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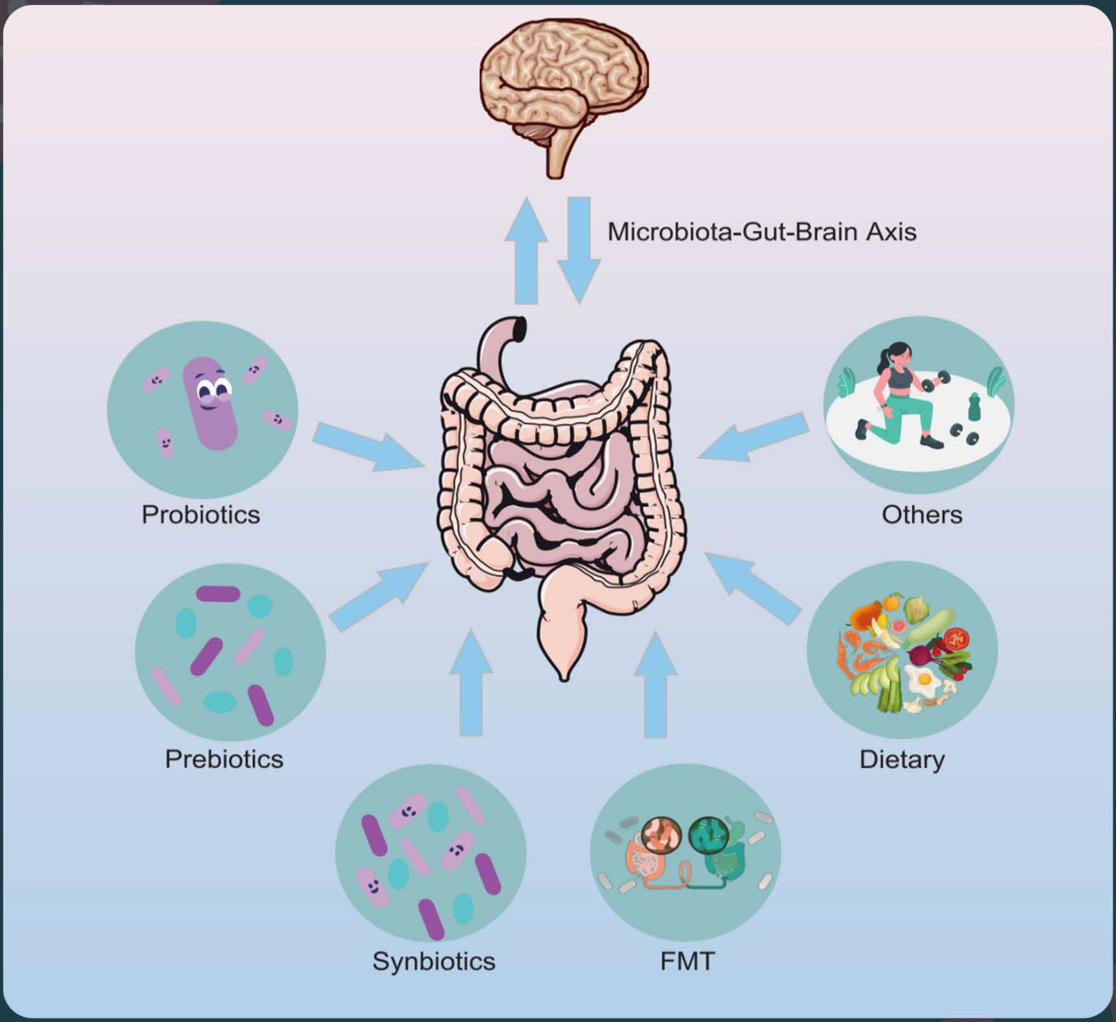

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REVIEW ARTICLE

Potential of medicinal mushroom extracts as adjunctive agents to enhance conversion therapy in colorectal cancer with liver and peritoneal metastases

Agustín Sánchez-Cobos*

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Abstract

Colorectal cancer (CRC) with liver metastases (LM) and peritoneal metastases (PM) presents a formidable challenge with poor prognosis despite advances in systemic chemotherapy and combined surgical approaches, including cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. Median overall survival (OS) for such unresectable cases ranges from 12 to 24 months with modern regimens (e.g., 5-fluorouracil [5-FU], leucovorin, and oxaliplatin/5-FU, leucovorin, and irinotecan + targeted therapies). However, conversion to resectable diseases remains limited by disease extent and treatment toxicity. This paper proposes that medicinal mushroom extracts, particularly polysaccharides (e.g., polysaccharide krestin and polysaccharopeptide) from *Trametes versicolor* and bioactive compounds from species, such as *Ganoderma lucidum*, could enhance conversion therapy outcomes in CRC with LM and PM. Evidence from pre-clinical and clinical studies demonstrates that these extracts exert anticancer effects through multiple mechanisms, including suppression of multidrug resistance, blockade of immune checkpoints (e.g., programmed cell death protein 1/programmed death-ligand 1), regulation of oncogenic signaling pathways (e.g., phosphoinositide 3-kinase/protein kinase B, mitogen-activated protein kinase, and Nuclear factor kappa-light-chain-enhancer of activated B cells), and robust immunomodulation. Meta-analyses of over 8,000 patients reported improved survival and tolerability when combining the extracts with chemotherapy post-surgery. Similarly, meta-analysis in the present study, involving 2,397 patients, reveals a 10% relative increase in the probability of survival at 5 years, with a survival risk ratio of 1.10 (95% confidence interval: 1.04–1.15), based on post-operative data without direct pre-operative validation. Integrating these extracts into neoadjuvant regimens could enhance chemotherapy efficacy, increase surgical eligibility, and mitigate toxicity, potentially extending OS. While direct evidence in unresectable CRC with dual metastases is lacking, the biological plausibility and safety profile of mushroom extracts warrant prospective trials to validate their role in conversion therapy.

Keywords: Colorectal cancer; Liver metastases; Peritoneal metastases; Conversion therapy; Medicinal mushrooms; *Trametes versicolor*; *Ganoderma lucidum*; Immunomodulation

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1. Background and disease context

Colorectal cancer (CRC) is a leading cause of cancer-related mortality, with metastases significantly impacting prognosis. The peritoneum and liver are common sites of metastasis, with peritoneal metastases (PM) occurring in 8–15% of patients during the first treatment cycle[1] and liver metastases (LM) in 15–25% of patients at diagnosis.[2] When both sites are involved, the disease is particularly challenging, traditionally associated with poor outcomes due to its extensive nature.

1.1. Assessment of the efficacy of systemic chemotherapy as a standalone treatment

Systemic chemotherapy, typically administered intravenously, aims to control cancer spread and prolong survival. For patients with peritoneal carcinomatosis, studies reported a median survival of 6–9 months without treatment,[3] extending to 12–24 months with systemic chemotherapy alone, depending on the regimen and patient characteristics.[1,4-7] Earlier studies using 5-fluorouracil (5-FU) and leucovorin showed disappointing results, with survival rarely exceeding 8 months.[3] For patients with both PM and LM, the prognosis is likely worse, with median survival potentially under 12 months at the lower end, though specific data for this subgroup are limited. Modern regimens, such as 5-FU, leucovorin, and oxaliplatin (FOLFOX) and 5-FU, leucovorin, and irinotecan (FOLFIRI), combined with targeted therapies, including bevacizumab and cetuximab, have improved outcomes, with a lower response in PM compared to LM.[8]

A pooled analysis from the Analysis and Research in Cancers of the Digestive CRC database, involving 17,924 patients, showed that compared to patients with LM (including those with additional sites, such as peritoneum) in the chemotherapy alone and chemotherapy + anti-vascular endothelial growth factor (VEGF) subgroups, non-LM patients had better overall survival (OS) and progression-free survival (PFS) within first-line and second-line settings.[9] However, among patients with *RAS* wild-type status in the chemotherapy + anti-epidermal growth factor receptor (EGFR) first-line and second-line subgroups, there was no significant difference in OS and PFS between non-LM and LM patients.

These findings suggest that while chemotherapy can induce responses depending on regimen and patient characteristics, the presence of multiple metastases, especially including LM and PM, reduces long-term survival. New treatment regimens are being developed to address the challenges of advanced, initially unresectable metastatic CRC (mCRC), such as the combination of folinic acid, 5-FU, oxaliplatin, and irinotecan (FOLFOXIRI) with bevacizumab. For example, the TRIBE trial, a large Phase III clinical study involving 508 patients, compared this regimen to FOLFIRI + bevacizumab. The results showed a median PFS of 12.1 months for the FOLFOXIRI group compared to 9.7 months for the FOLFIRI group, while the OS was 31.0 months versus 25.8 months, respectively.[10] The inclusion criteria for this treatment require an Eastern Cooperative Oncology Group (ECOG) performance status score of 2 or lower. Furthermore, the TRIBE trial included only patients aged 18–75 years, with those over 70 needing an ECOG score of 0. Meanwhile, an individual patient data meta-analysis conducted in 2020 with data from five eligible trials, including the TRIBE trial, concluded, with statistically significant results, that FOLFOXIRI + bevacizumab improves OS when compared with doublets + bevacizumab, with a median OS of 28.9 versus 24.5 months, longer PFS of 12.2 versus 9.9 months, and higher R0 resection rate, 16.4% versus 11.8%, respectively.[11] However, a higher burden of grade 3 and 4 adverse events was also observed, with some events being approximately double the rate compared to the control group.

According to the latest guidelines from the American Society of Clinical Oncology for patients with previously untreated, initially unresectable mCRC, doublet chemotherapy should be offered, while triplet therapy may be considered based on studies involving chemotherapy combined with anti-VEGF antibodies.[12] In the first-line setting, pembrolizumab is recommended for patients with mCRC that has microsatellite instability-high (MSI-H) or deficient mismatch repair (dMMR) tumors. For microsatellite stable (MSS) or proficient mismatch repair (pMMR) left-sided *RAS* wild-type mCRC, chemotherapy combined with anti-EGFR therapy is recommended, whereas chemotherapy combined with anti-VEGF therapy is recommended for MSS or pMMR right-sided *RAS* wild-type mCRC. For patients with previously treated *BRAF* V600E-mutant mCRC that has progressed after at least one line of therapy, encorafenib + cetuximab is recommended. In cases of colorectal PM, cytoreductive surgery (CRS) + systemic chemotherapy may be recommended for selected patients, although the addition of oxaliplatin-based hyperthermic intraperitoneal chemotherapy (HIPEC) is not recommended. For patients with liver oligometastases who are not candidates for resection, stereotactic body radiation therapy may be recommended following systemic therapy, while selective internal radiation therapy is not routinely recommended for patients with unilobar or bilobar LM. In addition, perioperative chemotherapy or surgery alone should be offered to patients with mCRC who are candidates for potentially curative resection of LM. The guidelines emphasize the importance of a multidisciplinary team management approach and shared decision-making to optimize patient care.

1.2. Combined treatment approaches and comparative outcomes

Given the limitations of chemotherapy alone, combined treatments have emerged as a more effective strategy. CRS aims to remove visible tumors, while HIPEC delivers heated chemotherapy directly into the abdominal cavity to target residual disease. For patients with both PM and LM studies showed significant survival benefits when CRS, HIPEC, and liver resection or ablation are used. For example, a systematic review and meta-analysis reported a median OS of 26.4 months with a 5-year OS rate of 25% for patients treated with CRS-HIPEC + local liver treatment, compared to 6–13 months with systemic chemotherapy alone, according to their metrics.[13] Another study, focusing on 565 patients, found that pre-operative systemic chemotherapy followed by combined surgery and HIPEC resulted in a 48-month median OS, highlighting the potential of multimodal strategies.[14] A total of 491 patients (91.9%) underwent pre-operative systemic chemotherapy before surgery to target peritoneal and/or LM before CRS with HIPEC and liver resection. The average interval between the diagnosis of LM or PM and the combined surgical intervention was 6 months, highlighting the urgent need for rapid adjunctive therapies to optimize the effectiveness of chemotherapy.

These outcomes contrast sharply with chemotherapy alone, suggesting that the efficiency of systemic chemotherapy is significantly enhanced when integrated into a multidisciplinary strategy. A clear example of this approach has been presented in a systematic review and pooled analysis of 11 studies regarding the potential of FOLFOXIRI + bevacizumab as a conversion therapy.[15] The eligibility for surgery after first-line chemotherapy in patients with unresectable mCRC was 39.1%, with 28.1% of patients achieving R0 resection.

1.3. Patient selection and challenges

Not all patients are candidates for combined treatments due to factors, such as disease extent, performance status, and comorbidities. The peritoneal cancer index (PCI) and completeness of cytoreduction score (CC score), as well as the number of liver lesions, are critical in determining eligibility. A higher PCI (>20), non-achievable complete cytoreduction (CC-0), or near-complete cytoreduction with residual tumor <2.5 mm (CC-1), as well as the presence of more than two or three liver lesions, often contraindicate CRS.[16] In addition, signet ring cell histopathology has also been considered a contraindication.[13] Systemic chemotherapy may serve as a bridge to surgery in some cases, converting unresectable disease into resectable, but its standalone efficiency remains limited for extensive multi-organ involvement. Given that approximately 25% of CRC cases are diagnosed at stage IV at first presentation,[17,18] there is an urgent need for adjunctive therapies that could increase the number of patients eligible for surgery.

1.4. Treatment-specific survival rates with peritoneal involvement

Survival rates vary significantly based on the treatment received, highlighting the importance of patient selection and multidisciplinary management. A 2021 review detailed outcomes of different treatment modalities for CRC patients with PM,[1] as summarized in Table 1. The review highlighted that CRS-HIPEC can significantly extend survival in selected patients, with median OS reaching up to 62.7 months in some case–control studies, compared to 12–24 months with systemic chemotherapy alone. However, the PRODIGE 7 trial in 2021 questioned the added value of oxaliplatin-based HIPEC over CRS alone, showing no significant difference in median OS (41.7 vs. 41.2 months), suggesting the need for a re-evaluation of the additional benefit of HIPEC within present practices and methodologies. In contrast, a Phase III randomized controlled trial (RCT) involving 245 ovarian cancer patients showed a HIPEC-benefited median OS (45.7 vs. 33.9 months); though differences in disease and protocol limit direct comparison.[19,20]

The design of the PRODIGE 7 trial has attracted criticism.[27,28] Methodological flaws included an overly optimistic sample size calculation that anticipated an 18-month survival benefit, a 12% cross-over rate from the CRS-only arm to HIPEC, and the potential for oxaliplatin resistance due to neoadjuvant FOLFOX treatment in 85% of patients. In addition, the post-chemotherapy assessment of the PCI may have misrepresented the initial disease burden. Pharmacologic issues were also noted, including an inadequate dose of 5-FU at only 400 mg/m^2, a limited HIPEC duration of just 30 min, which may have restricted oxaliplatin penetration and led to suboptimal bioavailability, and insufficient hyperthermia effect, even at 43°C.

To address these evidence gaps, the GECOP-MMC trial, a Phase IV, multicenter, randomized, open-label study, was initiated in March 2022.[29] This trial compares CRS-HIPEC with high-dose mitomycin-C (35 mg/m^2) to CRS alone in patients with limited PM from colon (not rectal) cancer, with a focus on preventing peritoneal recurrence. Study completion is estimated for 2029, representing a significant ongoing effort to clarify the efficacy of mitomycin-C HIPEC.

Table 1. Treatment-specific survival rates for peritoneal metastasis

Treatment	OS duration/rate	Study details/notes
Systemic chemotherapy alone	Median OS: 16.3 months	Limited effect in isolated PM, slightly improves OS[6]
Modern systemic chemotherapy	Optimal survival: 15.2 to 23.4 months	Reported for PM of colorectal origin with modern chemotherapy and supportive care[4,6]
CRS+mitomycin C-based HIPEC versus standard treatment	Median OS: 22.3 versus 12.6 months, p=0.032; 5-year OS: 54% for complete resection cases	RCT; 105 patients[21]
CRS+oxaliplatin-based HIPEC versus systemic therapy alone	Median OS: 62.7 versus 23.9 months, p<0.05; 5-year OS: 51% versus 13%	Case–control study post-FOLFOX introduction; 96 patients[22]
CRS+mitomycin C-based HIPEC versus systemic therapy alone	Median OS: 34.7 versus 16.8 months; p<0.001	Case–control study post-FOLFOX; 105 patients (38 in the control group)[23]
Surgery+EPIC (5-FU) versus FOLFOX alone	Median OS: 25 versus 18 months; p=0.04	Single-institution RCT; ended early with 48 eligible patients[24]
CRS+oxaliplatin-based HIPEC versus CRS alone	Median OS: 41.7 versus 41.2 months; p=0.99	PRODIGE 7 RCT; 265 patients; no significant difference[25]
Second-look surgery+prophylactic HIPEC versus standard follow-up	3-year OS: 79% versus 80%; 5-year OS: 68% versus 72%	PROPHYLOCHIP RCT; 150 patients; no significant difference[26]

Abbreviations: CRS: Cytoreductive surgery; EPIC: Early post-operative intraperitoneal chemotherapy; FOLFOX: Folinic acid, 5-fluorouracil, and oxaliplatin; HIPEC: Hyperthermic intraperitoneal chemotherapy; OS: Overall survival; PM: Peritoneal metastasis; RCT: Randomized controlled trial.

2. Anticancer mechanisms of selected medicinal mushrooms

Medicinal mushrooms have garnered attention for their potential anticancer properties, with *Coriolus versicolor* (now classified as *Trametes versicolor*), commonly known as turkey tail, standing out due to its extensive study and clinical use, particularly in Japan and China. Compounds, such as polysaccharide krestin (PSK) and polysaccharopeptide (PSP) from *T. versicolor* have been approved as adjuvant therapies in certain regions, suggesting their potential efficacy in enhancing treatment outcomes. This review explores the anticancer mechanisms of selected mushroom species—*T. versicolor* (turkey tail), *Ganoderma lucidum* (also known as *lingzhi* and *reishi*), *Grifola frondosa* (maitake), *Lentinula edodes* (shiitake), *Agaricus blazei* (sun mushroom), and *Inonotus obliquus* (chaga)—with an emphasis on evidence from *in vitro*, animal, and human studies. While *T. versicolor* is the focal point, findings from other species provide a broader context for mushroom-based therapies. A recent review, published in the *International Journal of Molecular Sciences* in 2022, highlights several anticancer mechanisms, targeting P-glycoprotein (P-gp)-mediated multidrug resistance, immune checkpoints (e.g., programmed cell death protein 1 [PD-1]/programmed death-ligand 1 [PD-L1] and cytotoxic T-lymphocyte-associated protein 4 [CTLA-4]/cluster of differentiation 80 [CD80; also known as B7-1]), and signaling pathways (e.g., phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), Wnt/β-catenin, mitogen-activated protein kinase [MAPK], and nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB]), while bolstering immunomodulation.[30] To clarify their contributions, these mechanisms were organized into subsections that reflect either direct antitumor effects, targeting cancer cell survival and progression, or immune enhancement effects, boosting immune responses, with signaling pathways and immunomodulation integrated across both categories.

2.1. Suppression of multidrug resistance

P-glycoprotein, also known as multidrug resistance protein 1, encoded by the *ABCB1* gene, is an ATP-dependent efflux pump that contributes to multidrug resistance by expelling chemotherapeutic agents from cancer cells, reducing their intracellular concentration and efficacy. Multidrug resistance is a major challenge in cancer treatment, and inhibiting P-gp is a strategy to enhance drug effectiveness. Medicinal mushrooms, known for their bioactive compounds, have been studied for various therapeutic properties, including potential effects on cancer and drug resistance. Some medicinal mushrooms can inhibit P-gp to restore drug sensitivity. For example, extracts of several species of *Ganoderma* have been shown to induce apoptosis in both drug-sensitive (H69) and multidrug-resistant (VPA) human small-cell lung cancer cells, reducing the IC_{50} of etoposide and doxorubicin.[31] In addition, polysaccharides from *G. lucidum* (GL-PS) suppress the persistent activation of NF-κB, leading to a decrease in the expression of P-gp in cancer cells.[30,32] Similarly, two edible mushrooms, *Agaricus bisporus* and *Pleurotus ostreatus*, have been studied for their tryptophan derivatives, specifically 5-hydroxy-*L*-tryptophan (5-HTP) and *L*-tryptophan (*L*-Trp). A 2021 study demonstrated that these compounds inhibit P-gp, with 5-HTP achieving 82% dye accumulation and *L*-Trp 47%.[33] Docking analysis revealed that serotonin, a 5-HTP metabolite, binds to P-gp with a minimum binding energy of −83.93 kcal/mol, while *L*-Trp shows a minimum binding energy of −64.38 kcal/mol. This suggests their potential as natural adjuvants to enhance chemotherapy by overcoming multidrug resistance. However, the study's *in vitro* focus and absence of *in vivo* or clinical cancer data limit immediate applicability, and bioavailability remains untested.

2.2. Blockade of immune checkpoints

Medicinal mushrooms can also enhance immune responses by inhibiting checkpoints that suppress T-cell activity, countering cancer immune evasion. β-glucan, a high-molecular-weight polysaccharide, serves as a significant pathogen-associated molecular pattern present in fungi, bacteria, and several other species,[34] enhancing both innate and adaptive immune responses. When used alongside PD-1/PD-L1 checkpoint-blocking antibodies, whole glucan particle β-glucan improves immune cell recruitment, balances T cell activation and tolerance, and delays tumor progression. This combination therapy has been shown to increase PFS in advanced cancer patients who previously discontinued anti-PD-1/PD-L1 therapy.[35] This finding is particularly relevant to MSI-H or dMMR tumors in CRC, where immunotherapy with agents, such as pembrolizumab—an anti-PD-1 antibody that inhibits the PD-1 receptor on T-cells, thereby enhancing the immune response against cancer cells—has demonstrated significant improvements in PFS compared to chemotherapy (median survival: 16.5 vs. 8.2 months; KEYNOTE-177 trial).[36] In addition, *G. lucidum* and its bioactive compounds can lower PD-1 protein levels in cultured human B-lymphocytes, indicating their potential for cancer prevention and treatment through immune modulation,[37] whereas PSP from *T. versicolor* inhibits CRC cell proliferation. Using human CRC cell lines (HCT116 and HT29), a 2022 study demonstrated that PSP significantly reduces cell growth by downregulating the expression of EGFR and PD-L1, along with key signaling molecules, such as signal transducer and activator of transcription 3 and c-Jun.[38] Moreover, PSP also enhanced T-cell-mediated killing of CRC cells by lowering PD-L1 levels, suggesting its potential as a prophylactic and therapeutic agent against CRC through immune modulation and EGFR pathway inhibition. Meanwhile, extracts from *I. obliquus* have also been shown to block the interactions between CTLA-4 and CD80, increasing T-cell activity to prevent immune evasion.[39]

2.3. Regulation of signaling pathways

Regulation of signaling pathways plays a dual role in cancer treatment. It contributes to direct antitumor effects by inducing apoptosis and inhibiting proliferation through pathways, such as PI3K/AKT, NF-κB, and Wnt/β-catenin. In addition, it enhances immune responses by modulating PD-L1 expression and immune cell infiltration through signaling, such as the MAPK pathway. Numerous signaling pathways are versatile, influencing both cancer and immune cells depending on the context.

