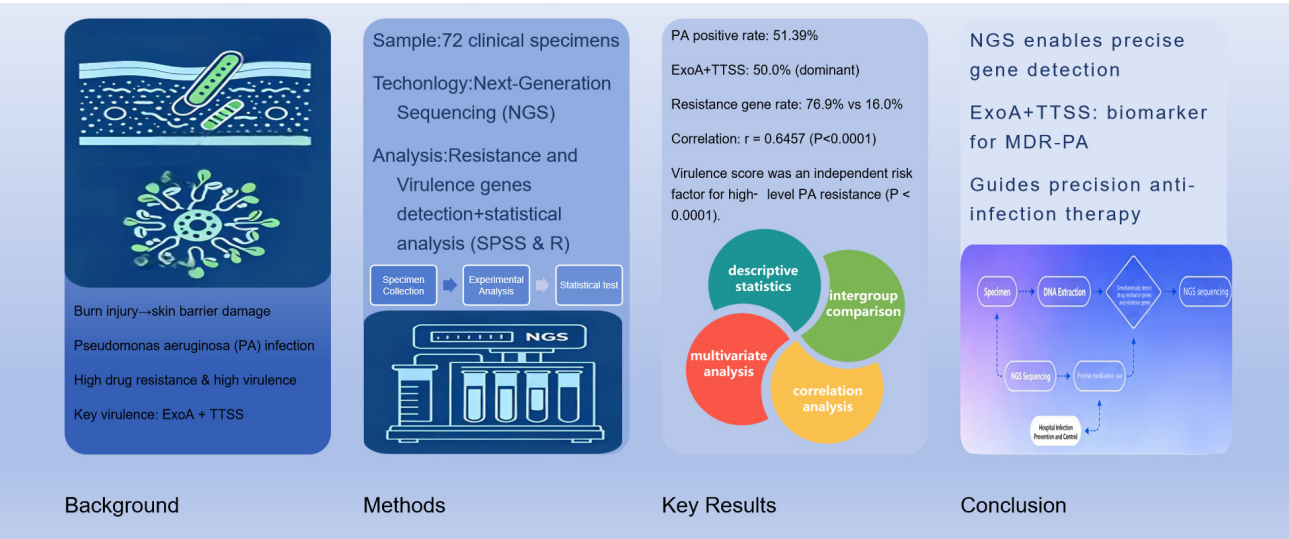
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Graphical Abstract



<https://doi.org/10.71321/dpqzt488>

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Analysis of the Correlation between *Pseudomonas aeruginosa* Resistance Genes and Virulence Genes in Burn Patients Using Next-Generation Sequencing

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Received: 2026-04-23 | Accepted: 2026-05-25 | Published online: 2026-07-14

Abstract

Objective: To analyze the distribution and correlation of drug resistance genes and virulence genes of *Pseudomonas aeruginosa* (PA) isolated from burn patients using next-generation sequencing (NGS), providing molecular evidence for precise clinical diagnosis and treatment.

Methods: A retrospective study was conducted on 72 suspected PA infection specimens and clinical data collected from the Department of Burn and Wound Repair Surgery at the First Affiliated Hospital of Anhui Medical University between January 2022 and December 2025. NGS was employed to detect drug resistance and virulence genes, with statistical analysis performed using SPSS 26.0 and R language.

Results: The positive detection rate of PA was 51.39% (37/72), with wounds being the primary infection site (positive rate: 83.8%). The infection rate was highest among moderate burn patients (58.3%). PA exhibited a 100.0% resistance rate to cefazolin, and among the 25 drug-sensitive strains, 36.0% were multidrug-resistant. A trend toward prevalence of PA resistant to carbapenems was observed. Among the 52 specimens tested for virulence genes, the overall carriage rate was 51.9% (27/52), with the ExoA+TTSS composite genotype being predominant (50.0%, 26/52). The detection rate of drug resistance genes in ExoA+TTSS-positive strains (76.9%) was significantly higher than in non-virulence gene-positive strains (16.0%; $\chi^2=19.9086$, $P<0.0001$). The virulence factor score showed a moderately strong positive correlation with the number of drug resistance genes ($r=0.6457$, $P<0.0001$) and was identified as an independent risk factor for drug resistance gene carriage (OR=2.1494–9.7401, $P<0.0001$).