Polysaccharides derived from edible and medicinal mushrooms enhance immune function and interact with various cell signaling pathways involved in cancer development, including NF-κB, MAPK, and PI3K/Akt. Given the limitations of present cancer treatments, these polysaccharides present a promising alternative due to their natural origin and low toxicity.[40] The PI3K/AKT pathway promotes cancer cell survival and resistance; hence, its inhibition induces apoptosis and sensitizes cells to therapy. It has been shown that ganoderic acid inactivates PI3K/AKT signaling, inducing apoptosis and autophagy in human glioblastoma cells.[41] Similarly, dysregulated Wnt/β-catenin signaling drives tumor growth and metastasis, and its inhibition reduces proliferation and enhances immune infiltration. Earlier studies demonstrated that *I. obliquus*-derived ergosterol peroxide reduces nuclear β-catenin in CRC cells, decreasing the expression of target genes, including *MYC*, *CCND1*, *CDK8*, and *VEGFA*.[42,43] The MAPK pathway contributes to oncogenesis and resistance to PD-1 blockade; therefore, its suppression enhances chemosensitivity and inhibits tumor growth. Previous research has found that the β-glucan lentinan, derived from *L. edodes*, suppresses cisplatin-induced PD-L1 expression through MAPK inhibition, restoring chemosensitivity in gastric cancer cells.[44] In addition, triterpene extracts from *G. lucidum* inhibit p38 MAPK phosphorylation, inducing autophagy in colon cancer cells.[45] Similarly, small molecules from *T. versicolor* inhibit the production of matrix metalloproteinase 3 (MMP-3), an enzyme that can facilitate tumor invasion and metastasis. This inhibition occurs indirectly by inactivating the p38 MAPK pathway.[46] It has also been reported that NF-κB promotes chemoresistance and tumor survival, and its inhibition induces apoptosis, thereby enhancing therapeutic efficacy. As mentioned earlier, GL-PS inhibits NF-κB, reducing P-gp expression.[32] Moreover, sulfated polysaccharides from *G. frondosa* induce apoptosis in HepG2 liver cancer cells by inhibiting Notch1 expression, preventing IκB-α degradation, blocking NF-κB/p65 translocation to the nucleus, and activating caspases 3 and 8, suggesting a Notch1/NF-κB/p65-mediated apoptotic pathway.[47]

2.4. Immunomodulation

Immunomodulation primarily enhances immune responses by stimulating immune cell activity and cytokine production through pathways, such as toll-like receptor (TLR) signaling and dectin-1. Some immunomodulatory compounds found in medicinal mushrooms directly inhibit cancer cell invasion, for example, by impairing the migratory capabilities of malignant cell lines. In relation to the immunomodulatory effects of medicinal mushrooms, *G. lucidum*-derived β-glucans and triterpenoids protect normal cells, reduce fatigue, and improve symptoms in patients with lung and breast cancer.[48,49] Similarly, extracts from *G. frondosa* have been shown to enhance the activity of natural killer (NK) cells and stimulate the production of various cytokines, including interferon (IFN)-γ and interleukin (IL)-12. A Phase I/II clinical trial involving breast cancer survivors demonstrated that oral administration of *G. frondosa* extract was well tolerated and resulted in significant immunological changes, with an optimal dose of 5–7 mg/kg/day associated with increased production of tumor necrosis factor (TNF)-α and both stimulatory (e.g., IL-2 and IFN-γ) and suppressive (e.g., IL-10) cytokines.[50] These findings suggest that *G. frondosa* extract may modulate immune responses, potentially benefiting cancer patients, although further research is needed to clarify its clinical significance and optimal dosing. Meanwhile, aqueous extracts of *L. edodes* can inhibit the proliferation of human breast cancer cells while enhancing the proliferation of rat thymocytes, indicating its potential as an immunostimulant.[51] Specifically, lentinan enhances cytotoxic T lymphocytes and macrophage proliferation.[52] Polysaccharides from *A. blazei* mitigate chemotherapy side effects in gynecological cancer patients receiving chemotherapy[53] and increase dendritic and T-regulatory cells in myeloma patients.[54]

Extracts from *T. versicolor*, especially PSP and PSK, exhibit significant immunomodulatory effects, enhancing antitumor immunity. PSP induces a pro-inflammatory cytokine profile, including TNF-α, IL-1β, IL-12, and IL-6, via pathways involving TLR2, Dectin-1, and TLR4, as evidenced by pre-clinical studies.[55] Notably, PSP increased TNF-α secretion >3.5-fold in human PBMCs *in vitro*,[56] demonstrating potent immunostimulation. Whereas under *in vivo* conditions, PSP-treated mice with H238-derived tumors showed elevated TNF-α expression in tumor specimens,[57] linking cytokine induction to tumor microenvironments and potential tumor necrosis effects. These findings underscore PSP's capacity to activate NK cells and overcome tolerogenic barriers, supporting its clinical use as an adjunct cancer therapy in Eastern cultures.

In contrast, small molecules derived from *T. versicolor* demonstrate significant immunomodulatory properties by inhibiting the production of TNF-α induced by lipopolysaccharide (LPS) while enhancing the levels of IFN-β induced by polyinosinic: polycytidylic acid in peripheral blood mononuclear adherent cells. These compounds exhibit both indirect anticancer effects by reducing MMP-3 production triggered by TNF-α in glioblastoma T98G cells and direct effects by impairing the invasive capabilities of various malignant cell lines, including T98G, A549, and MDA-MB-231.[46] The disruption of pro-inflammatory tumorigenic factors, as well as the anti-migratory effects of *T. versicolor* extract, have also been demonstrated in a wound healing assay, with a reduced production of IL-6, IL-8, and MMP-9 in cells stimulated by LPS, indicating the microenvironment-dependent modulatory capacity of these active compounds.[58] A small clinical trial involving 21 patients with advanced gastric cancer indicates that PSK can enhance OS, in part by inhibiting CD57$^+$ T cells, which are associated with a poor prognosis in this disease.[59] In another clinical trial, 30 rectal adenocarcinoma patients were randomly assigned to receive either standard chemoradiotherapy (CRT) or CRT with concurrent PSK administration. The findings indicated that the PSK group experienced a notable rise in NK cell counts in peripheral blood and cytotoxic T-cell counts in both peri-tumoral and normal mucosa, accompanied by a reduction in serum levels of immunosuppressive acidic protein. These findings suggest that combining PSK with pre-operative CRT may enhance immune function and reduce local recurrence, leading to improved survival outcomes.[60]

2.5. Induction of cell cycle arrest

Regarding cell cycle arrest, a critical mechanism for controlling cancer cell proliferation, research suggests that *T. versicolor* extracts can induce cell cycle arrest at various phases, impacting cancer cell division. PSP extracted from *T. versicolor* has been shown to induce S-phase cell arrest in HL-60 leukemia cells, enhancing the cell cycle-dependent activity and apoptotic cell death when combined with chemotherapy drugs, such as doxorubicin and etoposide.[61] The Memorial Sloan Kettering Cancer Center reported that *T. versicolor* blend inhibited cell proliferation and induced G2/M cell cycle arrest in an invasive breast cancer cell line, linked to suppression of urokinase plasminogen activator.[62,63] Another study noted that polysaccharides from *T. versicolor* induced cell cycle arrest at concentrations as low as 100 μg/mL *in vitro*, a significant effect given their large molecular weight.[64] These findings indicate that *T. versicolor* extracts potentially affect cell cycle regulatory genes, although the exact mechanisms may vary by cancer type and extract formulation.

Apoptosis induction, or programmed cell death, is a key strategy for eliminating cancer cells. Evidence leans toward *T. versicolor* extracts promoting apoptosis through multiple pathways. One example has already been highlighted in relation to cell cycle arrest, as these two mechanisms are interrelated.[61] Similarly, a commercially available PSP water extract has been shown to modulate apoptogenic and anti-apoptotic protein expression, inducing cell cycle changes and apoptosis in human leukemia HL-60 and U-937 cells.[65]

2.6. Anti-angiogenic properties

Anti-angiogenic effects involve inhibiting new blood vessel formation, which is essential for tumor growth and metastasis. *T. versicolor* extracts can impact angiogenesis by reducing pro-angiogenic factors. An *in vitro* study found that protein-bound polysaccharides from *T. versicolor* decreased the secretion of VEGF and monocyte chemoattractant protein-1 by breast cancer cells, thereby disrupting the tumor microenvironment and potentially inhibiting angiogenesis.[66] Similarly, an *in vivo* study directly showed that fungal PSP inhibited tumor angiogenesis in mice, reducing blood vessel growth and tumor expansion.[67]

2.7. Antimetastatic effects

Antimetastatic effects primarily target cancer cells directly by inhibiting invasion, migration, and angiogenesis, while also enhancing immune responses by activating immune cells. The protein-bound PSK derived from cultured mycelia of *T. versicolor* exhibits significant antimetastatic properties, as explored in a 1995 review by Kobayashi *et al.*[68] PSK, recognized for its significant role in inhibiting the early stages of cancer development, also demonstrates efficacy in impeding the dissemination of established tumors. Research indicates that PSK reduces metastasis in chemically induced sarcomas and the human prostate cancer cell line DU145M, as well as in lymphatic spread observed in murine leukemia P388, while concurrently enhancing survival rates in models of spontaneous metastasis. Furthermore, PSK has been shown to attenuate metastatic progression in controlled experimental settings involving rat hepatoma AH60C, mouse colon cancer (Colon 26), and mouse leukemia (RL male 1). The mechanisms underlying these effects are multifaceted—PSK inhibits tumor cell entry into the bloodstream by limiting invasion and adhesion, disrupts the binding of tumor cells to vascular endothelium by reducing platelet aggregation, restricts the motility of cancer cells post-circulation, and impedes growth at metastatic sites by obstructing angiogenesis, modulating immune signaling, and enhancing the activity of immune cells. In addition, PSK exhibits the capacity to neutralize deleterious superoxide radicals, thereby attenuating tumor aggressiveness in murine models. These properties highlight PSK's broad potential to combat metastasis, enhancing its value in cancer therapy. The review by Kobayashi *et al.*[68] also highlights the results from several murine models of LM, where suppression of liver lesions is observed after the administration of PSK. It also includes findings in mouse colon cancer (Colon 26), where a prolongation of the survival period is noted.[68-70]

Complementing this, a 2014 study examined the effects of aqueous extract from *T. versicolor* on metastatic breast cancer using the 4T1 mouse model.[71] Administering the extract at a daily dose of 1 g/kg for 4 weeks led to a 36% decrease in tumor weight and a 70.8% reduction in lung metastasis. In addition, the extract demonstrated protective effects on bone health, as indicated by increased bone volume observed in micro-computed tomography imaging. *In vitro* experiments revealed that the extract (at concentrations of 1–2 mg/mL) effectively inhibited the migration and invasion of 4T1 cells, significantly reducing MMP-9 activity. Furthermore, it enhanced immune function by increasing the production of cytokines, such as IL-2, IL-6, IL-12, TNF-α, and IFN-γ in spleen lymphocytes. Meanwhile, another study examined the direct effects of PSK on human colorectal adenocarcinoma cells (SW480 and HCT116) using protein microarrays.[72] After 96 h of PSK exposure, 14 proteins showed significant alterations, with ECA39 identified as a key candidate associated with PSK's antimetastatic effects. Together, these findings position *T. versicolor* as a promising agent against metastasis, targeting invasion, migration, angiogenesis, and immune modulation.

2.8. Clinical evidence and broader benefits

In the systematic review and meta-analysis by Zhong *et al.*,[73] the synergistic potential of *T. versicolor* and *G. lucidum* as adjunctive agents in cancer treatment was rigorously evaluated. This study, encompassing 23 RCTs and 4,246 cancer patients, demonstrated that natural products derived from these medicinal mushrooms significantly enhance therapeutic outcomes when combined with conventional cancer treatments. The meta-analysis revealed a reduced mortality risk (hazard ratio [HR]: 0.82; 95% CI: 0.72–0.94) and improved total efficacy (risk ratio [RR]: 1.30; 95% CI: 1.09–1.55) compared to control treatments, alongside immunomodulatory benefits, including elevated CD3 (mean difference [MD]: 9.03%; 95% CI: 2.10–16.50%) and CD4 (MD: 9.2%; 95% CI: 1.01–17.39%) levels. These effects are attributed to the synergistic action of bioactive compounds, such as polysaccharides (e.g., PSP and PSK) from *T. versicolor* and β-glucans and triterpenes from *G. lucidum*, all of which collectively enhance immune response and mitigate immunosuppression associated with chemotherapy and radiotherapy (RT). In addition, this combination therapy improved patients' quality of life with minimal adverse effects, underscoring its safety profile and offering a compelling foundation for integrating medicinal mushrooms into comprehensive cancer care strategies. While these findings positioned *T. versicolor* and *G. lucidum* as promising adjunctive agents across various cancer stages and types, the present study highlights the need for further large-sample-size, high-quality RCTs across different continents.

3. Medicinal mushrooms as adjunctive agents in cancer treatment: a summary

Medicinal mushrooms have emerged as valuable adjunctive agents in cancer management, particularly due to their diverse bioactive compounds. Notably, *T. versicolor* and *G. lucidum* have been extensively studied for their potential to enhance conventional cancer treatments. These fungi exhibit various mechanisms that contribute to their anticancer effects as potent biological response modifiers, as summarized in Table 2.

Certain mushroom extracts have been shown to counteract multidrug resistance by inhibiting P-gp, thereby increasing the effectiveness of chemotherapeutic agents. This property is particularly beneficial in overcoming resistance in cancer cells. The effects are mediated through several mechanisms:

(i) Immune system activation: The ability of these mushrooms to modulate immune responses is significant. They can inhibit immune checkpoint proteins, allowing for a more robust T-cell activity against tumors. This immunological enhancement can complement existing immunotherapies, improving patient outcomes.
(ii) Influence on cellular signaling: Bioactive compounds from medicinal mushrooms interact with critical cellular signaling pathways that regulate cancer cell survival and proliferation. By modulating these pathways, they can induce cell death and enhance the sensitivity of cancer cells to treatment.
(iii) Support for immune function: Extracts from various mushroom species have demonstrated the capacity to boost immune function, leading to increased production of cytokines and improved overall health in cancer patients. This support can alleviate treatment-related side effects and enhance quality of life.
(iv) Cell cycle regulation and apoptosis: Research indicates that certain mushroom extracts can induce cell cycle arrest and promote programmed cell death in cancer cells, further contributing to their therapeutic potential.
(v) Angiogenesis inhibition: By reducing the secretion of pro-angiogenic factors, medicinal mushrooms can disrupt the formation of new blood vessels that tumors rely on for growth and metastasis.
(vi) Antimetastatic effects: The protein-bound polysaccharides PSP and PSK from *T. versicolor* exhibit significant antimetastatic properties, limiting tumor spread and enhancing survival in various cancer models. In addition, extracts from *T. versicolor* have shown potential in reducing tumor weight and metastasis while optimizing immunological health.

Clinical studies have highlighted the synergistic effects of combining these mushroom extracts with standard cancer therapies, leading to improved survival rates and better therapeutic responses. However, further research is essential to establish standardized treatment protocols and confirm the clinical benefits of these natural products. The integration of medicinal mushrooms into cancer treatment regimens offers a promising strategy for enhancing therapeutic efficacy and patient well-being.

4. Review of human studies on medicinal mushroom compounds in cancer patients

A thorough investigation of the available literature has been conducted to assess the safety and efficacy of medicinal mushroom compounds in cancer patients, specifically focusing on *T. versicolor* and CRC. This investigation utilized several regularly updated review summaries on the subject, including those from the Memorial Sloan Kettering Cancer Center,[62] the Whole Health Library website from the United States Department of Veterans Affairs,[74] and the Physician Data Query summary provided by the National Cancer Institute.[75] These review summaries are comprehensive, evidence-based resources that offer information on various aspects of cancer, including prevention, diagnosis, treatment, and supportive care.

4.1. *G. lucidum (reishi), G. frondosa* (maitake) D-fraction, *L. edodes* extract (lentinan), and active hexose correlated compound (AHCC) (derived from various Basidiomycetes)

The use of bioactive compounds derived from *G. lucidum* as adjuncts to chemotherapy has long been suggested.[76] Patients receiving *G. lucidum* extracts in combination with chemotherapy and/or RT were found to have 1.27 times higher likelihood of responding compared to those receiving conventional treatment alone, according to a systematic review by the Cochrane Library, last updated in 2016.[77,78] An improvement in quality of life and a stimulation of host immune-mediated responses were observed, with a marked increase in CD3, CD4, and CD8

Table 2. Medicinal mushrooms and their anticancer mechanisms

Mushroom species	Mechanism of action	Details
Trametes versicolor (turkey tail)	Suppression of multidrug resistance	Small molecules reduce MMP-3 production via p38 MAPK inactivation. Reducing tumor invasiveness may decrease the selective pressure for multidrug resistance development[46]
	Blockade of immune checkpoints	PSP downregulates PD-L1 and EGFR, enhancing T-cell-mediated killing of CRC cells[38]
	Regulation of signaling pathways	Small molecules inhibit p38 MAPK, reducing MMP-3 production[46]
	Immunomodulation	PSP and PSK enhance antitumor immunity, increasing TNF-α, IL-1β, IL-12, and IL-6 via TLR2, Dectin-1, and partially TLR4 pathways.[55-57] Small molecules inhibit LPS-induced TNF-α while enhancing IFN-β.[46] Disruption of pro-inflammatory tumorigenic factors with a reduced production of IL-6, IL-8, and MMP-9 in cells stimulated by LPS, indicating microenvironment-dependent modulatory capacity.[58] PSK enhances NK and cytotoxic T-cell counts, reducing immunosuppressive acidic protein in rectal cancer patients[60]
	Induction of cell cycle arrest	PSP induces S-phase arrest in HL-60 leukemia cells in combination with chemotherapy drugs.[61] Blend induces G2/M arrest in breast cancer cells, suppressing urokinase plasminogen activator.[62,63] Polysaccharides achieve arrest at 100 μg/mL[64]
	Apoptosis induction	PSP modulates apoptogenic/anti-apoptotic proteins, inducing apoptosis in human leukemia HL-60 and U-937 cells[65]
	Anti-angiogenic properties	Protein-bound polysaccharides reduce VEGF and MCP-1 secretion in breast cancer cells.[66] PSP inhibits tumor angiogenesis in mice[67]
	Antimetastatic effects	PSK reduces metastasis in sarcomas, prostate (DU145M), leukemia (P388, RL male 1), hepatoma (AH60C), and colon cancer (colon 26) by limiting invasion, adhesion, motility, and angiogenesis, enhancing immunity, and neutralizing superoxide.[68] Aqueous extract (1 g/kg, 4 weeks) reduces tumor weight by 36% and lung metastasis by 70.8%, while boosting cytokines (IL-2, IL-6, IL-12, TNF-α, and IFN-γ) in spleen lymphocytes; inhibits 4T1 cell migration/invasion (1–2 mg/mL) *in vitro*; and suppresses MMP-9.[71] PSK alters ECA39 in CRC cell lines (SW480, HCT116)[72]
Ganoderma lucidum (*reishi*)	Suppression of multidrug resistance	Induces apoptosis in drug-sensitive (H69) and multidrug-resistant (VPA) small-cell lung cancer cells.[31] Polysaccharide from *G. lucidum* inhibits NF-κB, reducing P-gp expression[32]
	Blockade of immune checkpoints	Lowers PD-1 protein levels in B-lymphocytes[37]
	Regulation of signaling pathways	Ganoderic acid inactivates PI3K/AKT, inducing apoptosis/autophagy in glioblastoma cells.[41] Triterpenes inhibit p38 MAPK phosphorylation, inducing autophagy in colon cancer cells.[45] Polysaccharides inhibit NF-κB, reducing P-gp[32]
	Immunomodulation	β-Glucans and triterpenoids protect normal cells, reduce fatigue, and improve symptoms in lung/breast cancer patients.[48,49]
Grifola frondosa (maitake)	Regulation of signaling pathways	Sulfated polysaccharides inhibit Notch1/NF-κB/p65 pathway, inducing apoptosis in HepG2 cells via caspase 3/8 activation[47]
	Immunomodulation	Enhances NK cell activity and cytokine production (e.g., IFN-γ and IL-12). Phase I/II trial shows increased TNF-α, IL-2, IFN-γ, and IL-10 at 5–7 mg/kg/day in breast cancer survivors[50]
Lentinula edodes (shiitake)	Regulation of signaling pathways	Lentinan suppresses MAPK, reducing cisplatin-induced PD-L1 expression and restoring chemosensitivity in gastric cancer cells[44]
	Immunomodulation	Aqueous extracts inhibit breast cancer cell proliferation and enhance thymocyte proliferation.[51] Lentinan boosts cytotoxic T lymphocytes and macrophage proliferation[52]
Agaricus blazei (sun mushroom)	Suppression of multidrug resistance	Tryptophan derivatives (5-HTP and *L*-tryptophan) inhibit P-gp, with 5-HTP achieving 82% dye accumulation[33]; results from related species *Agaricus bisporus*
	Immunomodulation	Polysaccharides mitigate chemotherapy side effects in gynecological cancers[53] and increase dendritic/T-regulatory cells in myeloma patients[54]
Inonotus obliquus (chaga)	Blockade of immune checkpoints	Blocks CTLA-4/CD80 interaction, increasing T-cell activity[39]
	Regulation of signaling pathways	Ergosterol peroxide reduces Wnt/β-catenin signaling, decreasing *MYC*, *CCND1*, *CDK8*, and *VEGFA* in CRC cells[42,43]

Abbreviations: 5-HTP: 5-Hydroxy-*L*-tryptophan; AKT: Protein kinase B; CD80: Cluster of differentiation 80; CRC: Colorectal cancer; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; EGFR: Epidermal growth factor receptor; IFN: Interferon; IL: Interleukin; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MCP-1: Monocyte chemoattractant protein 1; MMP-3: Matrix metalloproteinase 3; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NK cell: Natural killer cell; P-gp: P-glycoprotein; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; PI3K: Phosphoinositide 3-kinase; PSK: Polysaccharide krestin; PSP: Polysaccharopeptide; TLR: Toll-like receptor; TNF-α: Tumor necrosis factor-alpha; VEGF: Vascular endothelial growth factor.

lymphocytes and a slight increase in NK cells. Notably, a few cases of minor adverse effects, including nausea and insomnia, were reported.

At present, there is an ongoing RCT sponsored by the Second Affiliated Hospital, Zhejiang University,[79] investigating the potential of *G. lucidum* spore powder as an adjunctive agent for osteosarcoma, a cancer with a low 5-year survival rate of <20%. This Phase II trial evaluates the efficacy of *G. lucidum* combined with doxorubicin and cisplatin chemotherapy in a multicenter, double-blind design. Previous research indicates that *G. lucidum* may inhibit tumor growth and metastasis without significant toxicity, supporting its use in combination therapy. For example, Japanese researchers investigated a water-soluble extract (MAK) from the mycelia of *G. lucidum* in patients with colorectal adenomas.[80] The study involved 123 patients receiving 1.5 g/day of MAK for 12 months, compared to 102 control patients who received no treatment. After 12 months, the treatment group showed a significant reduction in the number of adenomas (−0.42 ± 0.10) and a decrease in total adenoma size (−1.40 ± 0.64 mm). In contrast, the control group experienced an increase in both the number of adenomas (0.66 ± 0.10) and size (1.73 ± 0.28 mm) ($p<0.01$). These findings suggest that MAK may help suppress the development of premalignant colorectal adenomas. On the other hand, a *Ganoderma sinense* polysaccharide tablet, from a related species, has been approved in China since 2010 by the State Food and Drug Administration as an adjunctive therapy for addressing leukopenia and hematopoietic damage resulting from concurrent chemotherapy and radiation therapy.[81]

D-fraction from *G. frondosa*, a protein-bound polysaccharide containing β-glucans and protein, has demonstrated an improved response rate to chemotherapy, increasing from 12% to 28% in a Japanese non-randomized clinical study involving 165 patients suffering from various types of advanced cancers.[82,83] In a non-randomized case series, the effectiveness of a combination of *G. frondosa* D-fraction and whole *G. frondosa* powder was evaluated in cancer patients aged 22 to 57 with stages II–IV cancer.[84] The study found that 58.3% of liver cancer patients, 68.8% of breast cancer patients, and 62.5% of lung cancer patients experienced cancer regression or significant symptom improvement. In contrast, leukemia, stomach cancer, and brain cancer patients showed only a 10–20% improvement. In addition, the combination of *G. frondosa* extracts with chemotherapy enhanced immune-competent cell activities by 1.2–1.4 times compared to chemotherapy alone.

Lentinan, a polysaccharide extracted from the *L. edodes* mushroom, is recognized for its antitumor and immunostimulatory properties and is commonly used in cancer treatment. It has been approved as an adjuvant agent for cancer treatment in both China and Japan since the 1980s. An extensive review of over 9,474 patients from 135 independent studies in Chinese databases has summarized lentinan-associated cancer treatment cases over the past 12 years (2004–2016), utilizing data from China National Knowledge Infrastructure (CNKI), Chongqing VIP Chinese Scientific Journals Database (VIP), and Wanfang databases.[85] The results demonstrated clear improvements in quality of life and enhanced effects of chemotherapy and radiation therapy.

AHCC is a functional food supplement developed at the Faculty of Pharmaceutical Sciences, University of Tokyo, by Dr. Toshihiko Okamoto, in collaboration with researchers at Amino Up Chemical Co., Ltd. in Japan.[86] The primary component of AHCC is *L. edodes*, known for its long-standing reputation for healing properties. In addition, it contains various hybrids from the Basidiomycota phylum of fungi. A small randomized controlled study was conducted with 44 patients who had histologically confirmed advanced liver cancer, all receiving supportive care.[87] Among them, 34 patients received AHCC, while 10 received a placebo. The results indicated that patients treated with AHCC experienced significantly prolonged survival compared to the control group. In addition, quality of life improved in areas, such as mental stability and physical health after 3 months of treatment. Key clinical parameters, including albumin levels and lymphocyte percentages, also showed significant differences favoring the AHCC group. The study suggests that AHCC may enhance survival and prognosis in advanced liver cancer patients. Similarly, a pilot study examined the effects of AHCC in 11 patients with advanced cancer, including three each with prostate, breast, or ovarian cancer, and two with multiple myeloma.[88] All patients received conventional therapies alongside 3 g AHCC daily. After 2 weeks, 9 of the 11 patients exhibited a 2.5-fold increase in NK cell activity that sustained over time. In addition, the three prostate cancer patients showed a decline in prostate-specific antigen levels, indicating reduced malignancy. Normal levels were reached in 1–2 months post-treatment. Among the breast cancer patients, two experienced a significant decrease in CA-125 levels, with levels returning to normal 3–4 months after starting AHCC treatment. This study serves as a preliminary exploration of AHCC's potential effects in cancer therapy. Interestingly, encouraging results were obtained from a prospective cohort study of 269 hepatocellular carcinoma patients.[89] Of these, 113 received oral AHCC after curative surgery, showing a significantly prolonged recurrence-free period (HR = 0.639; 95% CI: 0.429–0.952; $p=0.0277$) and improved OS (HR = 0.421; 95% CI: 0.253–0.701; $p=0.0009$) compared to the control group.

4.2. *Coriolus/T. versicolor (turkey tail)*

With a long history in traditional Chinese medicine, *T. versicolor* is a medicinal fungus and is referred to as "*Yun Zhi*" in China. It is recognized for its immune-modulating properties, primarily through PSK and PSP, both of which are polysaccharides extracted from its mycelia. These extracts have been extensively researched for their potential as adjunctive agents in cancer therapies, enhancing the efficacy of conventional treatments, including chemotherapy and RT, while reducing adverse effects. In Asia, particularly Japan and China, these extracts have been integrated into clinical practices, with PSK approved for use in Japan since 1977 and PSP approved in China since 1987—when PSK's annual sales in Japan reached $357 million.[90,91] The PSP utilized in China is primarily derived from the COV-1 strain, while the PSK used in Japan comes from the CM-101 strain of *T. versicolor*. This review notes that PSK and PSP are used as adjuvant agents, with significant research from Asia focusing on colorectal, gastric, esophageal, lung, liver, and various advanced cancers.

A review of pre-clinical and clinical studies conducted in China, extracted from the Chinese VIP, CNKI, and Wanfang databases, concluded that PSP is an effective antitumor agent with minimal side effects, regardless of its administration method.[91] It helps alleviate adverse reactions from chemotherapy, such as bone marrow suppression, and significantly enhances patients' quality of life. A total of seven independent clinical studies were analyzed in this review, with one study from Japan, indicating that PSP has curative effects on various advanced cancers, including CRC, gastric, non-small cell lung, and primary liver cancers. It is commonly used in combination with chemotherapeutic drugs, such as XELOX (oxaliplatin + Xeloda) for advanced CRC, to reduce side effects, improve tolerance to treatment, and potentially prolong life.

Moreover, a systematic review and meta-analysis evaluated the efficacy of *T. versicolor* on the survival of cancer patients.[92] Analyzing data from 13 randomized, placebo-controlled trials, the study found that *T. versicolor* significantly improved survival rates compared to standard anticancer treatments alone, with a 9% absolute reduction in 5-year mortality. The effects on the overall 5-year survival rate were particularly notable in patients with breast cancer, gastric cancer, and CRC undergoing chemotherapy, while not so evident in esophageal and nasopharyngeal cancers. The analysis suggests that *T. versicolor* may enhance survival in certain cancer types, but further prospective studies are needed to optimize treatment protocols.

The 2015 systematic review by Fritz *et al.*[93] highlighted the promising potential of PSK, derived from the *T. versicolor* mushroom, as an adjuvant immunotherapy for lung cancer. Despite its established use in Japan, the review filled a significant gap by synthesizing English-language evidence on PSK's effects. The analysis included 31 reports from 28 studies, comprising six RCTs, five non-RCTs, and 17 pre-clinical studies. The majority of pre-clinical studies (15 out of 17) demonstrated PSK's anticancer effects through immunomodulation and direct tumor inhibition, while non-RCTs showed improvements in various survival metrics. Most RCTs reported beneficial impacts on immune parameters, tumor-related symptoms, and OS. Overall, the findings suggest that PSK may enhance immune function, reduce symptoms associated with tumors, and extend survival in lung cancer patients, underscoring the need for larger, more rigorous trials to further validate these encouraging results.

The need for further assessment and evaluation of relevant clinical data has also been highlighted in a 2022 systematic review published in the Cochrane Library, focusing on the use of PSK in CRC patients.[94] Although the report indicates that there is evidence suggesting a small effect on OS at 5 years, with an absolute risk reduction of 6% (95% CI: 1–11%), the impact of reduced adverse effects on this outcome could not be determined. Unfortunately, imprecision due to small sample sizes and indirectness related to the unknown relevance to present therapy standards downgraded the quality of evidence to "low-certainty," following the guidelines from the Grading of Recommendations Assessment, Development, and Evaluation system.[95]

Previous attempts to address the uncertainty in the field include a 2006 meta-analysis comprising 8,009 gastric cancer patients from eight RCTs, conducted by the Department of Epidemiological and Clinical Research Information Management, Kyoto University Graduate School of Medicine, to assess survival benefits after PSK adjunctive treatment.[96] The overall HR for eligible patients was 0.88 (95% CI: 0.79–0.98; p=0.018), with no significant heterogeneity observed (χ^2 [8] = 11.7; p=0.16). These findings indicate that adjuvant immunochemotherapy using PSK enhances the survival of patients following curative resection for gastric cancer. Another meta-analysis from the same year, with a focus on curatively resected CRC, reassessed the benefits of immunochemotherapy using the biological response modifier PSK.[97] This analysis included data from 1,094 patients enrolled in three clinical trials, all of whom were followed for at least 5 years post-surgery. The study compared outcomes of standard chemotherapy with chemotherapy + PSK, evaluating OS and disease-free survival through intent-to-treat analysis. The results indicated an OS RR of 0.71 (95% CI: 0.55–0.90; p=0.006) and a disease-free survival RR of 0.72 (95% CI:

0.58–0.90; p=0.003). These findings suggest that adjuvant immunochemotherapy with PSK can significantly improve both overall and disease-free survival in patients with curatively resected CRC.

In relation to CRC patients, two observational studies have reported significant results. In the retrospective analysis of 63 patients, 39 received post-operative uracil/tegafur (UFT) alone (control group) and 24 were treated with UFT + PSK (PSK group).[98] The results showed a significant improvement in the 3-year relapse-free survival rate, with 76.2% in the PSK group compared to 47.8% in the control group (p=0.041). In addition, the OS rate was notably higher in the PSK group at 80.8%, versus 52.8% in the control group (p=0.0498). Subset analyses indicated particularly favorable outcomes for patients with colon tumors and those with lower pre-operative lymphocyte percentages. Adverse drug reactions were minimal, with all reported reactions being grade 2 or lower. The second study by Sakai *et al.*[99] evaluated the long-term survival benefits of combining PSK with oral fluoropyrimidines in patients with curatively resected CRC. In this retrospective analysis, the 10-year OS rate for the PSK group was significantly higher at 81.9%, compared to 50.6% in the control group receiving only fluoropyrimidines. Notably, in Dukes' C cases, the PSK group also demonstrated superior survival rates. For patients with lymphatic invasion graded ly2–ly3 or venous invasion graded v2–v3, the PSK group achieved a 10-year survival rate of 80.6%, markedly better than the 25.9% in the control group. Cox's proportional hazard model analysis further confirmed a significant difference in prognosis between the two groups. The findings suggest that post-operative adjuvant immunochemotherapy with PSK significantly enhances long-term survival, particularly for patients with advanced disease features, and the authors recommend its use in these cases.

4.3. Efficacy of adjuvant immunochemotherapy with PSP/PSK: a meta-analysis of selected RCTs

This section highlights the key findings from the meta-analysis conducted in the present study that evaluates the efficacy of PSP and PSK as adjuncts to standard chemotherapy or RT in the post-operative treatment of patients with esophageal, gastric, and colon cancer, focusing on 5-year OS (detailed methodologies and data provided in the Supplementary File Section S1). The analysis initially considered 13 RCTs[59,100-111]; however, only 11 were finally included for evaluation after excluding two studies (Tsang *et al.*,[100] and Miyake *et al.*,[101]) that did not meet the inclusion criteria, as detailed in the "Relevant studies not evaluated in the meta-analysis" section of the supplementary documentation. The evaluated studies, listed in Table 3, span gastric, colorectal, and esophageal cancers, with seven RCTs selected for the final meta-analysis based on their reporting of 5-year OS.

In the meta-analysis, to estimate the pooled relative RR of survival, the following steps were followed[112]:

(i) The RR for each study, defined as the ratio of event probabilities (p_1/p_2), was log-transformed:

$$\ln\left(RR\right)=\ln\left(\frac{p_1}{p_2}\right) \tag{I}$$

(ii) The standard error (SE) of ln(RR) was derived from the 95% CI bounds:

$$\mathrm{SE}=\frac{\ln\left(\text{Upper CI}\right)\text{-}\ln\left(\text{Lower CI}\right)}{2\times 1.96} \tag{II}$$

(iii) The variance was calculated as the square of the SE:

$$\mathrm{Var}((\ln(RR)) = \mathrm{SE}^2 \tag{III}$$

(iv) Each study's weight (w_i) was computed as the inverse of the variance:

$$\mathrm{w_i}=\frac{1}{\mathrm{Var}\left(\ln\left(RR\right)\right)}=\frac{1}{\mathrm{SE}^2} \tag{IV}$$

(v) The pooled estimate was obtained by a weighted average of ln(RR_i) across studies:

$$\ln\left(RR_{\text{pooled}}\right)=\frac{\sum w_i \ln\left(RR_i\right)}{\sum w_i} \tag{V}$$

(vi) Heterogeneity was assessed using:

$$Q=\sum w_i\left(\ln\left(RR_i\right)-\ln\left(RR_{\text{pooled}}\right)\right)^2 \tag{VI}$$

(vii) The between-study variance was estimated, where k is the number of studies:

$$\tau^2=\frac{Q-\left(k-1\right)}{\sum w_i-\frac{\sum w_i^2}{\sum w_i}} \tag{VII}$$

τ^2 is set to 0 if $Q < k - 1$, indicating no additional variance beyond sampling error, with I^2 statistic as:

$$I^2=\frac{Q-\left(k-1\right)}{Q}\times 100\% \tag{VIII}$$

(viii) The SE of the pooled estimate was calculated as:

Table 3. Meta-analysis of selected randomized controlled trials

Study ID	Cancer type (study design)	Endpoint	Number of patients	Risk ratio (95% CI)	Normalized weight (%)
Akagi and Baba[59]	Gastric (CT+PSK vs. CT)	3-year OS	21	4.98 (0.97–25.56)	-
Niimoto *et al.*[102]	Gastric (CT+PSK vs. CT)	5-year OS	579	The risk ratio could not be obtained due to a lack of public access[b].	-
Torisu *et al.*[103a]	Colorectal (PSK vs. PSK-P [placebo])	5-year OS	111	1.47 (0.94–2.31)	1.28
Mitomi *et al.*[104a]	Colorectal (CT+PSK vs. CT)	5-year OS	448	1.12 (1.01–1.26)	21.17
Nakazato *et al.*[105a]	Gastric (CT+PSK vs. CT)	5-year OS	262	1.22 (1.02–1.45)	8.37
Ogoshi *et al.*[106a]	Esophageal (RT+PSK vs. RT)	5-year OS	87	1.06 (0.64–1.75)	1.02
Ogoshi *et al.*[106a]	Esophageal (RT+CT+PSK vs. RT+CT)	5-year OS	87	1.28 (0.70–2.34)	0.71
Toge and Yamaguchi[107a]	Gastric (non-stratified)	5-year OS	751	1.10 (0.99–1.22)	23.73
Toge and Yamaguchi[107a]	Gastric (stratified: granulocyte/lymphocyte≥2.0)	5-year OS	364	1.24 (1.05–1.46)	-
Ito *et al.*[108a]	Colorectal (CT+PSK vs. CT)	5-year OS	446	1.03 (0.95–1.13)	34.40
Ohwada *et al.*[109a]	Colorectal CT+PSK vs. CT)	5-year OS	205	1.13 (0.96–1.34)	9.31
Shichinohe *et al.*[110]	Colorectal (CT+PSK vs. CT)	3-year OS	256	0.98 (0.91–1.06)	-
Ogawa *et al.*[111]	Colorectal (CT+PSK vs. CT)	3-year DFS	186	1.13 (0.91–1.42)	-
Subtotal (pooled)[a]	-	5-year OS	2,397	1.10 (1.04–1.15)	100

Notes: [a]Studies that were included in the meta-analysis. [b]Survival rate significantly higher in the PSK group ($p<0.01$), with 5-year survival significantly increased ($p<0.05$).[102] The hazard ratio for overall survival is reported in the study by Zhong *et al.*[73]

Abbreviations: CT: Chemotherapy; DFS: Disease-free survival; OS: Overall survival; PSK: Polysaccharide krestin; RT: Radiotherapy.

$$\mathrm{SE}_{\ln\left(\mathrm{RR}_{\mathrm{pooled}}\right)} = \sqrt{\frac{1}{\sum w_i}} \qquad \text{(IX)}$$

(ix) The 95% CI for ln (RR_{pooled}) was computed as:

$$\ln\left(RR_{\mathrm{pooled}}\right) \pm 1.96 \times \mathrm{SE}_{\ln(RR\mathrm{pooled})} \qquad \text{(IX)}$$

These bounds were then exponentiated to yield the pooled RR and its 95% CI on the original scale.

By applying this methodology, the meta-analysis yielded a pooled RR of 1.10 (95% CI: 1.04–1.15), indicating a survival benefit for patients receiving PSP/PSK in combination with standard treatment compared to those receiving standard treatment alone. This result is visualized in a forest plot in Figure 1. The weights in the forest plot are allocated based on the inverse of the variance of the log-transformed RR ($1/SE^2$), reflecting each study's precision. Studies with smaller standard errors, such as that by Ito *et al.*[108] with a normalized weight of 34.40%, contribute more significantly to the pooled estimate due to their higher reliability, while those with larger standard errors, such as that by Ogoshi *et al.*[106] with normalized weights of 1.02% and 0.71% for the RT and RT + chemotherapy (CT) arms, respectively, contribute less.

Heterogeneity was assessed using Cochran's Q statistic, which yielded a value of 5.59 with seven degrees of freedom (8 comparisons minus 1), indicating no significant heterogeneity ($p=0.59$). The Q statistic heterogeneity test evaluates the variation in study results that exceeds what would be expected by chance. A non-significant p-value (>0.05) suggests that there is no evidence of heterogeneity, indicating that the effect sizes are consistent across studies. The inclusion of two independent comparisons from Ogoshi *et al.*[106]—RT + PSK versus RT and RT + CT + PSK versus RT + CT—increases the total number of comparisons to eight, justifying the adjustment in degrees of freedom. In addition, the I^2 statistic, at 0%, further reinforces the absence of heterogeneity, as it quantifies the proportion of variation due to between-study differences. A value of 0% implies that all variability is attributable to sampling error. This low heterogeneity enhances the reliability of the pooled RR and supports its generalizability across the included post-operative cancer cohorts. However, the consistency does not preclude potential subgroup-specific effects that could be explored in future analyses. These findings underscore the potential of PSP and PSK as adjuvant agents.

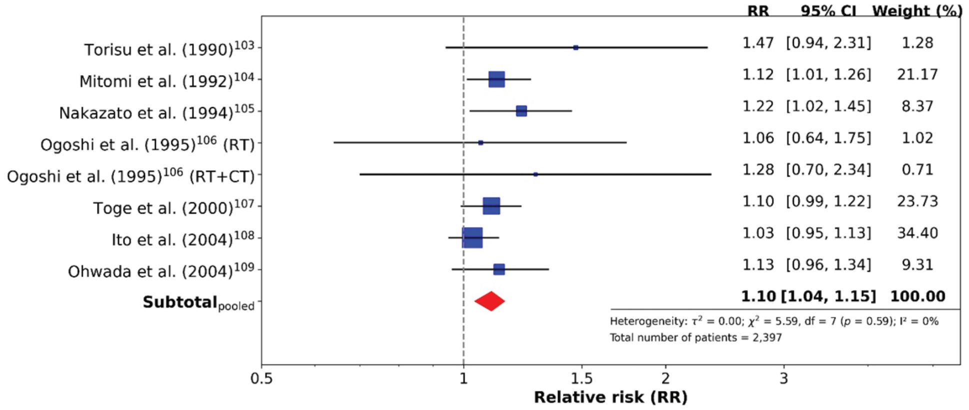

Figure 1. Forest plot of survival relative risk (RR) associated with polysaccharide krestin (PSK)/polysaccharopeptide (PSP). It summarizes data across seven studies, encompassing a total of 2,397 *patient*s. Each blue square represents the RR from an individual study, with the size of the square reflecting the study's weight. Each blue square represents the RR for an individual comparison between interventions in the meta-analysis. The horizontal lines indicate the 95% confidence intervals (CIs). The red diamond represents the pooled RR, which is 1.10 (95% CI: 1.04–1.15). The vertical dashed line at RR = 1 signifies no effect, while RR >1 favors the treatment group (standard treatment + PSP/PSK). Heterogeneity statistics displayed in the plot reveal no significant variability among the studies (p=0.59, I^2 = 0%), indicating a consistent effect of PSP/PSK on survival. Therefore, a random-effects model is unnecessary, as a fixed-effect model adequately represents the data.
Abbreviations: CT: Chemotherapy; RT: Radiotherapy.

5. Extraction methods and composition of medicinal mushroom-derived polysaccharides and other bioactive compounds

Medicinal mushrooms are known to contain a diverse array of bioactive compounds that contribute to their therapeutic potential. Among these, polysaccharides are particularly significant, including α-glucans and β-glucans, specifically β-1,3- and β-1,6-*D*-glucans. Other important polysaccharides include heteroglycans, peptidoglycans (e.g., ganoderan B and C), and polysaccharides (e.g., PSP and PSK). In addition to polysaccharides, terpenes and triterpenoids also play a crucial role in the bioactivity of medicinal mushrooms. Notable examples include ganoderic acids, ganodermic acids, ganodermic alcohols, lucidones, and lucinedic acids in the genus *Ganoderma*. Proteins and peptides are also essential components, with fungal immunomodulatory proteins recognized for their cytotoxic and immunomodulatory effects, and lectins, which specifically bind to mono- and oligosaccharides, being another important class. In addition, peptides, such as *G. lucidum* peptide, along with glycoproteins, such as *G. frondosa* glycoprotein-3a from *G. frondosa*, contribute to the bioactive profile of these mushrooms. Furthermore, phenolic compounds, known for their antioxidant properties, are present in various species of medicinal mushrooms. Fatty acids, including conjugated linoleic acid, further enhance the chemical diversity of these fungi. Steroids and their derivatives, including ergosterol and antcin A, are also noteworthy, while ergothioneine, an amino acid derivative, contributes separately to their bioactive spectrum. Diterpenoids, particularly erinacines A–I, are recognized for their bioactive properties, as are benzyl alcohol derivatives, such as hericenones C–H. Finally, other significant compounds include laccases, which are copper-containing oxidases, as well as nucleosides and nucleotides that can influence platelet aggregation. Flavonoids and the α-glucosidase inhibitor SKG-3 from *G. lucidum* further exemplify the wide range of bioactive compounds found in medicinal mushrooms, highlighting their diverse pharmacological potential.[113,114] The following section explores the main bioactive compounds used in clinical research and evaluates their pharmacological equivalence based on extraction methodologies and standardization techniques, with a primary focus on *T. versicolor*.