Conclusion: In burn patients, PA infections predominantly occur at the wound site, with prominent resistance and multidrug resistance phenomena; PA virulence genes exhibit a significant positive correlation with resistance genes, and strains with the ExoA+TTSS composite genotype demonstrate higher resistance; NGS enables simultaneous analysis of PA genetic characteristics, providing critical support for precision anti-infection therapy.

Keywords: Burn; *Pseudomonas aeruginosa* (PA); Next-generation sequencing (NGS); Drug resistance genes; Virulence genes

Introduction

Burns compromise the skin barrier and induce systemic immune dysfunction, rendering patients highly susceptible to invasive infections. *Pseudomonas aeruginosa* (PA) is the predominant opportunistic pathogen causing burn wound infections, frequently leading to severe sepsis, multiple organ dysfunction syndrome, and even patient mortality [1]. With the deepening of burn care and the wide application of broad-spectrum antibiotics, the clinical prevention and control of PA infections have become increasingly challenging. The clinical diagnosis and treatment of *Pseudomonas aeruginosa* infections present significant challenges, as the bacterial strains exhibit multidrug resistance (MDR) and high virulence. Particularly, the prevalence of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) has become a central therapeutic

challenge in global burn care [2]. In recent three years, the detection rate of CRPA in domestic burn wards has reached 45%–65%, showing a continuous upward trend, which seriously restricts the efficacy of clinical anti-infection treatment.

The pathogenicity of *Pseudomonas aeruginosa* is strictly regulated by virulence genes, among which exotoxin A (ExoA) and the type III secretion system (TTSS) serve as core virulence factors mediating host cell damage and bacterial dissemination. Diverse resistance genes confer resistance to β -lactams, aminoglycosides, quinolones, and carbapenems through mechanisms such as enzyme inactivation, overexpression of efflux pumps, and modification of target sites. Notably, an increasing number of clinical studies have indicated a potential synergistic relationship between virulence and resistance in clinically isolated PA strains. However, specialized studies investigating this correlation in burn patients—a population with unique wound microenvironments and immune states—remain scarce.

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Next-generation sequencing (NGS) enables high-throughput, simultaneous detection of bacterial resistance genes and virulence genes, demonstrating significantly superior accuracy and comprehensiveness compared to traditional drug susceptibility testing and single-gene PCR detection techniques [3]. At present, most domestic and foreign studies focus on the distribution of single type of genes or the correlation analysis in common clinical populations, while there is a lack of systematic research on the co-distribution and molecular correlation of resistance and virulence genes of PA in burn patients, and the exploration of synergistic mechanisms is also insufficient. To investigate the relevant experiments, this study employed NGS technology to analyze the distribution patterns of *Pseudomonas aeruginosa* resistance genes and virulence genes in burn patients and to investigate their correlation. The findings aim to provide a molecular biological basis for the precise diagnosis, individualized antimicrobial therapy, and targeted infection prevention and control of *Pseudomonas aeruginosa* infections in burn patients.

Materials and Methods

Study Subjects

A retrospective study was conducted on 72 specimens from patients suspected of PA infection admitted to the Department of Burn and Wound Repair Surgery at the First Affiliated Hospital of Anhui Medical University between January 2022 and December 2025, including wound tissue, blood, sputum, and central venous catheter specimens. Clinical data such as patient gender, age, burn area, burn depth, and infection site were collected.

Inclusion and Exclusion Criteria

Inclusion criteria: The strain was confirmed as PA through morphological examination, biochemical tests, VITEK MS mass spectrometry, and 16S rDNA sequencing; the strain was isolated from clinical specimens of burn patients with a purity >99% confirmed by subculture; the patient's clinical data were complete; the infection was solely due to burns; and no novel antimicrobial agents such as cefotaxime/avibactam or meropenem/flopiopenem were administered prior to strain isolation. **Exclusion criteria:** Contaminated strains or mixed infection strains; absence of critical clinical data; presence of severe underlying conditions such as malignant tumors or multiple organ dysfunction syndrome; homologous strains isolated repeatedly from the same patient during hospitalization (confirmed by whole-genome homology analysis).