5.1. Comprehensive analysis of medicinal compounds in selected mushrooms

This section, based on the most frequently utilized medicinal mushroom species or extracts in human clinical studies, provides a detailed examination of the medicinal compounds found in *G. lucidum*, *G. frondosa* D-fraction, lentinan (*L. edodes* extract), AHCC, and *T. versicolor*. The analysis aims to elucidate the chemical nature of these compounds, offering a thorough resource for understanding their pharmacological relevance.

5.1.1. G. lucidum (reishi)

G. lucidum, a widely studied medicinal mushroom, is known for its diverse bioactive compounds, particularly triterpenoids and polysaccharides. Triterpenoids, such as ganoderic acids (including A, C, F, H, and T), lucidenic acids, and ganodermanondiol, are derivatives of lanosterol, a tetracyclic triterpene, and are noted for their antitumor properties. GL-PS, including β-glucans (specifically β-1,3- and β-1,6-*D*-glucans) and α-1,3-*D*-glucans, as well as peptidoglycans ganoderan B and C, are sugar polymers with glucose as a major component, exhibiting strong immune-strengthening effects. GL-PS can be extracted from *G. lucidum* spores, fruiting bodies, mycelium, and fermentation broth using various methods, including hot water extraction, which is commonly employed to obtain the soluble polysaccharides.[115-118]

5.1.2. G. frondosa (maitake) D-fraction

The protein-bound D-fraction from *G. frondosa* is characterized as an acid-insoluble, alkali-soluble, and hot water-extractable fraction. It contains both a β-1,6-*D*-glucan with β-1,3 branches and a β-1,3 main chain having β-1,6 branches, forming a heavy proteoglycan with a molecular weight of approximately 1,000 kDa. This compound is a β-glucan conjugated with protein, known for its immune-activating properties, particularly in cancer therapy. It is standardized for oral administration and has been studied for its antitumor effects. This compound's safety and efficacy are supported by various studies, emphasizing its role as a biological response modifier.[82,83,119,120]

5.1.3. Lentinan

Lentinan, extracted from *L. edodes*, is a β-1,3-*D*-glucan with two β-1,6-glucopyranoside branches for every five β-1,3-glucopyranoside linkages. It is a polysaccharide with a molecular weight of approximately 500 kDa. It is known for its immunomodulatory and antitumor effects, often used as an adjuvant in cancer therapy, particularly in Japan and China. Its chemical structure is detailed in previous studies,[121,122] which discussed its host-mediated antineoplastic activity through immune stimulation. This compound's ease of availability and low toxicity make it a significant focus in medicinal mushroom research.[85]

5.1.4. AHCC

As mentioned earlier, AHCC is a functional food supplement synthesized at the University of Tokyo by Dr. Toshihiko Okamoto and researchers from Amino Up Chemical Co., Ltd. Its main ingredient is *L. edodes*, recognized for its healing properties, along with various hybrids from the Basidiomycota fungal phylum. AHCC has a composition rich in α-1,4-glucans (20–30%) with partially acylated sugar moieties. The product also includes various polysaccharides, such as β-1,3-glucan, along with trace amounts of protein, amino acids, lipids, and minerals, with a mean molecular weight of approximately 5 kDa. These components work synergistically to enhance immune responses, particularly the activity of NK cells and T cells. Its production involves a patented culturing process.[86,123,124]

5.1.5. T. versicolor (turkey tail)

Turkey tail, scientifically known as *T. versicolor*, previously classified as *C. versicolor*, is rich in polysaccharides, with PSP and PSK being the most commonly known. Both are protein-bound polysaccharides with a molecular weight of approximately 100 kDa. Some reports indicate that PSP has a polysaccharide-to-peptide ratio of 90:10, comprising mannose, xylose, galactose, and fructose, while PSK has a 60:40 ratio, comprising mannose, xylose, galactose, arabinose, and rhamnose; however, these ratios, as well as their monosaccharide compositions, might vary considerably across studies due to differences in extraction methods, fungal strains (e.g., COV-1 for PSP and CM-101 for PSK), and analytical techniques.

These structurally diverse β-glucan-based compounds, with a main primary partial structure as illustrated in Figure 2, are well-known for their immunomodulatory and anticancer properties.[55,64,90,113,125,126] Generally, in a broader polysaccharide fraction beyond PSP/PSK, *C. versicolor* polysaccharides (CVP) have a molecular weight of 500–1000 kDa, with a β-1,4/1,3-*D*-glucan main chain and β-1,6 branches. The monosaccharide composition of CVP predominantly contains glucose in the form of β-*D*-glucopyranose, along with small amounts of mannose, fructose, rhamnose, and fucose. In addition, the strain, processing technology, and extraction method of CVP influence its monosaccharide types, molecular weight, and chemical composition.[127] Further research is needed to investigate the pharmacological activities of small molecular weight compounds.[46,64]

Polysaccharides from *T. versicolor* are commercially extracted from mushrooms or mycelia grown on solid substrates, as well as from mycelial biomass produced through submerged fermentation. The primary clinically approved polysaccharide preparations, PSK and PSP, are derived from mycelial biomass cultivated under submerged conditions, processed into dried powder after hot water extraction.[127,128] The extraction and purification of CVP involve several techniques, each employing distinct chemical and physical principles to isolate these bioactive compounds from raw materials, such as fruiting bodies and mycelium. According to the study by Jing *et al.*,[127] the most commonly used extraction method for CVP is hot water extraction. The optimal extraction conditions are a solid-to-liquid ratio of 1:30 and an extraction temperature of 90°C for 120 min (twice), followed by ethanol precipitation, resulting in a yield of 5.38%. This yield can be increased to up to 16.7% with mechanical crushing and liquid nitrogen for particles under 200 mesh in size. Other methods include enzyme extraction, which is operationally more complex and can achieve yields of 9.58% at 52°C with an enzyme concentration of 2.50%, a pH of 5.5, and an enzymolysis time of 37 min, generally using cellulase, proteases, and pectinase. In addition, ultrasonic extraction can be optimized to achieve extraction rates up to 13.6% at a 1:45 solid-to-liquid ratio, 30 min of extraction time, and an ultrasonic power of 450 W through the response surface method, although high-intensity shock waves may have structural impacts. The purification process for crude CVP includes the removal of proteins and pigments through methods, such as ethanol fractionation, column chromatography, and ion exchange chromatography, resulting in a refined product suitable for further pharmacological use. These processes are commercially utilized in the production of high-quality, standardized extracts. It is important to note that although some of these polymers have distinct structures, they cannot be differentiated based on their physiological activity.[128]

5.2. Reproducibility and consistency of medicinal mushroom extracts for clinical applications

The therapeutic potential of medicinal mushrooms, including *T. versicolor*, *G. lucidum*, *G. frondosa*, and *L. edodes*, depends on the consistent delivery of bioactive compounds, such as polysaccharides and triterpenoids, for clinical applications.[113] Variability in fungal raw materials and extraction processes poses significant challenges to achieving reproducible and reliable extracts. This section examines these challenges and proposes strategies to ensure consistency, balancing scientific rigor with economic feasibility for clinical adoption.

Variability in fungal source material is a primary obstacle to reproducibility. Differences in strains, growth substrates, and environmental conditions, such as temperature or cultivation methods, can significantly alter bioactive compound profiles. Extraction methods, including hot water, ultrasonic, or enzyme-assisted techniques, further contribute to variability by affecting yield and molecular integrity. In addition, inconsistent analytical methods for characterizing compounds can also lead to discrepancies in reported compositions, hindering standardization.[114,117,127,128]

Standardized cultivation practices are critical for addressing these issues. Controlling substrate composition and environmental parameters minimizes variability in chemical profiles. Among the cultivation practices, two common techniques for mycelium cultivation are solid-state fermentation and submerged fermentation. Solid-state fermentation often utilizes substrates, such as sawdust combined with rice bran or wheat bran. On the other hand, submerged fermentation tends to be more efficient, resulting in greater mycelial productivity within a shorter duration, requiring less physical space for production, and enabling improved quality control.[119]

Optimized, uniform extraction protocols ensure consistent yields and bioactivity. Analytical tools, such as high-performance liquid chromatography and mass spectrometry, enhance quality control by enabling precise characterization of bioactive fractions.[127] In applications requiring high consistency, purified compounds offer reliable solutions; however, the associated purification costs may pose challenges to scalability. Economically, while crude extracts are less expensive, they tend to exhibit greater variability and are not suitable for clinical use. Purification costs for these extracts can be substantial, covering raw materials, solvents, energy for extraction, and specialized equipment costs. Furthermore, labor

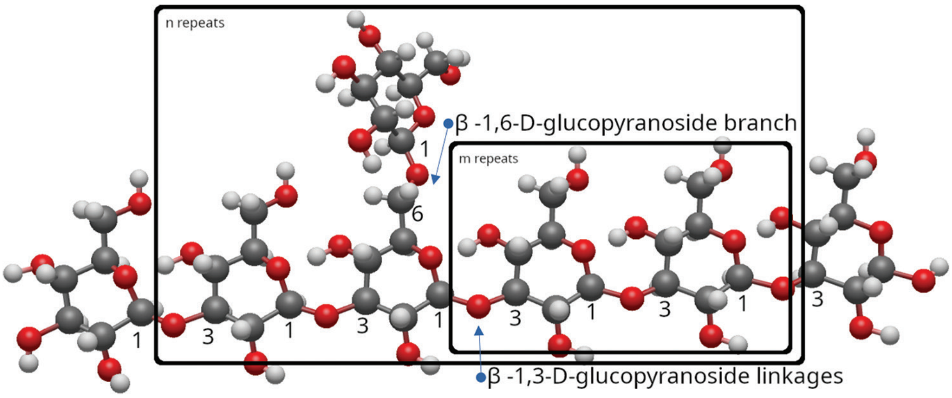

Figure 2. Repeat unit of the partial structure of polysaccharide krestin/polysaccharopeptide, featuring m and n repeats.[55] The β-*D*-glucan molecular structure was generated using the Avogadro software. Carbon atoms in black, oxygen in red, and hydrogen in white.

for production and testing, as well as laboratory analysis for purity and potency, adds to the expense.[129] Costs can be further increased due to adherence to certifications for organic or good manufacturing practices (GMP) standards. While exact figures are difficult to assess due to their proprietary nature and vary by scale and species, these costs are often reflected in the higher price of premium mushroom supplements. Recent biotechnological advances, such as precision fermentation and omics-based strategies,[130] may provide more affordable solutions in the near future, enhancing scalability.

In clinical settings, where predictable therapeutic outcomes are essential, reproducibility is paramount. Standardized extracts, such as *T. versicolor*-derived PSP and PSK, exhibit significant immunomodulatory and anticancer potential. Their integration into medical practices requires robust standardization. Future research should prioritize cost-effective standardization and synthetic analogs to facilitate routine clinical use, unlocking the full therapeutic benefits of medicinal mushrooms. Rigorous quality control, including batch testing for bioactivity and adherence to GMP, is critical for clinical-grade reliability.

6. Surgical eligibility enhancement: a proposal for the use of medicinal mushroom extracts as an adjunctive therapy to optimize pre-operative systemic chemotherapy outcomes

6.1. Proposal

The author posits that medicinal mushroom extracts, which encompass a variety of bioactive compounds, particularly PSP and PSK derived from *T. versicolor*, as well as polysaccharides, alongside small molecules, from species, such as *G. lucidum*, *G. frondosa*, *L. edodes*, *A. blazei*, and *I. obliquus*, may serve as adjunctive agents to enhance pre-operative systemic chemotherapy in patients with initially unresectable mCRC involving LM and PM. This approach aims to increase tumor response rates, improve surgical eligibility (e.g., achieving R0 resection via CRS and liver resection/ablation), and mitigate treatment-related toxicities, ultimately extending OS beyond present benchmarks. The hypothesis leverages the mushroom extracts' multifaceted anticancer properties to optimize conversion therapy outcomes in this challenging dual-metastasis subgroup.

6.2. Clinical context, rationale, and limitations of present therapies

CRC with LM (15–25% at diagnosis) and PM (8–15% during initial treatment) is associated with poor prognosis, with a median OS of 6–9 months untreated and 12–24 months with modern systemic chemotherapy alone (e.g., FOLFOX and FOLFIRI) or targeted therapies. Although aggressive regimens, such as FOLFOXIRI + bevacizumab, improve outcomes, with an OS of 31.0 months and a PFS of 12.1 months as demonstrated in the TRIBE trial involving 508 patients, it is important to note that these results are not limited to patients with LM and PM and only apply to patients who are fit to receive this treatment. Nevertheless, conversion to resectable disease within this cohort occurs in only 39.1% of cases, with an R0 resection rate of 28.1%, underscoring the importance of patient selection in optimizing therapeutic strategies. Combined approaches, such as CRS-HIPEC with liver resection, extend OS to 26–48 months in selected patients, but eligibility is restricted by high PCI (>20), multiple liver lesions (>2–3), and incomplete cytoreduction (CC >1). Therefore, enhancing pre-operative tumor shrinkage is critical to broaden surgical candidacy (refer to Section 1 for citations).

6.3. Medicinal mushroom extracts: mechanisms and evidence

Medicinal mushroom compounds, such as PSP/PSK and small molecules from *T. versicolor*, GL-PS and triterpenoids from *G. lucidum*, β-glucans from *G. frondosa*, lentinan from *L. edodes*, polysaccharides from *A. blazei*, and ergosterol peroxide from *I. obliquus* collectively exhibit potent anticancer effects, as detailed in pre-clinical and clinical studies. Their activity as biological response modifiers can suppress multidrug resistance (e.g., P-gp inhibition), block immune checkpoints (e.g., PD-L1 and CTLA-4), regulate oncogenic pathways (e.g., PI3K/AKT, Wnt/β-catenin, MAPK, and NF-κB), and enhance immunomodulation (e.g., NK cell activation and Th1 cytokine production). Furthermore, they can induce apoptosis, inhibit angiogenesis, and reduce metastasis. Clinical trials and meta-analyses of over 8,000 patients (e.g., Oba *et al.*,[96] 8,009 gastric cancer patients; Zhong *et al.*,[73] 4,246 patients across cancers; and Sakamoto *et al.*,[97] 1,094 CRC patients), have reported improved OS (e.g., the meta-analysis in the present review indicates a 4–15% relative increase in the probability of survival at 5 years) and tolerability when these compounds were used in combination with chemotherapy, primarily in post-operative CRC and other cancers.

6.4. Potential in conversion therapy

While most clinical data on mushroom extracts pertain to post-operative settings, their ability to enhance chemotherapy efficacy suggests a pre-operative role. For example, the immune-enhancing effects and chemosensitizing properties of PSP/PSK could amplify tumor responses to FOLFOXIRI + bevacizumab, potentially increasing the 39.1% surgical eligibility rate observed in systematic reviews. In patients with LM and PM, these extracts might counteract liver immunosuppression (e.g., regulatory T-cells) and peritoneal tumor burden, reducing PCI and lesion counts to surgically manageable levels. Synergy with PD-1 inhibitors further suggests compatibility with modern therapies, such as pembrolizumab in MSI-H mCRC, though this requires further validation.

6.4.1. Safety and practical integration

Mushroom extracts, such as PSP/PSK (1–3 g/day), lentinan, and AHCC, are well-tolerated, with only mild side effects (e.g., nausea) and extremely rare severe events, as evidenced by extensive use in Japan and China. Their compatibility with chemotherapy supports their integration into pre-operative treatment regimens; however, standardization is crucial to ensure consistency. This includes standardizing extraction methods—such as hot water extraction—and the source materials used, including fruiting bodies, mycelium, and submerged fermentation broths, with the latter typically preferred in industrial settings to maximize yield and production. Combining these extracts with present regimens may mitigate toxicities (e.g., grade 3–4 events doubled with FOLFOXIRI), thereby improving patient tolerance, treatment response, and treatment duration.

6.4.2. Implementation and testing

The author argues that a Phase II trial in patients with unresectable mCRC with PM/LM would be highly valuable. Participants should be randomized to receive standard chemotherapy (e.g., FOLFOXIRI + bevacizumab) with or without a mushroom extract cocktail (e.g., PSP/PSK 2–3 g/day or GL-PS 1–2 g/day) for a duration that allows for adequate assessment of treatment response, potentially ranging from 24 to 48 weeks. This proposed Phase II trial aims for a 10–15% increase in the conversion rate to resectable disease (e.g., from 39% to 49–54%), with 80% power and a two-sided alpha of 0.05.

The primary endpoint, conversion rate, is defined by a PCI <20, 2–3 liver lesions, and a CC of 0/1. The baseline conversion rate in the control arm for patients who are fit to undergo a FOLFOXIRI + bevacizumab regimen was 39%, as highlighted in the systematic review and pooled analysis of prior studies, including the TRIBE trial.[15] It is important to consider varying conversion rates for different patient cohorts.

A two-sample proportion test (e.g., Chi-square) will be used to compare conversion rates across arms. The sample size is calculated using the following formula[131]:

$$n = \frac{\left(Z_{1-\alpha/2} + Z_{1-\beta}\right)^2 \cdot \left(p_1\left(1-p_1\right) + p_2\left(1-p_2\right)\right)}{\left(p_1 - p_2\right)^2} \qquad \text{(XI)}$$

Where n is the sample size per arm, $Z_{1\text{-}\alpha/2}$ is 1.96 for a two-sided α of 0.05, $Z_{1\text{-}\beta}$ is 0.84 for 80% power, p_2 represents the control arm conversion rate set at 0.39, and p_1 represents the experimental arm set at 0.49 or 0.54 for a 10% or 15% increase, respectively. For a 10% increase (p_1=0.49), the required sample size is approximately 383 per arm, while it is approximately 170 per arm for a 15% increase (p_1=0.54). To account for a 10% dropout rate, the sample size is inflated by dividing by 0.9. Thus, ultimately, for a 10% increase, the adjusted sample size is 426, while it is 189 for a 15% increase.

A sample size of 200–250 patients per arm is proposed, prioritizing a 15% increase for feasibility in a Phase II setting, while acknowledging that detecting a 10% increase requires a larger sample. The range reflects practical constraints and variability in effect size assumptions. The sample size is powered for the primary endpoint of conversion rate to resectable disease, with overall response rate and PFS as exploratory endpoints. Secondary endpoints include OS, toxicity (e.g., a comparative analysis of grade 3–4 adverse reactions per Common Terminology Criteria for Adverse Events v5.0), and quality of life. If several endpoints are analyzed, adjustments for multiple comparisons (e.g., Bonferroni correction) may be applied to control the overall alpha level.

6.4.3. Predicted outcomes and implications

If effective, this adjunctive therapy could elevate R0 resection rates beyond the 28.1% observed with FOLFOXIRI + bevacizumab, potentially aligning OS with CRS-HIPEC outcomes (26–48 months) or even surpassing them in a broader population. By enhancing tumor shrinkage and reducing immunosuppression, mushroom extracts could redefine conversion therapy, offering a low-toxicity, biologically plausible adjunct to multidisciplinary care.

6.4.4. Limitations and future directions

The absence of direct pre-operative trials for mCRC limits present evidence, requiring extrapolation from post-operative data. Variability in extract composition and the unique challenges of dual metastases (e.g., liver vascularity and peritoneal spread) necessitate tailored studies. Future research should assess the potential of mushroom extracts in combination with targeted therapies (e.g., anti-VEGF, anti-EGFR), immunotherapies (e.g., pembrolizumab in MSI-H cases), or other bioactive compounds (Supplementary File Section S2) to enhance efficacy across mCRC subtypes.

7. Conclusion

This review examines the challenges of managing mCRC with LM and PM, where systemic chemotherapy alone yields a median OS of 12–24 months, and conversion to resectable disease remains limited (e.g., 39.1% in patients eligible for FOLFOXIRI + bevacizumab, not limited to those with LM and PM). Combined approaches, such as CRS-HIPEC extend OS to 26–48 months in selected cases, but eligibility is often constrained by disease extent. The author proposes that medicinal mushroom extracts (e.g., PSP/PSK from *T. versicolor* and polysaccharides from *G. lucidum*) may enhance pre-operative chemotherapy outcomes by increasing tumor response, surgical eligibility, and patient tolerability. This approach is supported by pre-clinical data on multidrug resistance suppression, immune modulation, and pathway regulation, among others, as well as clinical evidence from meta-analyses (>8,000 patients) showing survival benefits. The meta-analysis in this study, presented in Supplementary File Section S1 and involving 2,397 patients, indicates a 10% relative increase in 5-year survival with the application of PSP/PSK compared to standard treatment (RR = 1.10; 95% CI: 1.04–1.15), based on post-operative data. The review proposes a Phase II trial to evaluate this approach. While promising, the lack of direct pre-operative data and variability in metastatic site responses highlight the need for further research to validate and optimize this strategy in mCRC management.

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Conflict of interest

The author declares no conflict of interest.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

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Availability of data

All data supporting the findings of this study's meta-analysis are provided in the Supplementary File.

Further disclosure

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REVIEW ARTICLE

Decoding phosphoinositide signaling in viral pathogenesis and autoimmune disorders

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Abstract

Phosphoinositide (PIP) lipids are master regulators of cellular signaling, membrane dynamics, and trafficking. Dysregulation of PIP signaling contributes critically to autoimmune disorders, where it disrupts immune tolerance and promotes inflammatory tissue damage. Similarly, viruses extensively exploit host PIP signaling pathways for entry, replication, and immune evasion. However, a comprehensive analysis linking PIP dysregulation across specific viral infections to autoimmune mechanisms is lacking. This review bridges this gap by decoding the intricate roles of PIP signaling in both viral pathogenesis and autoimmunity. We detail how eight distinct viral pathogens manipulate PIP networks and explore the resulting implications for autoimmune initiation or exacerbation. We synthesize findings on key PIP species, their effector proteins, and the modulated immune pathways central to both disease contexts. By elucidating these shared and unique mechanisms, this review identifies promising PIP-centric targets for therapeutic intervention in viral diseases and autoimmune disorders.

Keywords: Phosphoinositides; Viral pathogenesis; Autoimmune diseases; Stress signaling

1. Introduction

Phosphoinositides, or phosphatidylinositol phosphates (PIPs), transcend their role as minor membrane phospholipids to function as master regulators of cellular signaling.[1] Derived from phosphatidylinositol (PI) through combinatorial phosphorylation of its inositol ring, the seven PIP species create spatiotemporally defined lipid codes that orchestrate vesicular trafficking, cytoskeletal dynamics, cell survival, and immune responses.[2] These lipids recruit effector proteins through specialized domains to precisely control fundamental processes.[3] Critically, the PIP landscape is dynamically sculpted by kinases, phosphatases, and phospholipases, an enzymatic equilibrium essential for cellular homeostasis.[1,2,4]

The centrality of PIPs in immune regulation, governing antigen recognition, phagocytosis, lymphocyte activation, and cytokine signaling, renders this system vulnerable to pathogenic exploitation.[5-7] Viruses, as obligate intracellular parasites, strategically hijack PIP networks to facilitate entry, construct replication organelles, suppress host defenses, and evade immune surveillance.[8] Simultaneously, dysregulation of specific PIP pathways underpins autoimmune disorders.[7] Aberrant PIP metabolism, whether through genetic mutations, altered enzyme activity, or chronic inflammatory cues, drives loss of self-tolerance by hyperactivating B-cell receptor (BCR) and T-cell receptor (TCR), impairing regulatory T-cell function, and sustaining inflammatory tissue damage.[6,9,10]

This review synthesizes emerging insights into the dual role of PIP signaling at the intersection of viral infection and autoimmune pathogenesis. We explore how viral commandeering of PIP nodes (e.g., PI 3-kinase [PI3K]-protein kinase B [AKT] activation, PI 4-phosphate [PtdIns4P] manipulation) not only enables infection but may inadvertently prime autoimmune pathways. In addition, we examine how autoimmune-associated PIP dysregulation (e.g., phosphatase and tensin homolog [PTEN]/Src Homology 2 domain-containing inositol phosphatase [SHIP] deficiency, PI3K hyperactivity) mirrors viral subversion strategies. By decoding these shared mechanisms, where pathogens exploit and autoimmune disorders dysregulate convergent PIP hubs, we highlight novel therapeutic targets for modulating immune hyperactivity across disease spectra.

1.1. Phosphoinositide signaling

Phosphoinositides, also known as PIPs, are a specialized class of signaling lipids derived from PI (Figure 1).[11] Chemically, PI consists of a diacylglycerol lipid backbone linked to the cyclic polyalcohol myo-inositol through a phosphodiester bond.[1] Although PI represents a small fraction of cellular phospholipids, it serves as the precursor for the PIP family and plays a pivotal role in diverse biological functions.[1,12]

The phosphorylation of hydroxyl groups at the D-3, D-4, and/or D-5 positions of the inositol ring by specific lipid kinases generates seven key PIP species: PI 3-phosphate (PtdIns3P), PtdIns4P, PI 5-phosphate (PtdIns5P), PI 4,5-bisphosphate (PtdIns[4,5]P_2), PI 3,4-bisphosphate (PtdIns[3,4]P_2), PI 3,5-bisphosphate (PtdIns[3,5]P_2), and PI 3,4,5-trisphosphate (PtdIns[3,4,5]P_3).[1,12] This combinatorial phosphorylation pattern generates a unique "molecular barcode" for each PIP species.

Far beyond their roles as membrane components, PIPs form a dynamic and sophisticated signaling network. They define membrane identity and recruit effector proteins through specific lipid-binding domains, such as Pleckstrin Homology (PH),[13,14] Phox Homology,[15] Fab1, YOTB, Vac1, and EEA1 (FYVE),[16] and Epsin N-terminal Homology domains.[17]

Conventionally, PIP signaling has been considered membrane-associated and concentrated at the plasma membrane in response to agonist stimulation.[12] However, accumulating evidence reveals that PIP signaling extends to endomembrane systems, including endosomes, mitochondria, the Golgi apparatus, and the endoplasmic reticulum.[12,18,19] Moreover, PIPs have recently been recognized to function in non-membranous structures, such as the cytoskeleton, nucleoplasm, nuclear speckles, nuclear lipid islets, nucleoli, and chromatin, where they complex with proteins.[4,11,18,20-24]

In these diverse cellular contexts, PIPs act as second messengers, regulating critical processes, such as vesicular trafficking (endocytosis, exocytosis, and autophagy), cytoskeleton dynamics, cell survival, proliferation, migration, and immune cell activation.[2,4,7,10,12,25] One representative pathway is the PI3K-AKT signaling cascade: Upon agonist-stimulation and receptor activation (e.g., growth factor/cytokine receptors), class I PI3Ks phosphorylate PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3.[26,27] This lipid second messenger recruits AKT and phosphoinositide-dependent kinase 1 (PDK1) to the membrane through their PH domains.[26,28] PDK1 then phosphorylates AKT at Thr308, while full activation requires Ser473 phosphorylation of AKT by mammalian target of rapamycin (mTOR) complex 2.[26,29] Activated AKT governs a wide array of cellular processes, including cell motility, survival, proliferation, and metabolism. Key pathways regulated by AKT include Arf-GTPase activating protein with coiled-coil, ankyrin repeat, and PH domain-containing protein 1 (ACAP1)-mediated endosomal trafficking, Forkhead box O (FOXO)-dependent transcriptional programs, mechanistic target of rapamycin complex 1 (mTORC1)-driven metabolic adaptation, and cytoskeletal remodeling through Vimentin and Rac1.[2,26,30] Together, these pathways modulate cellular responses to environmental cues and contribute to immune regulation.

Dysregulation of this PI3K-AKT axis and PIP signaling broadly has been implicated in numerous diseases, including cancer (via hyperactivation), neurodegeneration

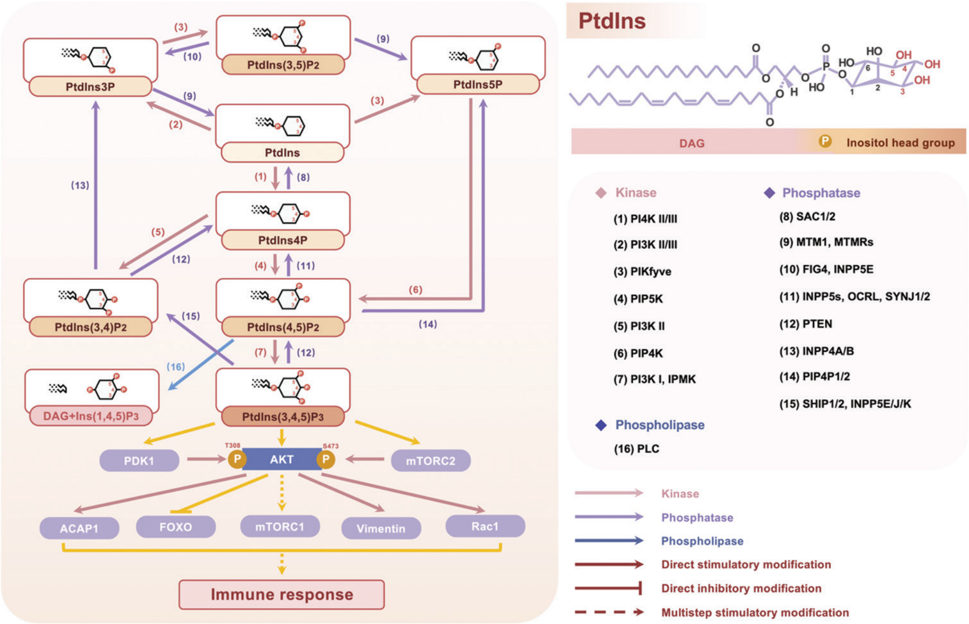

Figure 1. Phosphoinositide modification and regulation of the PI3K-AKT signaling axis. Reversible phosphorylation at the three hydroxyl positions of the inositol ring generates seven spatially distinct PIP species, defined by their phosphate group number and distribution. A set of tightly regulated enzymes, including lipid kinases, such as PI3K, phosphatases, and phospholipases, maintains the dynamic balance of this system. Among these, PtdIns(3,4,5)P_3 acts as a pivotal signaling lipid, triggering downstream cascades by recruiting proteins, including AKT. AKT activation requires sequential phosphorylation by PDK1 and mTORC2 in a PtdIns(3,4,5)P_3-dependent manner. Once fully activated, AKT regulates a broad spectrum of cellular processes, including ACAP1-mediated endosomal trafficking, FOXO-dependent transcriptional programs, mTORC1-driven metabolic adaptation, and cytoskeletal reorganization via Vimentin and Rac1. Together, these pathways modulate cellular responses to environmental cues and contribute to immune regulation. Image created by authors using Microsoft PowerPoint Software Version 16.66.

Abbreviations: ACAP1: Arf-GAP with coiled-coil, ankyrin repeat, and PH domain-containing protein 1; AKT: Protein kinase B; DAG: Diacylglycerol; FIG4: Factor-induced gene 4; FOXO: Forkhead box O; INPP: Inositol polyphosphate; mTOR: Mammalian target of rapamycin; MTM1: Myotubularin 1; MTMRs: Myotubularin-related proteins; OCRL: Oculocerebrorenal syndrome protein of Lowe; PDK1: 3-phosphoinositide-dependent protein kinase-1; PI3K: Phosphatidylinositol 3-kinase; PI4K: Phosphatidylinositol 4-kinase; PIKFYVE: Phosphoinositide kinase, Fab1, YOTB, Vac1, and EEA1; PIPK: Phosphatidylinositol phosphate kinase; PtdIns(3,4,5)P_3: phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P_3); PtdIns(4,5)P_2: Phosphatidylinositol 4,5-bisphosphate; PtdIns3P: Phosphatidylinositol 3-phosphate; PtdIns4P: Phosphatidylinositol 4-phosphate; PtdIns5P: Phosphatidylinositol 5-phosphate; PTEN: Phosphatase and tensin homolog; SHIP: Src Homology 2 domain-containing inositol phosphatase; SYNJ: Synaptojanin.

(altered synaptic PIP dynamics), chronic inflammation, and autoimmune disorders, highlighting its potential as a therapeutic target.[6,7,31-34]

1.2. Autoimmune diseases

Autoimmune diseases encompass a diverse group of disorders caused by dysregulated immune responses against self-tissues (Figure 2).[35] This response stems from a failure of immune tolerance, leading to recognition of self-antigens as pathogenic and subsequent immune activation, resulting in sustained inflammation, autoantibody production, and progressive tissue damage.[36] More than 80 distinct autoimmune diseases are currently recognized,[36] affecting approximately 8.5% of the global population based on epidemiological studies and contributing significantly to disability and disease burden, including both systemic disorders (e.g., systemic lupus erythematosus [SLE], Sjögren's syndrome [SS]) and organ-specific diseases (e.g., type 1 diabetes [T1D], multiple sclerosis [MS]).[37]

The pathogenesis of autoimmunity involves a complex interplay of genetic predisposition, environmental triggers, and immune dysregulation.[38] Genetic factors include polymorphisms in *HLA* genes and immune regulators, such as protein tyrosine phosphatase non-receptor type 22 (affecting lymphocyte signaling), cytotoxic T-lymphocyte-associated protein 4 (a checkpoint inhibitor), and signal transducer and activator of transcription 4 (promoting pro-inflammatory responses).[39-41] Environmental triggers include infections (e.g., Epstein–Barr virus [EBV]), hormonal changes (e.g., estrogen fluctuations), smoking, vitamin D deficiency, and psychological stress, which may dysregulate immune responses and exacerbate disease susceptibility.[42] These factors converge to disrupt immune tolerance mechanisms, including central tolerance in the thymus and bone marrow and peripheral tolerance mediated by regulatory T cells (Tregs).[43] Aberrant activation of immune cells, driven by dysregulated signaling pathways, culminates in

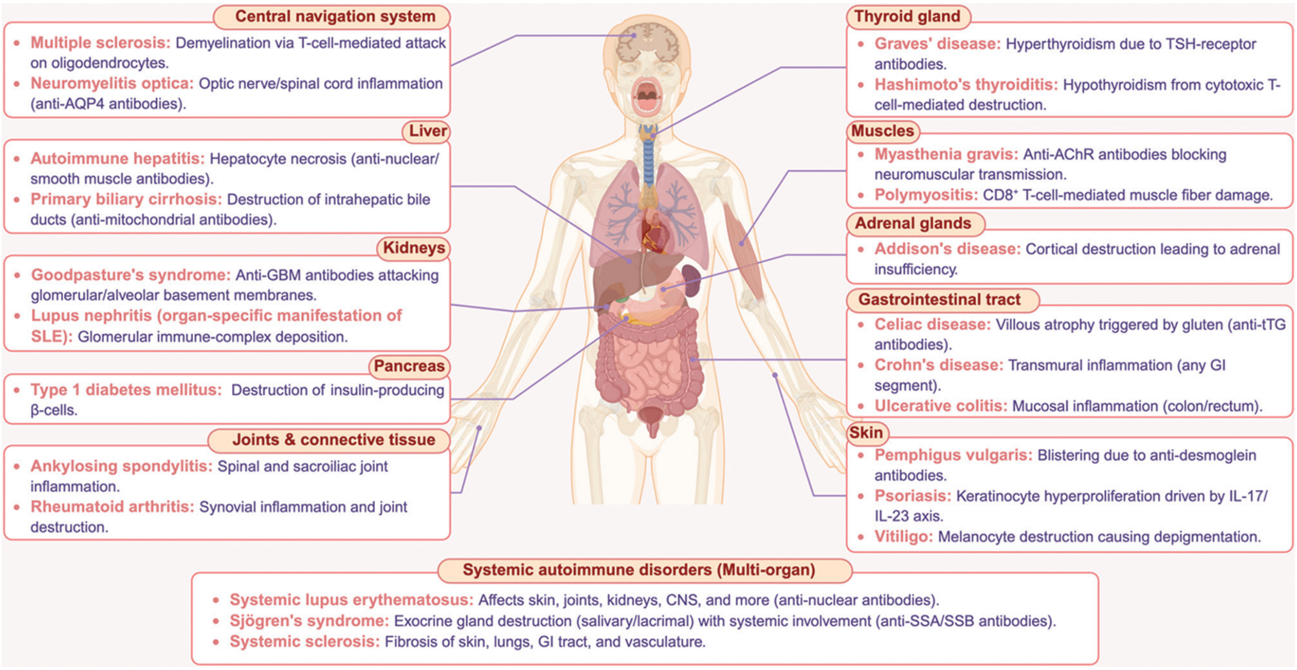

Figure 2. Major organs and associated autoimmune diseases. The schematic diagram outlines the human body's main organs and their related autoimmune diseases. Autoimmune diseases are diseases in which the immune system mistakenly attacks its own tissues. It can be divided into two categories based on the organs and scope involved: organ-specific and systemic. Created with BioRender. Chen, M. (2025) https://BioRender.com/0ssapnq.

autoantibody production, immune complex deposition, and tissue destruction.[44]

Phosphoinositide signaling plays a pivotal role in immune cell activation and tolerance.[7] Dysregulation of PIP metabolism, through altered activity of PI3Ks, PTEN, SHIP, and phospholipases, impairs key processes, such as BCR and TCR signaling, cytokine production, Treg function, and phagocytosis.[45] The hyperactivation of pathways, such as PI3K-AKT-mTOR, is frequently observed in autoimmune diseases,[46-48] promoting lymphocyte hyperresponsiveness, impaired Treg function, and inflammatory tissue damage.[49] Understanding these PIP-mediated dysfunctions is critical for unraveling autoimmune pathology and identifying therapeutic targets.

1.3. Viral infections and autoimmunity

The intricate relationship between viral infections and the development or exacerbation of autoimmune disorders represents a significant area of immunological research. While a robust and swift immune response during viral infection is essential for antigen clearance, immune regulatory mechanisms can sometimes fail, leading to the breakdown of self-tolerance and the development of autoimmune diseases. Present research indicates that viral infections can induce autoimmune diseases through various mechanisms, among which molecular mimicry, bystander activation, and epitope spreading are the most well-documented.[50]

Molecular mimicry serves as a classic paradigm, where structural or sequential homology between viral antigens and host self-antigens deceives the immune system, leading to cross-reactive T-cell activation or autoantibody production.[51] Large-scale virome analyses reveal that this phenomenon is particularly prevalent in the Herpesviridae and Poxviridae families, where short linear mimics frequently target host proteins involved in cellular replication and inflammatory pathways.[52] A well-documented example is the homology between the EBV nuclear antigen-1 and several lupus-associated autoantigens, such as SS-related antigen A and Smith antigen, contributing to SLE pathogenesis.[53] Similarly, cross-reactivity between Coxsackievirus B4 (an enterovirus) peptides and the glutamate decarboxylase 65 antigen in pancreatic islet cells is implicated in the initiation of T1D.[54]

Bystander activation provides another critical pathway. During an acute viral infection, intense local inflammation releases sequestered self-antigens and a milieu of pro-inflammatory cytokines (e.g., interleukin [IL]-1, IL-6, IL-8, and tumor necrosis factor [TNF]-α).[55-57] This "inflammatory storm" can non-specifically activate nearby autoreactive T lymphocytes that were previously ignorant or anergic, even in the absence of direct antigenic recognition of the virus itself.[55,58] The sheer magnitude of inflammation lowers the threshold for activation of these autoreactive cells.[59] This mechanism is particularly relevant in tissues targeted by viruses, as demonstrated in the rat insulin promoter-glycoprotein transgenic mouse model of T1D, where lymphocytic choriomeningitis virus infection triggers both virus-specific and bystander activation of autoreactive CD8$^+$ T cells through inflammatory cytokines, such as IL-21 and IL-15, leading to destruction of pancreatic β-cells.[60]

Epitope spreading further amplifies the autoimmune response over time, especially during persistent infections. As an initial autoimmune response (potentially triggered by mimicry or bystander effects) causes tissue damage, new self-antigens are released and presented to the immune system. This leads to the activation of lymphocytes specific for these newly exposed epitopes, broadening the autoimmune attack beyond the initial target.[50,55,61] A prime example of this phenomenon is observed in Theiler's murine encephalomyelitis virus (TMEV) infection, where the virus persistently infects glial cells, including astrocytes, microglia, and oligodendrocytes.[62] In this model, the chronic infection drives an initial CD4$^+$ T cell response against the dominant myelin proteolipid protein (PLP) epitope 139–151, which then progressively expands to target the less immunogenic PLP 178–191 epitope through epitope spreading.[63-65] This intramolecular epitope spreading exemplifies how persistent viral infections can drive the diversification of autoimmune responses, ultimately leading to more widespread tissue damage and disease progression. Epidemiological and clinical evidence robustly links specific viruses to distinct autoimmune conditions.

Beyond the well-established link of EBV with MS[66-69] and its debated association with SLE,[70-74] and the connection between enteroviruses (e.g., Coxsackievirus B [CVB] and T1D,[75,76] potentially via molecular mimicry), other notable viral-autoimmune associations include: Human cytomegalovirus (HCMV), implicated in systemic sclerosis (SSc)[68,70] and proposed as a T1D risk factor;[69] hepatitis C virus (HCV), the primary cause of mixed cryoglobulinemia (MC);[77,78] human herpesvirus 6 (HHV-6), detected in MS plaques[79] but less consistently associated than EBV; congenital rubella virus (RuV) infection is a recognized T1D risk factor;[80,81] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), temporally linked to autoimmune sequelae in long coronavirus disease 2019 (COVID-2019) (e.g., Guillain-Barré syndrome [GBS],[82] antiphospholipid syndrome [APS],[83] and immune thrombocytopenia [ITP][83]) through mimicry or immune dysregulation. TMEV, though not a human pathogen, is a key model for MS-like demyelination.[84,85] Investigating how these viruses manipulate host PIP signaling (e.g., PI3K-AKT-mTOR) to disrupt immune tolerance forms a core focus of this review.

2. Viral infection triggered phosphoinositide signaling in autoimmunity

This intricate relationship between viral infections, PIP signaling, and autoimmunity underscores the critical role of PIP pathways in bridging infection-induced immune dysregulation and autoimmune disease progression (Table 1). To elucidate the intricate relationship between viral infections and PIP signaling dysregulation in autoimmunity, we focus on eight representative viruses—CVB, EBV, HCMV, HCV, HHV-6, RuV, SARS-CoV-2, and TMEV. These pathogens were highlighted based on three key criteria. First, they span five distinct viral families (*Picornaviridae*, *Herpesviridae*, *Flaviviridae*, *Togaviridae*, and *Coronaviridae*), enabling broad mechanistic comparisons across evolutionary lineages. Second, each virus exhibits well-documented associations with autoimmune disorders and demonstrates specific strategies to hijack host PIP signaling pathways, providing a robust framework to explore shared and unique mechanisms. Third, while most are highly prevalent in humans (e.g., EBV infects >90% of adults), TMEV—though non-human-infecting—is included due to its established murine model for studying MS, offering critical insights into PIP-mediated demyelination. By analyzing how these viruses distinctly perturb PIP networks and downstream immune responses, we aim to decode their contributions to autoimmune pathogenesis.

2.1. Coxsackievirus B

Coxsackieviruses, members of the *Enterovirus* genus within the *Picornaviridae* family, are small RNA viruses characterized by a positive-sense, single-stranded genome.[134] They are categorized into two groups—Coxsackievirus A and CVB—each linked to a variety of diseases[135], including hand, foot, and mouth disease, T1D, acute myocarditis, and autoimmune myocarditis.[75,76,86,87,136-138] Among the six known serotypes of CVB (CVB1–CVB6), CVB1, CVB3, and CVB5 have been particularly associated with viral myocarditis.[139] Myocarditis, which involves inflammation of the heart muscle, can be triggered by a range of causes, both infectious and non-infectious, with viruses being the most frequently identified agents.[140,141] Clinical studies have reported that nearly half of patients with dilated cardiomyopathy (DCM) test positive for CVB-specific antibodies, implying a potential autoimmune component involving CVB and host cardiac antigens in the onset of myocarditis and DCM.[142-145]

Table 1. Viral pathogens' relative autoimmune diseases and impact on phosphoinositide signaling.