Detection Method

NGS detection was performed using the Illumina MiSeq platform and supporting Nextera XT DNA Library Preparation Kit (Illumina Inc., Model: FC-131-1096). The detection of PA resistance genes (β -lactamase genes, aminoglycoside-modifying enzyme genes, fluoroquinolone resistance genes) and virulence genes (ExoA, TTSS) was performed strictly following the kit instructions.

Statistical Analysis

Data analysis was performed using SPSS 26.0 and R 4.2.1 software. Categorical data were expressed as counts (%). In-

tergroup comparisons were analyzed using the χ^2 test or Fisher's exact probability method; correlation was assessed with Spearman's rank correlation analysis; and multivariate analysis was conducted using logistic regression analysis. A P-value <0.05 was considered statistically significant.

Results

Clinical Distribution Characteristics of PA

Among the 72 specimens, PA positivity was detected in 37 cases, yielding a detection rate of 51.39% (37/72). The majority of specimens (56 cases, 77.8%) were wound tissues, with a PA positivity rate of 83.8% (31/37) (Figure 1). Among the 45 burn patients, those with moderate burns (body surface area 10%–30%) exhibited the highest PA infection rate (58.3%), as detailed in Table 1.

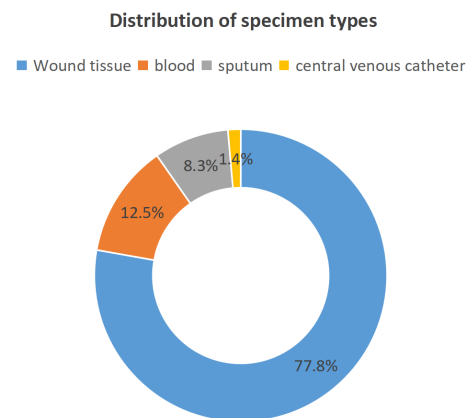


Figure 1. Distribution of specimen types.

The pie chart displays the proportion of different specimen types. Wound tissue accounts for 77.8%, blood 12.5%, sputum 8.3%, and central venous catheter 1.4%.

Table 1. Summary of Descriptive Statistics for *Pseudomonas aeruginosa* Detection Samples.

project	quantity	percentage (%)
Total number of specimens	72	100.0
Wound tissue	56	77.8
blood	9	12.5
sputum	6	8.3
central venous catheter	1	1.4
Burn patient	45	62.5
Non-burn patients	27	37.5
Mild burn (<10%)	2	2.8
Moderate burns (10%–30%)	14	9.4
Severe burns (>30%)	29	40.3
Resistant gene detected	35	48.6
No drug-resistant genes were detected.	37	51.4
Virulence factors were detected	27	51.9
No virulence factors were detected.	25	48.1

Note: This table summarizes the descriptive statistics for 72 *Pseudomonas aeruginosa* detection samples, covering specimen type composition, burn/non-burn patient distribution, burn severity grading, and detection proportions of drug-resistant genes and virulence factors.

PA Resistance Characteristics

Among the 25 strains with complete antibiotic susceptibility profiles, the resistance rates of PA were 100.0% to cefazolin, 83.3% to gentamicin, 77.8% to ciprofloxacin, 75.0% to meropenem, and 72.7% to cefoperazone-sulbactam (Figure 2). Multidrug-resistant strains accounted for 36.0% (9/25), with one highly resistant strain exhibiting resistance to 11 antimicrobial agents. A total of 24 resistance genes were identified, among which the combination of FosA, OXA-50, APH(3')-IIb, and catB7 showed the highest detection rate (8.1%, 3/37).