Viral pathogen	Relative autoimmune diseases	Effects on phosphoinositide signaling
CVB	AM,[86,87] T1D[75,76]	CVB's replication relies on the host PI4K/PtdIns4P,[88] and its infection activates the PI3K-AKT-mTOR signaling. [89]
EBV	APS,[90,91] MS,[66-69] RA,[92-94] SLE,[70-74] SS,[95,96] SSc[97]	EBV infection triggers the activation of PI4K, with the latent protein EBNA1 further enhancing PI4K and PIPK activities, resulting in elevated levels of PtdIns4P and PtdIns(4,5)P_2.[98] The EBV latent proteins LMP1 and LMP2A stimulate the PI3K-AKT signaling pathway and promote IRF4 activity. [99,100] LMP1 also suppresses PTEN expression by upregulating miR-21, thereby intensifying PI3K-AKT signaling. [101] Similarly, EBV-miRNA-BART7-3P supports PI3K-AKT activation by inhibiting PTEN.[99] Moreover, the EBV envelope protein gp350 binds to the CR2/CD21 receptor, further activating PI3K.[102,103]
HCMV	RA,[104-106] SLE,[107-109] SSc, [104,110,111] T1D[104,112]	HCMV infection upregulates the PI3K-AKT pathway, and inhibiting PI3K activity effectively suppresses its viral replication and downstream signaling. [113]
HCV	AITD,[114] MC[77,78]	The activation of PI3K-AKT signaling helps the virus enter cells, [115] possibly by altering cell polarity. [116] HCV's NS5A protein activates PI4KIIIα, creating PtdIns4P-rich membranes that attract OSBP lipid transporters, forming ideal replication platforms. [117] This process is enhanced when NS5A's BAAPP domain binds to PtdIns(4,5) P_2, strengthening interaction with the host factor TBC1D20.[118]
HHV-6	AITD,[119] IBD,[120] MS,[79] SSc, [121] T1D[122]	Inflammatory factors, such as TNF-α and IL-1 β in HHV-6-positive MS patients activate the downstream NF-κB signaling pathway, [123,124] which may further regulate the cellular inflammatory response through the PI3K-AKT pathway.
RuV	T1D[80,81]	RuV infection activates PI3K-AKT signaling, which is required for cell survival and virus production. [125]
SARS-CoV-2	APS,[83] AT,[126] GBS,[82] ITP[83]	The activation of the PI3K-AKT-mTOR signaling pathway promotes the replication of SARS-CoV-2, inhibits cell apoptosis, and increases cytokine release. [127,128] The viral ORF3a protein upregulates miR-155 to suppress SHIP1, and enhances the production of PtdIns(3,4,5)P_3, which activates AKT and NF-κB, leading to the amplification of pro-inflammatory cytokines. [129-131] Inhibiting PIKFYVE effectively blocks the fusion of the virus with endosomal membranes, preventing the release of the virus into the cytoplasm and thus inhibiting virus infection. [132]
TMEV	MS[84,85]	PI3K-AKT signaling participates in the progression of clinical symptoms in the TEMV model of MS.[133]

Abbreviations: AITD: Autoimmune thyroid diseases; AKT: Protein kinase B; AM: Autoimmune myocarditis; APS: Antiphospholipid syndrome; AT: Autoimmune thyroiditis; BAAPP: Basic amino acid PtdIns(4,5)P_2 pincer; CR2: Complement receptor type 2; CVB: Coxsackievirus B; EBNA1: Epstein–Barr virus nuclear antigen 1; EBV: Epstein–Barr virus; GBS: Guillain-Barré syndrome; HCMV: Human cytomegalovirus; HCV: Hepatitis C virus; HHV-6: Human herpesvirus 6; IBD: Inflammatory bowel disease; IL: Interleukin; IRF4: Interferon regulatory factor 4; ITP: Immune thrombocytopenia; LMP: Latent membrane protein; MC: Mixed cryoglobulinemia; MS: Multiple sclerosis; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NS5A: Non-structural protein 5A; ORF3a: Open reading frame 3a; OSBP: Oxysterol-binding protein; PI3K: Phosphatidylinositol 3-kinase; PI4K: Phosphatidylinositol 4-kinase; PIKFYVE: Phosphoinositide kinase, Fab1, YOTB, Vac1, and EEA1; PIPK: Phosphatidylinositol phosphate kinase; PtdIns(3,4,5)P_3: phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P_2: Phosphatidylinositol 4,5-bisphosphate; PtdIns4P: Phosphatidylinositol 4-phosphate; PTEN: Phosphatase and tensin homolog; RA: Rheumatoid arthritis; RuV: Rubella virus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SHIP: Src Homology 2 domain-containing inositol phosphatase; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; SSc: Systemic sclerosis; T1D: Type 1 diabetes; TBC1D20: TBC1 domain family member 20; TMEV: Theiler's murine encephalomyelitis virus; TNF: Tumor necrosis factor.

Coxsackievirus B replication is known to depend on host phosphatidylinositol 4-kinase (PI4K) activity and its product PtdIns4P.[88] Besides, CVB3 infection exhibits a sophisticated temporal regulation of the PI3K-AKT-mTOR signaling axis, demonstrating upstream PI3K-AKT activation coupled with downstream mTOR suppression. This strategic division facilitates viral replication through stage-specific modulation of host cell processes. Following cellular entry and initial replication steps, CVB3 activates AKT in a PI3K-dependent manner,[146] creating a biphasic apoptotic response that initially promotes cell death to facilitate viral release before transitioning to anti-apoptotic effects that preserve the cellular environment for sustained viral production.[146,147] Pharmacological inhibition of PI3K significantly reduces viral protein 1 (a major capsid protein that plays a crucial role in the virus's structure, antigenicity, and pathogenesis) expression while enhancing autophagy and suppressing viral replication, highlighting the critical role of this pathway in CVB3 life cycle completion.[89] Paradoxically, despite PI3K-AKT activation, CVB3 infection simultaneously suppresses mTOR activity, resulting in markedly elevated autophagy levels that paradoxically enhance viral replication, suggesting the virus has evolved mechanisms to uncouple these normally coordinated pathways.[89] Complementary studies with curcumin demonstrate additional complexity, as its antiviral effects against CVB3 appear mediated through PI3K-AKT-dependent nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, which amplifies pro-inflammatory cytokine production (TNF-α, IL-6, IL-1β) and potentiates host immune responses against the infection.[148]

This multilayered manipulation of PI3K-AKT-mTOR signaling underscores CVB3's remarkable capacity to differentially exploit various nodes within a single signaling cascade to optimize its replication strategy while modulating host defense mechanisms. Nevertheless, targeting the PIP signaling network presents a promising avenue for therapeutic intervention in CVB-related autoimmune diseases. Continued investigation is warranted to unravel this complex relationship and identify actionable targets within the pathway.

2.2. Epstein–Barr virus

Epstein–Barr virus, the first recognized human tumor virus,[149] is widespread globally, persisting as a lifelong infection in over 90% of adults.[68,150] While typically latent, EBV can act as a conditional pathogen when host-virus balance is disrupted,[151] and it is strongly associated with various human cancers.[152] These include epithelial-derived carcinomas, such as gastric[153] and nasopharyngeal carcinoma,[154] as well as B and T cell-derived lymphomas,[99,155-158] including Hodgkin lymphoma,[159] Burkitt lymphoma,[160] diffuse large B cell lymphoma,[161] plasmablastic lymphoma,[156,162] primary effusion lymphoma,[163] natural killer/T-cell lymphoma,[164] and post-transplant lymphoproliferative disorder.[165] This association underscores the virus's profound interaction with the immune system. Evidence suggests that EBV plays a role in autoimmune diseases,[155,166-168] as patients with conditions, such as APS,[90,91] MS,[66-69,169-171] rheumatoid arthritis (RA),[92-94,167] SLE,[70-74,171] SS,[95,96,171,172] and SSc[97,173] exhibit EBV antibodies in their plasma.

Reactivation of latent EBV, often linked to compromised immune surveillance, leads to abnormal disease pathogenesis and cellular proliferation.[174] EBV relies on host PIP metabolism for efficient replication, with PI4K and PtdIns4P being key components in this process.[88] Notably, EBV miRNAs and latent proteins significantly influence these pathways. For instance, EBV nuclear antigen 1 stimulates PI4K and PIP kinase activity, increasing PtdIns4P and PtdIns(4,5)P_2 levels in B cells.[98] Latent membrane protein 1 (LMP1) and latent membrane protein 2A (LMP2A), other latent EBV proteins, activate the PI3K-AKT pathway[175] and enhance interferon regulatory factor 4 activity,[31,52] which is essential for immune cell differentiation.[176,177]

In addition, LMP1 suppresses PTEN expression through miR-21 upregulation, amplifying PI3K-AKT signaling.[101,178] Similarly, EBV-miRNA-BART7-3P contributes to this pathway by inhibiting PTEN.[99,179] EBV employs gp350, its envelope-associated protein, to attach to the complement receptor type 2/CD21 receptor,[180-182] influencing pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-2, and IL-6.[102,183] The NF-κB pathway in gp350-treated cells is activated through protein kinase C and PI3K, impacting IL-1β and IL-6 expression.[102,103,184,185]

Although research on EBV-induced inflammation and its link to PIP signaling remains limited,[68,183] emerging evidence highlights a potential connection that warrants further exploration, particularly regarding autoimmune diseases and PIP metabolism (Figure 3).

2.3. HCMV

HCMV, a prototypical member of the *Betaherpesvirinae* subfamily,[186,187] is a widespread pathogen capable of infecting individuals across all age groups. A defining feature of HCMV is its ability to establish lifelong latency following primary infection, during which it exerts broad modulatory effects on both innate and adaptive immunity. These immunomodulatory properties make HCMV a compelling candidate in the etiology of various autoimmune diseases.[188,189]

Accumulating evidence suggests that HCMV is involved in the pathogenesis of several autoimmune conditions, including RA,[104-106] SLE,[107-109] SSc,[104,110,111] and T1D.[104,112] Proposed mechanisms for HCMV-associated autoimmunity include molecular mimicry, chronic inflammatory responses, and non-specific activation of B cells.[190-192] One frequently observed phenomenon is the elevation of autoantibody levels in HCMV-infected individuals, particularly in SLE and SSc, suggesting a potential role for the virus in driving or exacerbating autoimmune processes.[193] In some SLE and SSc patients, HCMV infection has been linked to increased serum concentrations of pro-inflammatory cytokines, such as interferon-γ, IL-4, and IL-2, along with expanded memory T-cell populations.[194] These immune alterations may contribute to fibrotic responses and vascular injury, hallmark features of these diseases.

Emerging evidence indicates that type II interferon signaling pathways orchestrate immune responses by modulating the PI3K-AKT-mTOR signaling axis.[195,196] Moreover, the PI3K-AKT pathway may play distinct regulatory roles during different phases of HCMV infection. During latency, viral proteins UL7 and UL138 activate the PI3K-AKT signaling cascade through epidermal growth factor receptor engagement.[197-199] This activation maintains

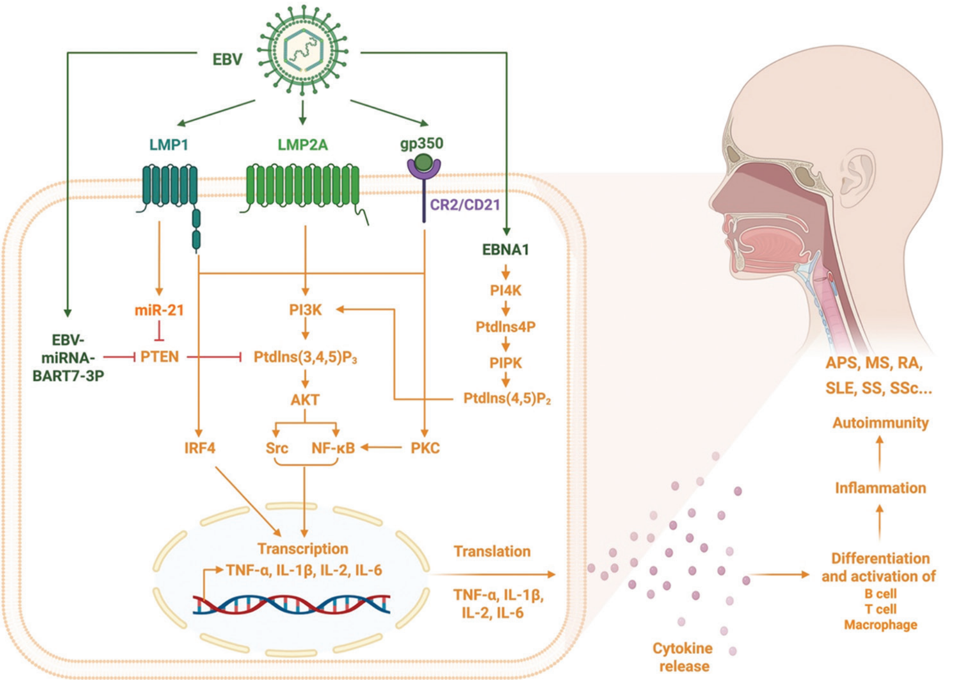

Figure 3. Proposed mechanism of EBV-induced PIP signaling in autoimmune diseases. EBV infection triggers PIP signaling through multiple interconnected pathways. EBV-miRNA-BART7-3P promotes PI3K-AKT signaling by suppressing PTEN. The latent viral proteins LMP1 and LMP2A further enhance PI3K-AKT and IRF activity, with LMP1 downregulating PTEN expression via miR-21 upregulation, thereby intensifying the PI3K-AKT cascade. The EBV envelope protein gp350 interacts with the complement receptor type 2/CD21 receptor, activating PI3K and PKC. Concurrently, the EBV protein EBNA1 stimulates PI4K and PIPK, increasing the synthesis of PtdIns4P and PtdIns(4,5)P$_2$. The cumulative activation of the PI3K-AKT pathway drives Src and NF-κB signaling, in combination with the effects of active IRF4, leading to the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-2, and IL-6. These cytokines facilitate the differentiation and activation of B cells, T cells, and macrophages, resulting in an amplified inflammatory response that may contribute to the onset of autoimmune diseases. Created with BioRender.com. Chen, M. (2025) https://BioRender.com/fq68o3e.
Abbreviations: AKT: Protein kinase B; APS: Antiphospholipid syndrome; CR2: Complement receptor type 2; EBNA1: Epstein-Barr virus nuclear antigen 1; EBV: Epstein-Barr virus; IL: Interleukin; IRF4: Interferon regulatory factor 4; LMP: Latent membrane protein; MS: Multiple sclerosis; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K: Phosphatidylinositol 3-kinase; PI4K: Phosphatidylinositol 4-kinase; PIPK: Phosphatidylinositol phosphate kinase; PtdIns(4,5)P$_2$: Phosphatidylinositol 4,5-bisphosphate; PtdIns4P: Phosphatidylinositol 4-phosphate; PTEN: Phosphatase and tensin homolog; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; SSc: Systemic sclerosis; TNF: Tumor necrosis factor.

viral genome silencing through multiple mechanisms: (i) Suppression of FOXO transcription factors to prevent their nuclear translocation and subsequent activation of lytic genes; (ii) induction of anti-apoptotic pathways that enable viral persistence in short-lived myeloid cells, such as monocytes; and (iii) promotion of CD34$^+$ progenitor cell differentiation toward myeloid lineages, thereby creating a stable cellular niche for latent maintenance.[199-201] In stark contrast, during lytic replication, the viral UL38 protein activates mTORC1 through both tuberous sclerosis complex 2-dependent and -independent mechanisms.[202,203] This leads to the degradation of insulin receptor substrate 1, consequent inhibition of PI3K-AKT signaling, and subsequent nuclear translocation of FOXO3a. This triggers viral lytic gene expression and drives the viral replication cycle.[204] This biphasic regulation of PI3K-AKT signaling exemplifies HCMV's sophisticated strategy to temporally control host cell pathways for optimal viral persistence and replication.

Although direct links between PIP signaling and HCMV-induced autoimmunity remain to be clarified, the involvement of PI3K-AKT-mTOR in HCMV latency and Lytic replication, T cell activation, and cytokine secretion suggests that PIP signaling could play a role in mediating HCMV-driven immune dysregulation. This potential connection represents a promising direction for future investigation.

2.4. HCV

HCV, first identified in 1989,[205] is an enveloped positive-sense single-stranded RNA virus belonging to the genus *Hepacivirus* of the family *Flaviviridae*.[206] Upon infection, HCV's positive-sense single-stranded RNA genome directly functions as mRNA, enabling immediate viral protein translation and subsequent hijacking of host cells for replication and virion assembly.[206] Despite the World Health Organization's 2030 elimination target, HCV still caused approximately 221,000 global deaths in 2022.[207]

HCV infection is associated with a range of liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma, as well as numerous extrahepatic diseases, such as kidney diseases, metabolic disorders, and autoimmune diseases.[208] These autoimmune manifestations include MC[77,78] and autoimmune thyroid diseases (AITD).[114] Under the persistent antigenic stimulation of HCV, B lymphocytes significantly increase the secretion of IgM antibodies with rheumatoid factor (RF) activity.[209] These RF-IgM antibodies bind to polyclonal IgG to form immune complexes that precipitate at low temperatures, resulting in MC, particularly type II MC, which is characterized by immune complexes composed of monoclonal IgM and polyclonal IgG.[210] In more severe pathological processes, the HCV E2 protein binds to the CD81 receptor on B-cell surfaces, inducing *BCL-2* gene rearrangement and t(14;18) chromosomal translocation, promoting malignant transformation, ultimately leading to B-cell lymphomagenesis.[211,212] Concurrently, HCV infection leads to T cell dysfunction, manifested as an increase in helper T cells (Th) and a decrease in Tregs.[213,214] Combined with antigenic similarity between HCV proteins and thyroid tissue components, immune dysfunction leads to thyroid tissue damage and subsequent development of AITD.[215,216]

The PIP signaling network plays stage-specific roles throughout HCV pathogenesis.[217] During early infection, the HCV envelope glycoprotein E2 interacts with its coreceptors CD81 and claudin-1 to rapidly activate AKT signaling, thereby enhancing viral entry efficiency,[115] possibly through deregulation of cell polarity.[116] In the viral replication process, HCV enhances neuroblastoma rat sarcoma viral oncogene homolog (NRAS) protein accumulation within detergent-resistant membranes (the site of viral replication complex assembly), consequently activating the NRAS-PI3K-AKT-mTOR signaling cascade. This pathway sustains basal viral replication while concurrently inhibiting host cell apoptosis. This dual mechanism facilitates persistent infection,[218] though the exact molecular details of this regulatory network require further characterization. Furthermore, HCV may alternatively activate the PI3K-AKT signaling pathway by perturbing host cellular miRNA networks (e.g., upregulation of miR-483-3p and miR-320c). This represents an additional layer of viral manipulation of host signaling cascades to facilitate persistent infection.[219] Simultaneously, HCV non-structural protein 5A (NS5A) protein activates PI4KIIIα to generate PtdIns4P-enriched membranes that recruit oxysterol-binding protein lipid transporters, creating optimal replication platforms.[117] This process is further enhanced when NS5A's basic amino acid PtdIns(4,5)P_2 pincer (BAAPP) domain binds PtdIns(4,5)P_2, inducing conformational changes that strengthen interaction with the host factor TBC1 domain family member 20.[118]

Many PIP signal inhibitors have shown promising anti-HCV effects;[220,221] thus, they may also inhibit HCV-induced autoimmunity. Besides, the broad cellular expression of PIP pathways and HCV's capacity to infect and replicate in immune cells suggest these signaling molecules may directly regulate immune functions in HCV-induced autoimmunity, though direct proof is currently absent.[222]

2.5. HHV-6

HHV-6 belongs to the β-herpesvirus subfamily of the *Herpesviridae*, which is the same subfamily as HCMV.[223] Since its first isolation in 1986 from patients with lymphoproliferative disorders, two main variants, HHV-6A and HHV-6B, have been recognized.[224,225] Available data indicate that HHV-6B accounts for the majority of infections in children, whereas HHV-6A appears to be associated with lower pathogenicity.[226] HHV-6 exhibits a broad cellular tropism and has been reported to infect T and B lymphocytes, among other cell types. It can establish long-term latency and may reactivate under conditions of immunosuppression or other stimuli, events that have been linked to chronic inflammation and sustained immune stimulation.[227,228]

The PIP signaling pathway is thought to be important for maintaining immune homeostasis and immune responses. The activation of the PI3K-AKT signaling pathway has been implicated in promoting cell proliferation, differentiation, and memory T cell formation, as well as regulating the differentiation of Th cell subsets and Treg functions.[229] In B lymphocytes, PIP signaling can be engaged through BCR-associated co-receptors to modulate survival and plasma-cell differentiation.[230] Collectively, these observations raise the possibility that HHV-6 might contribute to autoimmunity through the interaction with PIP-dependent pathways.

Previous studies have explored potential links between HHV-6 and autoimmune diseases, including MS,[79] SSc,[121] AITD,[119] T1D,[122] and inflammatory bowel disease (IBD).[120] Among these, the association with MS has received the most attention. HHV-6 nucleic acids and proteins have frequently been detected in peripheral blood mononuclear cells, cerebrospinal fluid, and brain tissue from MS patients, and some studies report that their abundance correlates with disease activity or relapse.[79,231-233] Seropositive MS patients have been found to display elevated levels of TNF-α, IL-1β, and IL-6, cytokines that can activate NF-κB signaling and, in turn, may influence the PI3K-AKT cascade.[123,124,234,235] Due to the association of other viruses with MS, research investigated the interactions between neurotropic viruses. Emerging evidence suggests that HHV-6 could trans-activate EBV, potentially modulating MS progression through PIP-related pathways.[236]

A meta-analysis on the cumulative incidence (incidence ratio) of HHV reactivation in COVID-19 patients also found the presence of HHV-6 virus reactivation,[237] highlighting the need to investigate post-viral autoimmune sequelae further. Moreover, in AITD, elevated HHV-6 levels have been reported in thyroid tissue, leading to the hypothesis that the virus might initiate disease through infection of thyroid or immune cells.[238] Similarly, HHV-6B has been detected in pancreatic islets of T1D patients, and it has been postulated that transient reactivation could trigger β-cell-directed autoimmunity.[122]

Although the precise inter-relationship among HHV-6 infection, PIP signaling, and autoimmune pathogenesis remains to be fully elucidated, their intertwined interactions warrant continued investigation, which may offer new insights into disease mechanisms.

2.6. Rubella virus

Rubella virus, the only member of the *Rubivirus* genus within the *Togaviridae* family,[239] is widely regarded as the causative agent of rubella, commonly referred to as German measles. Although widespread immunization efforts have contributed to a global decline in RuV incidence, the virus continues to circulate in regions with insufficient vaccine coverage.[240] Persistent, chronic RuV infections involving multiple organ systems—as well as the accompanying generation of autoantibodies—have been suggested to contribute to the development of autoimmune diseases.[241,242]

Growing evidence has associated RuV, along with other viruses, such as HCMV and CVB, with an increased risk of T1D.[75,76,80,81,104,112] Two principal mechanisms have been hypothesized by which viruses may contribute to T1D pathogenesis: Direct cytolytic damage to pancreatic β cells and virus-induced immune responses that trigger autoimmunity and subsequent β cell destruction.[242] Persistent RuV infection has been correlated with the presence of islet cell autoantibodies, suggesting a potential role in initiating autoimmune T1D.[243-245] Preserving β-cell viability and metabolic activity appears critical for preventing and treating T1D.[246] The PI3K-AKT signaling pathway, known for its regulatory functions in cell survival, proliferation, and metabolism, has been proposed as a promising target in this context. RuV has been reported to activate the PI3K-AKT pathway during infection, a process thought to support both host cell survival and efficient viral replication.[125] This pathway may also modulate β-cell function, possibly delaying the onset of autoimmune destruction.

Therapeutic strategies to enhance PI3K-AKT signaling in β cells have shown preliminary promise. For example, FhHDM-1—a 68-amino-acid peptide secreted by the parasitic helminth *Fasciola hepatica*—has been observed to directly stimulate PI3K-AKT signaling in β cells, promoting survival and protecting against cytokine-induced apoptosis without triggering uncontrolled proliferation.[247,248] In addition, gamma-aminobutyric acid has been reported to exert both anti-apoptotic and proliferative effects on β cells, suggesting its potential utility as an adjunct in islet transplantation therapies.[249]

While conclusive evidence linking RuV to PI3K-pathway dysregulation in autoimmune disease is still lacking, available data indicate that both factors may contribute to disease progression. Investigating the interplay among RuV infection, PI3K signaling, and autoimmunity may provide insights into disease mechanisms and inform novel therapeutic approaches. Continued research will be necessary to clarify these complex relationships and to determine their clinical relevance.

2.7. SARS-CoV-2

SARS-CoV-2, a member of the *Coronaviridae* family, has caused the global COVID-19 pandemic, affecting millions of individuals worldwide since its emergence in late 2019. This virus is composed of four structural proteins—spike, envelope, membrane, and nucleocapsid—and a single-stranded positive-sense RNA genome.[250] While the primary manifestation of SARS-CoV-2 infection is respiratory illness, emerging evidence suggests that the virus can also induce a range of autoimmune disorders through complex mechanisms involving molecular mimicry (where viral proteins resemble human antigens), epitope spreading (exposure of hidden self-antigens), and bystander activation (non-specific T-cell stimulation).[251] These mechanisms have been implicated in the emergence of conditions, such as GBS,[82] multisystem inflammatory syndrome in children,[252] autoimmune thyroiditis (AT),[126] APS,[83] and ITP.[83]

Central to the pathogenesis of SARS-CoV-2 infection is the viral S protein, which mediates host cell infection by binding to the angiotensin-converting enzyme 2 receptors on the cell surface.[253] This binding event triggers a cascade of intracellular signaling pathways, including the PI3K-AKT-mTOR pathway, which has been shown to promote viral replication and enhance the severity of COVID-19.[127] Targeting mTORC1, a key component of the PI3K-AKT-mTOR pathway, has been proposed as a potential therapeutic strategy to suppress the metabolic reprogramming induced by the virus, thereby inhibiting disease progression.[254] The PI3K-AKT-mTOR pathway also plays a crucial role in modulating the immune response during SARS-CoV-2 infection. Activation of this pathway promotes protein synthesis, inhibits apoptosis, and enhances cytokine release, all contributing to the development of an autoimmune storm.[128] This inflammatory cascade is further exacerbated by the viral open reading frame 3a (ORF3a) protein, which upregulates miR-155 to suppress SHIP1, a suppressor of the PI3K pathway by dephosphorylating PtdIns(3,4,5)P_3.[129,130] This suppression enhances the production of PtdIns(3,4,5)P_3, which activates AKT and NF-κB, leading to the amplification of pro-inflammatory cytokines, such as IL-6 and TNF-α. These cytokines are key drivers of the hyperinflammatory state observed in severe COVID-19 cases and may contribute to the development of autoimmune disorders.[131]

Recent studies have also identified phosphoinositide kinase (PIK)FYVE, a PtdIns3P 5-kinase,[2] as a potential therapeutic target.[132] PIKFYVE inhibitors have been shown to effectively block viral fusion with endosomal membranes, preventing the release of the virus into the cytoplasm and thus inhibiting SARS-CoV-2 infection. This suggests that targeting specific components of the PIP signaling pathway may provide a novel approach to combating COVID-19.[132]

Notably, in addition to the PIP signaling pathway, the sphingolipid signaling pathway also plays a significant role in SARS-CoV-2 infection. Ceramide can facilitate viral entry and release by modulating membrane fluidity, making it easier for the virus to infect host cells. Conversely, sphingosine-1-phosphate has been shown to suppress viral infection and replication by regulating inflammatory responses and promoting cell survival.[255] This highlights the complex interplay between lipid signaling pathways and viral pathogenesis.

In conclusion, the PIP signaling pathway, particularly the PI3K-AKT-mTOR axis, plays a pivotal role in SARS-CoV-2 infection by promoting viral replication and driving autoimmune and inflammatory responses (Figure 4). Targeting this pathway offers significant therapeutic potential for mitigating COVID-19 severity and preventing associated autoimmune complications. Further research on the interplay between viral proteins and host signaling pathways is essential for developing effective treatments.

2.8. Theiler's murine encephalomyelitis virus

Theiler's murine encephalomyelitis virus is a naturally occurring picornavirus that infects rodents and is widely used as an experimental model for studying virus-triggered autoimmune demyelinating diseases, particularly MS.[84,85,256] In the development of TMEV-induced demyelinating disease (TMEV-IDD)—a well-established model that mimics aspects of MS—initial viral infection of brain endothelial cells plays a pivotal role in disease initiation.[133]

Research has indicated that autoantibodies may intensify the inflammatory cascade during TMEV-IDD, highlighting the contribution of immune-mediated mechanisms to demyelination.[257] The brain endothelium is central to this pathological process. Cyclooxygenase-2 (COX-2), an enzyme known to mediate inflammation in central nervous system (CNS) disorders, including MS, is expressed by brain endothelial cells and has been implicated in TMEV pathology.[258] Inflammatory cells, such as macrophages and microglia, in active demyelinating lesions—as well as degenerating oligodendrocytes—also express COX-2, contributing to elevated levels of COX-derived prostaglandins observed in the CNS of MS patients.[259,260]

Emerging data suggest that cannabinoids may help mitigate the progression of TMEV-IDD, potentially through signaling pathways involving PIPs.[258] One such example is the synthetic cannabinoid agonist WIN 55212-2, which has been shown to activate the PI3K-AKT signaling axis, subsequently increasing the expression of COX-2 and prostaglandin E2. This upregulation alleviates disease symptoms in TMEV models by modulating cerebral blood flow and dampening intracerebral immune responses.[133]

Autoimmune disorders driven by TMEV have profound effects on neurological and psychological health. Targeting the PI3K signaling pathway may offer promising strategies for preventing or treating these conditions. Ongoing research into this pathway could unlock novel therapeutic options and deepen our understanding of virus-induced autoimmune diseases.

3. Discussion and future perspectives

The intricate relationship between viral infections and autoimmune diseases has long been a focal point in immunological research. This review delves into the pivotal role of PIP signaling in this complex interplay, uncovering how viruses exploit PIP pathways to facilitate their lifecycle while simultaneously triggering autoimmune responses.

The functional specialization of individual PIP species and their regulatory enzymes underpins their consequential roles in immune regulation beyond generic signaling cascades. PtdIns4P, concentrated at the Golgi and endosomal membranes, governs vesicular trafficking and organelle identity. Its exploitation by viruses, such as CVB and HCV (via PI4KIIIα activation by HCV NS5A),

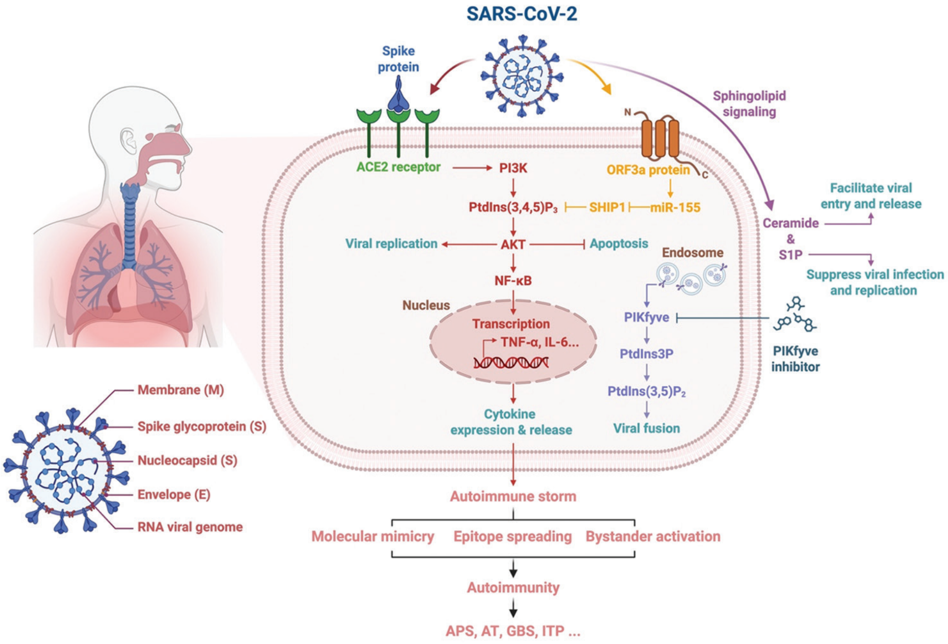

Figure 4. Key lipid signaling pathways in SARS-CoV-2 pathogenesis and autoimmunity. This schematic depicts key host signaling pathways exploited by SARS-CoV-2 to drive pathogenesis and autoimmune complications. SARS-CoV-2 infection hijacks host lipid signaling pathways, primarily the PI3K-AKT-mTOR cascade, to promote viral replication, inhibit apoptosis, and drive pro-inflammatory cytokine release (TNF-α, IL-6), contributing to a hyperinflammatory "autoimmune storm." Viral proteins like ORF3a further enhance PI3K signaling by suppressing PtdIns(3,4,5)P_3 phosphatase SHIP1 via miR-155. Concurrently, sphingolipid signaling influences infection: ceramide facilitates viral entry/release, while S1P suppresses it. Molecular mimicry, epitope spreading, and bystander activation link infection to autoimmune disorders (such as APS, AT, GBS, and ITP). Targeting key nodes in these pathways, such as mTORC1 or PIKFYVE (blocking endosomal viral fusion), represents a promising therapeutic strategy to mitigate the inflammatory state. Created with BioRender.com. Chen, M. (2025) https://BioRender.com/eyln0kh.

Abbreviations: ACE: Angiotensin-converting enzyme 2; AKT: Protein kinase B; APS: Antiphospholipid syndrome; AT: Autoimmune thyroiditis; GBS: Guillain-Barré syndrome; IL: Interleukin; ITP: Immune thrombocytopenia; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; ORF3a: Open reading frame 3a; PI3K: Phosphatidylinositol 3-kinase; PIKFYVE: Phosphoinositide kinase, Fab1, YOTB, Vac1, and EEA1; PtdIns(3,4,5)P_3: phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P_3); PtdIns(4,5)P_2: Phosphatidylinositol 4,5-bisphosphate; PtdIns3P: Phosphatidylinositol 3-phosphate; PTEN: Phosphatase and tensin homolog; S1P: Sphingosine-1-phosphate; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SHIP: Src Homology 2 domain-containing inositol phosphatase; TNF: Tumor necrosis factor.

facilitates the assembly of viral replication organelles, directly linking PtdIns4P abundance to viral persistence. Conversely, PtdIns(3,4,5)P_3—a key cell membrane and nuclear resident lipid messenger—acts as a master regulator of immune cell activation by recruiting PH domain-containing effectors, such as AKT and PDK1. In autoimmunity, excessive PtdIns(3,4,5)P_3 production due to PTEN/SHIP deficiency or PI3K hyperactivation (e.g., in SLE T cells) lowers activation thresholds for BCR/TCR signaling, promoting loss of self-tolerance. Viruses, such as EBV, exacerbate this through latent proteins (LMP1/LMP2A) and miRNAs (BART7-3p) that suppress PTEN, thereby amplifying PI3K-AKT-driven inflammatory responses and bystander activation.

The spatial restriction of PIPs (e.g., nuclear PIPs modulating transcription vs. plasma membrane-endomembrane pools controlling receptor signaling) and the substrate specificity of kinases (PI3K vs. PI4K) and phosphatases (PTEN dephosphorylating PtdIns[3,4,5]P_3) explain why dysregulation of distinct PIP nodes has non-redundant consequences: PtdIns4P manipulation primarily subverts intracellular membrane dynamics for viral replication, while PtdIns(3,4,5)P_3 dysregulation directly skews immune cell fate decisions, bridging viral pathogenesis to autoimmune initiation.

Different PIP signaling pathways play distinct roles in immune regulation. Viral infections can induce markedly divergent activation patterns and immunomodulatory effects even within the same pathway. Despite their distinct tissue tropisms, diverse viruses have evolved sophisticated strategies to hijack the host PI3K-AKT signaling pathway, a pivotal regulatory hub. Our systematic analysis reveals that these pathogens employ unique molecular mechanisms to activate PI3K-AKT signaling, thereby inducing cellular changes conducive to viral replication, including apoptosis inhibition, metabolic reprogramming, and membrane restructuring. Strikingly, different virus families have developed highly specific regulatory tactics: EBV achieves sustained pathway activation through miRNA-mediated PTEN silencing; SARS-CoV-2 induces SHIP1 degradation through ORF3a-driven miR-155 production; while HCMV demonstrates exquisite spatiotemporal control, employing distinct effector proteins during latent and lytic phases to modulate pathway activity precisely.

These differential activation patterns directly shape viral replication strategies: HCV maintains basal pathway activity to sustain persistent infection; CVB3 employs phase-specific regulation to induce autophagy, favoring viral release; and rubella virus ingeniously utilizes transient activation to protect host cells. These differential regulatory patterns lead to distinct immunopathological outcomes: Ranging from EBV-associated systemic autoimmunity to SARS-CoV-2-triggered acute neuroimmune disorders, and even to neuroprotective effects in the TMEV model. These findings collectively establish the PI3K-AKT pathway as a conserved metabolic hub essential for viral replication, and more importantly, as a decisive regulator of post-infection immune equilibrium, with outcomes critically dependent on virus-specific modulation patterns.

The shared exploitation of PIP signaling nodes by diverse viruses and their disruption of immune homeostasis present compelling therapeutic opportunities. Among these nodes, the PI3K-AKT, PI4K, and PIKFYVE pathways are particularly critical, as emerging evidence highlights their dual roles in viral pathogenesis and immune dysregulation. The PI3K-AKT pathway, a well-established target in oncology, has already yielded multiple FDA-approved inhibitors (e.g., Idelalisib, Copanlisib, Duvelisib, Alpelisib, and Umbralisib) for cancer therapy.[261] Although initially focused on oncology, PI3K inhibitors are now being explored for their potential in autoimmune diseases.[262] While no PIKFYVE inhibitors have yet reached the market, the first small-molecule inhibitor targeting PIKFYVE has advanced to a Phase 2a clinical trial (NCT05163886) for ALS. In addition, pre-clinical studies have highlighted the role of PIKFYVE inhibitors in suppressing viral infections.[263,264] In contrast, although PI4K plays a critical role in HCV infection, the development of PI4P-targeting inhibitors remains in the pre-clinical stage. These challenges underscore the need for further research to optimize the safety and efficacy of PIP-targeted therapies—particularly through isoform-selective drug design and combinatorial strategies that address both viral persistence and immune dysregulation.

While this review highlights the central role of PIP signaling at the virus-autoimmunity interface, several critical knowledge gaps and technical challenges remain. First, present research predominantly focuses on proteomic analyses of PIP regulatory proteins (particularly kinases and phosphatases) in virus-induced autoimmunity, while the spatiotemporal dynamics of individual PIP species during acute versus persistent infection remain poorly resolved. Advanced PIP biosensors enabling real-time tracking of lipid dynamics at organelle resolution are urgently needed to map viral-induced PIP rewiring in living systems.[265] Second, the field lacks isoform-selective chemical tools targeting key PIP regulators (kinases, phosphatases, transporters, and phospholipases) with optimal specificity and safety profiles, as individual PIP-metabolizing enzymes play distinct roles under specific physiological and pathological conditions.[266] Structure-guided development of inhibitors against virus-hijacked PIP nodes (e.g., HCV-NS5A-activated PI4KIIIα or EBV-suppressed PTEN) could achieve pathogen-selective disruption while preserving physiological signaling.

Moreover, existing pre-clinical studies rely heavily on cell models (particularly cancer cell lines), which poorly recapitulate systemic aspects of viral infection and autoimmunity. Tissue-specific conditional knockout models targeting PIP-modifying enzymes in virally infected hosts are crucial for establishing causal mechanisms. Finally, integrating phosphoinositide omics with proteomics and single-cell omics in human cohorts—correlating viral load, PIP flux, and autoimmune signatures—will enable patient stratification, while clustered regularly interspaced short palindromic repeats screening in human organoids can identify virus-specific PIP dependencies. Addressing these challenges will advance mechanistic insights and facilitate precision therapies that disentangle viral pathogenesis from autoimmune cascades.

4. Concluding remarks

This review bridges the understanding of PIP signaling in viral pathogenesis and autoimmune disorders, offering a comprehensive framework for future research and therapeutic development. By targeting the shared PIP-centric mechanisms, we may unlock new strategies to combat both viral infections and their autoimmune sequelae, ultimately improving patient care and outcomes.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Associations between HIV, sexually transmitted diseases, and antimicrobial resistance in the era of combination antiretroviral therapy and antibiotics

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Abstract

The advancement of combination antiretroviral therapy (cART) has dramatically transformed the management of human immunodeficiency virus (HIV), significantly reducing morbidity and mortality rates. However, the coexistence of sexually transmitted diseases (STDs) and antimicrobial resistance (AMR) presents interconnected challenges that compromise these advances in this era. This review explores the intricate associations between HIV, STDs, and AMR in the framework of extensive cART and antibiotic usage. Published literature on epidemiological data was analyzed to identify the patterns of co-infection and resistance trends, examining how the suppression of HIV influences the prevalence and treatment outcomes of concurrent STDs. Furthermore, the impacts of antibiotic overuse or misuse on the emergence of resistant strains of common bacterial STDs were investigated, particularly focusing on pathogens such as *Neisseria gonorrhoeae* and *Mycoplasma genitalium* among HIV-infected individuals. The findings highlight the critical need for integrated surveillance, antimicrobial stewardship, expanded vaccination, and culturally sensitive public health strategies. By enhancing our understanding of these interactions, this review intends to inform alterations in the existing public health policies and to upgrade previously optimized treatment protocols in the near future to improve patient outcomes in the era of cART and antibiotics.

Keywords: HIV; Sexually transmitted disease; Antimicrobial resistance; Combination antiretroviral therapy; Antibiotic era

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1. Introduction

The global human immunodeficiency virus (HIV) epidemic remains a significant public health challenge, with millions of people affected worldwide, particularly in low- and middle-income countries. Despite the success of combination antiretroviral therapy (cART) in controlling HIV progression, the spread of sexually transmitted diseases (STDs) has surged in many regions.[1] Co-infection with STDs increases the risk of HIV transmission, leading to a vicious cycle of infection. Compounding this issue is the alarming rise of antimicrobial resistance (AMR) in common STD pathogens, such as *Neisseria gonorrhoeae*.

The emergence of AMR is undermining the effectiveness of antibiotics, making STD treatment more difficult and threatening to complicate HIV management, especially in individuals co-infected with resistant strains. Together, these trends pose a critical challenge to global public health efforts in controlling HIV and STDs, followed by the burning issue of AMR. Antibiotics play a crucial role in treating bacterial STDs, helping to prevent complications and reduce transmission. However, the overuse and misuse of antibiotics have led to the emergence of AMR, rendering standard treatments less effective and complicating STD management. Thus, several interconnecting factors need to be studied to understand the dynamics for effective management and treatment of these diseases.[2]

2. Methodology

To ensure a comprehensive review of the literature, multiple databases and sources, including PubMed, Google Scholar, ScienceDirect, and published reports, were searched. Boolean operators, such as AND, OR, and NOT, were applied to optimize search results and capture the most relevant and up-to-date information. Review articles, research papers, and reports were selected based on their relevance to HIV-STD co-infection and the emergence of AMR. The selected articles were summarized and analyzed to provide a thorough overview of the current status of HIV-STD co-infection and the role of the immune system at various stages of disease progression. The validity and reliability of the articles were assessed based on their sample sizes. In addition, factors influencing or confounding the management and severity of HIV-STD co-infection were included.

3. HIV and STDs

3.1. Development of HIV-STD co-infection

Initially, in the rise of the epidemic, Wasserheit[3] identified the link between HIV and traditional STDs that produce the irritation of the mouth or ulcers, which he coined as "epidemiologic synergy." Recent research has focused extensively on biological pathways to understand how STDs facilitate the transmission of HIV by disrupting natural defenses and amplifying viral replication. STDs, particularly those causing genital ulcers or inflammation, such as syphilis and herpes, damage mucosal barriers, making it easier for HIV to enter the body during sexual contact. Inflammation during STDs triggers immune activation, attracting immune cells, such as cluster of differentiation 4-positive ($CD4^+$) T cells, to the infection site. This increases the number of susceptible cells in the genital tract, facilitating HIV acquisition. These investigations indicate that STDs increase the contagiousness of HIV-positive individuals and the susceptibility of HIV-negative individuals. Furthermore, recent studies have highlighted that the alterations in the viral phenotype of HIV variants, particularly those enhancing viral fitness and transmissibility, play a critical role in the increasing spread of the virus. These phenotypic changes can lead to higher viral replication rates and elevated viral loads in genital secretions, especially vaginal fluid, thereby significantly increasing the risk of sexual transmission. Such adaptations not only facilitate more efficient person-to-person transmission but may also contribute to the persistence and resurgence of the epidemic in certain populations.[4]

In general, HIV R5-tropic (C-C chemokine receptor type 5-using) viruses are more commonly transmitted than X4-tropic (C-X-C chemokine receptor type 4-using) viruses. Transmitted/founder viruses often display higher replicative fitness and resistance to host restriction factors. Moreover, viruses with low glycosylation and neutralizing antibody sensitivity are more likely to establish infection during transmission.[5,6] Epidemiological studies and meta-analyses have quantified the increased risk of HIV acquisition in the presence of STDs. For example, genital ulcer diseases (e.g., syphilis and herpes) increase the risk of HIV acquisition by 2–5 times, primarily by disrupting mucosal barriers. Non-ulcerative STDs (e.g., gonorrhea and chlamydia) can increase HIV transmission risk by 1.5–3 times, mainly through inflammation and immune cell recruitment.[7]

Sexual transmission is one of the primary routes of HIV spread, with infection resulting from exposure to blood, pre-ejaculate semen, and vaginal fluids.[8] STDs, such as gonorrhea, syphilis, and herpes, can accelerate HIV progression by causing chronic inflammation and immune system activation. This immune activation increases the number of HIV target cells, facilitating viral replication and increasing viral loads, thereby leading to faster disease progression. In addition, HIV can be transmitted from an infected mother to her infant during pregnancy, at childbirth through contact with maternal blood or vaginal fluids, or during breastfeeding.[9] HIV is present in body fluids as free virus particles and as cell-associated virus within infected immune cells.[10] Maternal antiretroviral therapy (ART) reduces the risk of vertical HIV transmission to <1% when started early and maintained throughout pregnancy and breastfeeding, while pre-exposure prophylaxis (PrEP) is safe and effective for HIV-negative pregnant women with high-risk partners.[11,12] Furthermore, early ART initiation helps reduce the size and diversity of the latent HIV reservoir, according to multiple clinical studies at various places.[13-15]

As HIV weakens the immune system, the body becomes more vulnerable to infections, making STDs more persistent, severe, and difficult to treat. This interplay between HIV and STDs creates a vicious cycle that not only worsens individual health outcomes but also increases the likelihood of transmission. HIV-STD co-infection can increase HIV viral load in genital secretions, further raising the risk of transmission to sexual partners. Conversely, when the viral load of a person is undetectable, sexual transmission of HIV through condomless intercourse is exceedingly rare.[16]

Epidemiological data demonstrated that regions with high HIV prevalence, such as sub-Saharan Africa and Southeast Asia, also reported high STD burden, with co-infections particularly common among key populations such as men who have sex with men (MSM), sex workers, and adolescents.[17,18] These synergistic epidemics not only amplify disease transmission but also complicate diagnosis and treatment, underscoring the need for integrated surveillance and prevention strategies.[19,20]