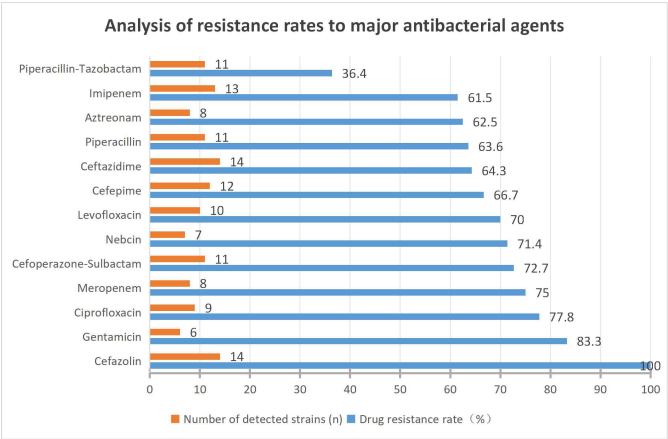


Figure 2. Analysis of resistance rates to major antibacterial agents. The bar chart shows PA resistance rates to common antibiotics: cefazolin 100.0%, gentamicin 83.3%, ciprofloxacin 77.8%, meropenem 75.0%, cefoperazone-sulbactam 72.7%.

Distribution Characteristics of PA Virulence Genes

Among the 52 specimens for which virulence gene testing was completed, the overall carriage rate of virulence genes was 51.9% (27/52); the detection rate of the ExoA+TTSS composite genotype was 50.0% (26/52), the detection rate of TTSS alone was 1.9% (1/52), and no virulence genes were detected in 48.1% (25/52) of the specimens (Figure 3). The detection rates of virulence genes across different specimen types are presented in Table 2.

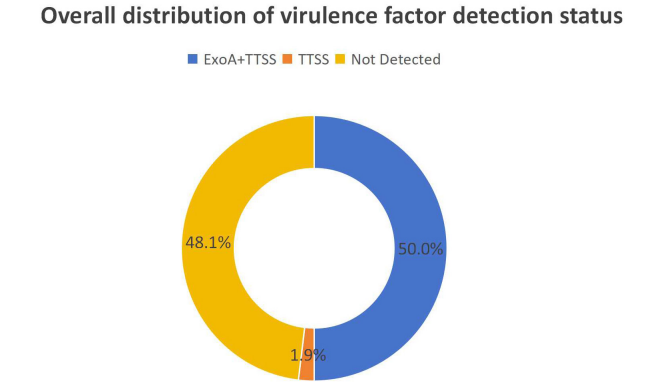


Figure 3. Overall distribution of virulence factor detection status. The chart shows PA virulence gene carriage rate: total 51.9%, ExoA+TTSS 50.0%, TTSS alone 1.9%, no virulence genes 48.1%.

Correlation between virulence genes and drug resistance genes

The detection rate of drug resistance genes in ExoA+TTSS

Table 2. Detection rates of virulence genes in various specimen types.

Specimen Type	Total sample size	Virovirulence gene positive	Virovirulence gene negative	relevance ratio (%)
blood	9	0	9	0.00
central venous catheter	1	0	1	0.00
sputum	5	5	0	100.00
Wound tissue	37	22	15	59.46
Overall	52	27	25	51.92

Note: This table shows the detection rates of virulence genes in 52 valid samples across different specimen types (blood, central venous catheter, sputum, wound tissue), as well as the overall virulence gene detection rate.

co-positive strains was 76.9% (20/26), significantly higher than that in non-virulence gene strains (16.0%, 4/25), with a statistically significant difference ($\chi^2=19.9086$, $P<0.0001$) (Table 3). This study established a virulence factor scoring system to classify samples into three groups: Group 0: no relevant virulence genes detected; Group 1: only the virulence gene TTSS detected; Group 2: both ExoA and TTSS detected. A count-based indicator for the number of drug resistance genes was constructed, measuring the total number of drug resistance gene types carried by each strain. The virulence factor score showed a moderately strong positive correlation with the number of drug resistance genes ($r=0.6457$, $P<0.0001$) (Figure 4). Logistic regression analysis demonstrated that the virulence factor score was an independent risk factor for the presence of PA drug resistance genes ($OR=2.1494-9.7401$, $P<0.0001$).

Table 3. Presence rates of drug resistance genes in strains with different virulence factor types.

Type of virulence factor	Resistant gene-positive	Negative for drug resistance genes	amount to	relevance ratio (%)
ExoA+TTSS complex-positive	20	6	26	76.9
No virulence factors were detected.	4	21	25	16.0
TTSS alone is positive	0	1	1	0.0
amount to	24	28	52	-

Note: This table presents the detection rates of drug resistance genes in 52 *Pseudomonas aeruginosa* strains with distinct virulence factor types, illustrating the relationship between virulence factor profiles and drug resistance gene carriage.

Discussion

Clinical Distribution Characteristics of *Pseudomonas aeruginosa* in Burn Patients

Among the 72 suspected infection specimens in this study, the positive detection rate of *Pseudomonas aeruginosa* was 51.39%, consistent with epidemiological surveillance data

The scatter plot shows a moderately strong positive correlation ($r=0.6457$, $P<0.0001$), regression equation $y=3.14x+0.37$.

Among the 52 specimens with completed virulence gene testing, the overall carriage rate of virulence genes was 51.9%.

In addition, the hypoxic and highly inflammatory microenvironment of burn wounds can induce PA stress response, up-regulate the activity of the QS system, and further amplify the synergistic effect of resistance and virulence, increasing the difficulty of clinical treatment.

Research Limitations and Clinical Implications

This study has certain limitations: First, it is a single-center retrospective study with a relatively small sample size, which may introduce selection bias and limit the generalizability of the findings; second, virulence gene detection only covered *exoA* and *ttss*, excluding adhesion-related (*pilA*, *algD*) and protease-related (*lasB*, *aprA*) virulence genes, potentially overlooking other virulence-resistance regulatory pathways; finally, no *in vitro* functional validation experiments (e.g., gene knockout, transcriptomic analysis) were conducted, making it impossible to directly elucidate the molecular regulatory mechanisms underlying the association between these factors.

Despite the aforementioned limitations, the findings of this study still hold significant clinical value: NGS technology enables rapid and simultaneous analysis of the drug resistance and virulence gene profiles of *Pseudomonas aeruginosa*, providing support for the precise management of antimicrobial therapy in burn patients; the ExoA+TTSS genotype can serve as a molecular biomarker for identifying high-risk multidrug-resistant *Pseudomonas aeruginosa* infections, guiding early clinical intervention and infection control. Clinically, this biomarker can be used for routine screening of PA isolates from burn patients. For ExoA+TTSS-positive strains, clinicians should avoid using highly resistant antibiotics such as cephalosporins, and prioritize selecting sensitive antibiotics combined with anti-virulence adjuvant therapy to optimize individualized treatment plans and reduce the risk of nosocomial infection spread. Future large-scale, multicenter prospective studies combined with functional genomics experiments are required to elucidate the molecular mechanisms underlying the virulence-resistance coupling in *Pseudomonas aeruginosa*, thereby facilitating the development of targeted therapeutic strategies for burn-associated *Pseudomonas aeruginosa* infections.

Conclusion

In burn patients, PA infections are centered around the wound site, with severe bacterial resistance and multidrug resistance phenomena; the PA ExoA+TTSS composite virulence genes exhibit a significant positive correlation with resistance genes, markedly increasing the risk of resistance in highly virulent strains [14]. NGS technology enables simultaneous and precise detection of PA resistance and virulence genes, providing critical molecular evidence for the accurate diagnosis of PA infections in burn patients, personalized selection of antimicrobial agents, and hospital infection prevention and control.

Abbreviations

CARST: National Bacterial Resistance Surveillance Network; CRPA: carbapenem-resistant *Pseudomonas aeruginosa*; ExoA: exotoxin A; NGS: next-generation sequencing; PA: *Pseudomonas aeruginosa*; TTSS: type III secretion system.

Author Contributions

Zijian Zhang: Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Formal anal-

ysis. Delin Hu: Supervision, Writing – review & editing, Project administration.

Acknowledgments

Not applicable.

Funding Information

Not applicable.

Ethical Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data supporting the conclusions of this article are included within the article. The authors may be contacted for additional data related to this paper if required.

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