The widespread use of cART has led to effective suppression of HIV viral load, substantially reducing the risk of HIV transmission. However, this success has had mixed effects on the dynamics of STD transmission. Individuals with undetectable viral loads may engage in riskier sexual behaviors due to a perceived reduction in HIV transmission risk, potentially leading to increased incidence of other STDs, such as syphilis, gonorrhea, and chlamydia. Moreover, while HIV suppression reduces systemic immune activation, it does not prevent the acquisition or transmission of bacterial or viral STDs. This interplay of behavioral, virological, and immunological factors in the era of HIV suppression necessitates the continued emphasis on regular STD screening, safer sex practices, and integrated sexual health services to control STD transmission in populations living with HIV.[21]

3.2. Insights into HIV transmission and prevention

The introduction of cART has significantly improved the life expectancy and quality of life for people living with HIV. It has also influenced the prevalence and treatment of co-infections, underscoring the importance of understanding how cART interacts with treatments for other STDs. Primary HIV infection refers to the early stage following the acquisition of HIV and represents a critical opportunity for expedited diagnosis and rapid initiation of ART. Early treatment can improve immune functions, reduce the size of the viral reservoir, and limit onward transmission. Primary HIV infection may sometimes last for 2–3 weeks, during which the virus infects susceptible CD4$^+$ T cells.[22] Rapid viral replication produces a burst of viremia, enabling widespread dissemination to lymphoid organs, the brain, and other tissues.[23]

Acute retroviral syndrome, which typically lasts for 2–3 weeks, marks the onset of HIV-specific immune responses. It often presents with infectious mononucleosis-like symptoms and usually resolves spontaneously.[24] During this phase, a transient decline in CD4$^+$ T cell counts may occur, which then stabilizes for a period before beginning a gradual decline.[25] Acute HIV infection is mostly unrecognizable in primary care settings because the non-specific symptoms of acute retroviral syndrome frequently resemble common viral illnesses such as influenza.[26]

During the asymptomatic phase of HIV infection, the body mounts a robust cellular and humoral immune response. However, the virus escapes immune-mediated clearance, developing chronic infection as the virus is never fully eliminated. This asymptomatic stage may last for 8–10 years. With therapy, cell-mediated immunity gradually recovers, and the CD4$^+$ cell count increases.[27]

Progression to the symptomatic phase of HIV infection is marked by opportunistic infections and non-infectious complications, such as early immunodepletion, persistent generalized lymphadenopathy, intermediate and severe immunodeficiency, tuberculosis, and ultimately acquired immunodeficiency syndrome.[28] HIV exists as two major types: HIV-1 and HIV-2.[29] A key accessory protein of HIV-1, viral protein R (Vpr), is a 14-kDa, 96-amino acid virion-associated protein highly conserved among primate lentiviruses, including HIV-1 and HIV-2.[30] Vpr is incorporated into virions through the p6 region of Gag, and its abundance is closely linked to Gag expression.[31] Vpr displays karyophilic properties, localizing to the nucleus despite lacking a canonical nuclear localization signal. It is required for the nuclear import of the HIV-1 genome and plays a critical role in pre-integration complex formation, enabling efficient replication in macrophages.[32] Beyond replication, Vpr arrests host cells at the G2/M phase of the cell cycle and contributes to apoptosis and HIV-associated neurotoxicity. Recent studies have explored Vpr as a therapeutic target, including inhibitors and gene-editing strategies (e.g., clustered regularly interspaced short palindromic repeats/Cas9) designed to disrupt its expression.[33(p1)-35]

As HIV infection continues to rise, prevention remains paramount, since no permanent cure, universally effective drug, or widely accessible preventive vaccine is yet available—particularly in developing countries. Information, education, and communication programs targeting men, women, and adolescents, with special emphasis on high-risk groups, are essential. These efforts should be complemented by accessible facilities for the detection and treatment of other STDs, promotion of condom use, and dissemination of accurate information free from stigma and discrimination. HIV-infected persons usually experience recurrent illnesses and complications that require medical and nursing care, including periodic hospitalization. Even during periods of minimal infection, clinical care should include pain relief and treatment for common opportunistic infections. Effective care also requires trained healthcare providers and a reliable supply of essential medicines. With comprehensive clinical care, the lives of HIV-infected persons can be substantially prolonged and their quality of life greatly improved.

Healthcare workers, such as doctors, nurses, and paramedical staff, face a heightened risk of accidental HIV infection if safety measures are not strictly followed. Infected healthcare personnel may, in turn, pose a transmission risk to uninfected patients if appropriate precautions are neglected. Such risks can be avoided through adherence to universal precautions, such as safe handling and disposal of sharps, proper decontamination of instruments and equipment, frequent hand hygiene, and the use of protective barriers to prevent direct contact with body fluids. Implementation of universal personal protective equipment protocols is essential to reduce occupational exposure, particularly in healthcare settings managing HIV, STDs, and other infectious diseases. Routine surveillance for needlestick injuries and timely access to post-exposure prophylaxis are both critical for protecting healthcare workers. Furthermore, vaccination coverage, particularly against the hepatitis B virus (HBV) and seasonal influenza, further enhances biosafety measures. In addition, expanding the use of point-of-care diagnostic technologies minimizes unnecessary handling of infectious specimens, thereby reducing exposure risks. Vaccination against human papillomavirus (HPV) and HBV remains a cornerstone of STD prevention. In low-resource settings, various programs led by the World Health Organization (WHO) have improved HPV vaccine access, especially for girls aged 9–14.[36]

3.3. Insights into STD transmission and prevention

STDs are diseases transmitted primarily through sexual intercourse. Their transmission requires a causative agent that spreads from one person to another. STDs can affect the genital tract and other parts of the body participating in sexual interaction, including infections such as syphilis, gonorrhea, chancroid, donovanosis, non-gonococcal urethritis, genital warts, and genital herpes. STDs have a tremendous impact on public health, contributing to maternal morbidity, ectopic pregnancies, infant illness and mortality, malignancies, infertility, and increased susceptibility to HIV infection. They are a major cause of infertility in both men and women and play a significant role in adverse pregnancy outcomes, including fetal death, abortions, and low birth rates. The risk of acquiring STDs and subsequent development of cervical and other genital cancers is directly associated with the number of lifetime sexual partners, partner turnover, and sexual network dynamics.

Among viral STDs, HBV and HPV are particularly important due to their oncogenic potential. While the incidence of bacterial STDs has declined in many developed countries, the global HIV pandemic continues to spread relentlessly, exacerbating the overall burden of STDs. This problem is further compounded by the emergence of AMR in pathogens such as *N. gonorrhoeae*, herpes simplex virus (HSV), and HIV. Rising drug resistance highlights the need for continuous monitoring, novel therapeutic strategies, and adaptive clinical guidelines. In addition, early pathogen detection with advanced diagnostic technology is essential for the timely initiation of treatment.[37]

The transmission of STDs is strongly influenced by sexual behaviors and sociocultural factors. Early marriage, multiple sexual partners, rapid partner change, and high-risk sexual practices are key determinants of STD risk, while education, religion, and cultural practices further shape sexual behaviors. Pathogens such as HIV, HBV, *N. gonorrhoeae*, and *Chlamydia trachomatis* have a more efficient male-to-female than female-to-male transmission, largely due to extended mucosal exposure following sexual contact.

Viruses such as HPV are transmitted through direct sexual contact, regardless of visible wart presentation. The incubation period typically ranges from 2 to 3 months, though longer durations may occur. HPV thrives in warm, moist environments but does not survive well outside the human body, which explains its lack of transmission through inanimate objects. Genital warts may also occur in the mouth following oral sex, and vertical transmission from mother to infant can occur during delivery. In such cases, caesarean section is often recommended.[38]

Emerging challenges in STD control include the rise of AMR gonorrhea, HPV-related cancers, evaluation of HPV vaccine impact, increasing syphilis incidence among MSM, and the use of nucleic acid amplification tests for earlier and more accurate diagnosis. Newly recognized sexually transmitted pathogens, such as the hepatitis C virus and *Mycoplasma genitalium*, have also expanded the scope of concern.

The prevention of STDs aims to reduce both behavioral and biological risks. The primary steps include comprehensive risk assessments through sexual histories, behavioral screening, and testing for biological markers associated with HIV acquisition and transmission.[39] Routine STD screening is an essential component of risk assessment: women under 25 years, older women with risk factors, and sexually active bisexual and MSM should undergo regular testing for infections such as chlamydia and gonorrhea.[40]

Preventive risk-reduction strategies include not engaging in sexual activity, reducing the number of sexual partners,[41] and promoting consistent condom use. However, sociocultural barriers significantly influence prevention. In some settings, religious conservatism may discourage condom use and hinder comprehensive sex education, while low literacy levels limit understanding of disease transmission and protective measures. In addition, gender norms and stigma surrounding STDs further deter individuals from seeking testing or disclosing infection status, thereby delaying both diagnosis and treatment and increasing transmission risks.[42,43] To overcome these barriers, intervention should include school-based and community-based education programs to raise awareness, conditional cash transfers to discourage early marriage, and youth-friendly STD clinics to provide accessible and confidential care for adolescents.[44,45]

4. Management strategies of the most common bacterial and viral STDs

4.1. Management of chlamydia

Chlamydia is caused by *C. trachomatis*, which is a gram-negative intracellular bacterium belonging to the family Chlamydiaceae, and is the most commonly reported STD in the United States (US),[46] with the highest burden among adolescents and young adults. Studies have reported that untreated *C. trachomatis* infection in women can develop into pelvic inflammatory disease (PID),[47] which is associated with serious reproductive health consequences. Approximately 20% of women with PID develop infertility, 18% experience chronic pelvic pain, and 9% have life-threatening tubal pregnancies. Symptoms of chlamydial infection are often mild or absent, contributing to underdiagnosis and delayed treatment.

In neonates, chlamydia arises primarily from perinatal exposure to an infected maternal cervix. Diagnosis in women is typically made using urine specimens or swabs from the endocervix or vagina, while in men, it is confirmed through urethral swabs or urine specimens. Rectal infections can be diagnosed using rectal swabs. The preferred diagnostic methods include nucleic acid amplification tests, cell culture, direct immunofluorescence, and nucleic acid hybridization.[48] Chlamydia treatment involves antibiotics such as azithromycin, doxycycline, erythromycin, and levofloxacin. Timely therapy not only cures the infection but also prevents progressive tissue damage and long-term complications.[39]

4.2. Management of gonorrhea

Gonorrhea is caused by *N. gonorrhoeae*, a gram-negative diplococcus with an incubation period of 3–14 days. The highest prevalence is observed among females aged 15–19 years and males aged 20–24 years. Gonorrhea cases in the US have been increasing in all races and ethnic groups.[49] Transmission occurs primarily through unprotected sexual contact with an infected partner. Common sites of infection include the urethra, vagina, cervix, rectum, pharynx, and conjunctiva. Because *N. gonorrhoeae* does not survive well outside the human host, transmission through inanimate objects such as toilet seats is highly unlikely. Vertical transmission during childbirth can result in severe conjunctivitis.[50]

Symptoms of gonorrhea vary from symptomatic urethritis, epididymitis, vaginal and pelvic discomfort, and abnormal vaginal discharge to asymptomatic infections. Both symptomatic and asymptomatic infections can progress to PIDs, leading to complications such as tubal infertility, ectopic pregnancy, and chronic pelvic pain. In rare cases, disseminated gonococcal infection can occur, resulting in arthritis, meningitis, or endocarditis.

Diagnosis of gonorrhea infection in symptomatic men can be achieved by Gram staining of urethral specimens, which typically reveal polymorphonuclear leukocytes with intracellular Gram-negative diplococci. However, in asymptomatic men, a negative Gram stain result does not exclude infection. Other diagnostic methods include culture, nucleic acid hybridization tests, and nucleic acid amplification tests.

Treatment of gonorrhea includes the use of antibiotics such as ceftriaxone, often combined with azithromycin as dual therapy. However, the emergence of AMR in *N. gonorrhoeae* poses a growing threat. Patients treated for gonorrhea should undergo follow-up testing to ensure microbiological cure and prevent further transmission.[50] Currently, increasing resistance to ceftriaxone and azithromycin has been reported. While high-level azithromycin resistance (minimum inhibitory concentration ≥256 mg/L) remains rare, its emergence threatens the long-term efficacy of empirical treatment strategies.[51,52]

4.3. Management of HPV infections

HPV is one of the most common STDs, transmitted through sexual contact and nonsexual skin-to-skin contact. HPV comprises a large family of small, double-stranded DNA viruses that cause warts in different body parts depending on the strain. The types of warts include genital warts, common warts, plantar warts, and flat warts. Moreover, some types of HPV are implicated in cervical cancer and oral cancer.[53]

Most HPV infections are asymptomatic and transient. According to the Centers for Disease Control (CDC), 90% of infections resolve spontaneously within 2 years. However, persistent infections may lead to the development of precancerous lesions and malignancies. Importantly, HPV-related cancers are often asymptomatic until late stages, underscoring the importance of regular screening. The HPV types that cause warts are different from those that cause cancer; thus, the presence of warts does not necessarily indicate increased cancer risk.

Diagnosis of HPV infection varies by sex. In women, regular pap tests help to identify abnormal cervical cells and assess cancer risk. Colposcopy may be used for further evaluation. In men, there are currently no Food and Drug Administration-approved HPV tests; however, anal pap tests are sometimes used to screen for anal cancer.[53]

There is no permanent treatment for HPV infection, as most cases resolve spontaneously. Some warts, such as genital warts, can be treated with medications, electrocautery, or cryotherapy with liquid nitrogen. Nevertheless, wart removal does not eradicate the underlying virus, and recurrence is common. For precancerous lesions, treatment may involve chemotherapy, radiation therapy, or surgical excision.

4.4. Management of HSV infections

HSV is one of the most prevalent STDs.[54] Two major types exist: HSV-1, which is commonly acquired during childhood and primarily causes orolabial lesions, and HSV-2, which is more frequently associated with genital infections.[55(p2)] Notably, HSV-1 can be transmitted through oral secretions during oral-genital contact. Asymptomatic HSV infection is common and is known to account for over 75% of viral transmission.[56] The incubation period of HSV usually ranges from 1 to 26 days.

Primary HSV infection often presents with painful genital or anal ulcers and bilateral tender inguinal lymphadenopathy. The initial stages of HSV-1 and HSV-2 infections are usually the same.[57] Systemic flu-like symptoms may occur, and in rare cases, sacral radiculomyelopathy can develop. In women, genital and urethral lesions may cause transient urinary retention. Severe complications of HSV infection include pneumonitis, disseminated infection, hepatitis, meningitis, and encephalitis.[58]

Diagnosis of HSV infection is achieved using viral culture, cell culture, and polymerase chain reaction (PCR). Among these methods, cell culture and PCR are the most widely preferred in patients presenting with genital ulcers or mucocutaneous lesions,[59] with PCR commonly used for the detection of HSV DNA in cerebrospinal fluid during suspected central nervous system infection. Meanwhile, viral culture and serology assays based on glycoprotein G1 (HSV-1) and glycoprotein G2 (HSV-2) help to detect specific HSV types.[60] For the treatment of HSV infections, the recommended regimens include acyclovir 400 mg taken orally three times daily for 7–10 days, famciclovir 250 mg taken orally three times daily for 7–10 days, or valacyclovir 1 g taken orally twice daily for 7–10 days.[61]

4.5. AMR

Antimicrobial drugs have revolutionized modern healthcare, providing effective treatment for life-threatening bacterial, viral, fungal, and parasitic infections. However, increasing levels of AMR have threatened the health benefits achieved with antibiotics, emerging as a global crisis.[62,63] Although often described as a new phenomenon, AMR has existed long before the clinical use of antimicrobials. Ancient bacterial samples, estimated to be 2000 years and even 30,000 years old, have shown resistance to ampicillin and vancomycin, respectively.[64,65] In addition, *Staphylococcus aureus* has been known to have resistance to penicillin since the introduction of the antibiotic into clinical use.[66]

Today, AMR to traditional treatments, such as azithromycin and doxycycline, is being actively monitored through global surveillance programs, including the WHO's Gonococcal Antimicrobial Surveillance Program and the CDC's Gonococcal Isolate Surveillance Project. These initiatives track resistance trends in pathogens such as *N. gonorrhoeae* and *M. genitalium*. In response to rising resistance, treatment guidelines are shifting toward resistance-guided therapy, incorporating molecular diagnostics to tailor antibiotic selection and reduce empirical treatment failures.[52]

The misuse and overuse of antimicrobials in both human medicine and agriculture have further accelerated the spread of resistance.[67] The National Institute of Animal Health has reported that 7.5–8.6 billion chickens, 60–92 million pigs, and 275–292 million turkeys are fed with various types of antibiotics, underscoring the global scale of antimicrobial pollution. In European countries, antibiotic-resistant bacteria are responsible for an estimated 30,000 deaths annually, with Italy and Greece having the highest number of cases.[68] In low- and middle-income countries across Asia, Africa, and South America, multidrug-resistant infections have higher mortality and morbidity rates.[69,70] Projections suggest that annual global deaths attributable to antibiotic-resistant infections may rise from 700,000 in 2014 to 10 million by 2050.[71]

4.6. Roles of antibiotics in HIV and STD management and their contribution to AMR

Antibiotics play a crucial role in managing opportunistic infections in people living with HIV and in treating bacterial STDs, such as gonorrhea, syphilis, and chlamydia. However, their frequent and often empirical use in these settings contributes significantly to the development of AMR. In HIV-positive individuals, recurrent infections and prophylactic antibiotic use can exert selective pressure, leading to the emergence of resistant strains. Similarly, the widespread and sometimes inappropriate use of antibiotics for STDs, particularly in the absence of antimicrobial susceptibility testing, has accelerated resistance in pathogens such as *N. gonorrhoeae* and *M. genitalium*. This growing AMR burden threatens the effectiveness of standard treatments, complicating care for co-infected patients and highlighting the urgent need for antibiotic stewardship programs.[72]

4.7. Mechanisms of drug resistance

AMR is the ability of microorganisms to survive in the presence of antimicrobial agents. The mechanisms of AMR are conserved across prokaryotes and eukaryotes and usually act by limiting the uptake of antimicrobial drugs, inactivating them, modifying their targets, or actively pumping them out of the cell.[73] For example, bacterial cells can resist antibiotic action by reducing the permeability of outer membranes, altering porin activity, or increasing multidrug efflux pumps.[74] Pathogens such as *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and fungal species such as *Candida lusitaniae* exemplify organisms that deploy such mechanisms.[75] Among them, the so-called ESKAPE pathogens (*Enterococcus faecium, S. aureus*, *Klebsiella pneumoniae*, *A. baumannii, P. aeruginosa*, and *Enterobacter* spp.) are responsible for the majority of hospital-acquired infections and exhibit high levels of AMR.[76]

In both prokaryotes and eukaryotes, the presence of antibiotic resistance genes (ARGs) is the main cause of bacterial resistance. These genes are often carried on plasmids, integrons, and transposons, which spread through horizontal gene transfer among strains of the same species and across different species. Even after resistant bacteria die, extracellular DNA containing ARGs stays active in the environment for a long duration, contributing to the environmental pool of resistance determinants.[77,78] In some cases, multiple resistance mechanisms coexist within a single bacterial cell, conferring high-level resistance to diverse classes of antibiotic compounds.[79] Moreover, some bacteria possess intrinsic resistance due to the lack of specific metabolic pathways, the lack of an antibiotic target, or the absence of enzymes needed for the activation of drugs.[80] For example, genes coding for a novel tripartite resistance-nodulation-cell division pump were found to be carried on a plasmid together with genes encoding for an antibiotic-targeting enzyme.[81] Furthermore, overexpression of efflux pumps is frequently observed in clinical isolates of *P. aeruginosa* and *S. aureus* from systemic infections, highlighting the importance of multidrug-resistance efflux pumps as the major resistance mechanism of AMR.[82] All such mechanistic approaches can now be identified using recently developed diagnostic technologies, which enable earlier detection of AMR.[48]

Beyond intrinsic resistance, microorganisms can also exhibit adaptive resistance, a transient, reversible phenotype triggered by environmental cues such as stress, pH changes, nutrient limitation, or exposure to subinhibitory levels of antibiotics. Adaptive resistance is often mediated through epigenetic regulation, including DNA methylation by DNA adenine methyltransferase, which drives heterogeneity in gene expression within bacterial populations. This allows subpopulations to survive antibiotic exposure and revert once the stressor is removed.[83] Mutations in drug targets also play a critical role—for example, mutations in DNA gyrase (*gyrA*), topoisomerase (*parC*) result in resistance to fluoroquinolones, the beta subunit of RNA polymerase (*rpoB*) mutations for rifampin resistance, 16s ribosomal RNA (rRNA) mutations for tetracycline and aminoglycosides resistance, and 23s rRNA mutations for linezolid resistance. Other antibiotic resistance mechanisms include the modification of target molecules. For example, in Gram-negative bacteria such as *A. baumannii*, resistance to polymyxins—cyclic antimicrobial peptides with long, hydrophobic tails that bind to bacterial lipopolysaccharides (LPSs) to disrupt cell membranes—is linked to modifications of LPS, where the addition of phosphoethanolamine to lipid A reduces colistin binding by decreasing net negative charge.

Bacteria such as *N. gonorrhoeae*, *C. trachomatis*, and *Treponema pallidum* have shown increasing resistance to first-line antibiotics, posing significant challenges to treatment protocols. *N. gonorrhoeae*, in particular, has developed resistance to multiple drug classes, including penicillins, tetracyclines, fluoroquinolones, and even extended-spectrum cephalosporins. The underlying mechanisms include mutations in penicillin-binding proteins, ribosomal targets, horizontal gene transfer, action of efflux pumps, and reduced permeability to antibiotics. The growing prevalence of multidrug-resistant strains underscores the importance of surveillance and timely diagnostics to monitor and control the spread of AMR in STD pathogens. Without sustained monitoring and stewardship, AMR not only threatens individual health but also amplifies the transmission risks, including the spread of HIV through co-infection.

5. Behavioral and biological pathways linking STDs and increased HIV transmissibility

STDs significantly amplify the risk of HIV transmission through both behavioral and biological mechanisms**.** Behaviorally, individuals with STDs often engage in high-risk sexual practices, such as inconsistent condom use, multiple sexual partners, or transactional sex, all of which increase exposure opportunities to HIV. Biologically, STDs like gonorrhea, chlamydia, syphilis, and HSV-2 cause mucosal inflammation, ulceration, and disruption of epithelial barriers, facilitating HIV entry. In addition, these infections recruit activated CD4$^+$ T cells and dendritic cells—primary targets for HIV—to the genital mucosa, further enhancing susceptibility. In HIV-positive individuals, concurrent STDs can increase genital HIV shedding, raising the risk of transmission. Together, these interlinked pathways act synergistically, fueling the dual epidemics of HIV and STDs and underscoring the need for integrated prevention strategies.[84]

6. Novel ways to tackle AMR

AMR is one of the most pressing global health threats, and no permanent solution has yet been achieved. Despite intensive scientific efforts, progress has been slow, with current strategies focusing primarily on improving diagnostic tools, optimizing antibiotic-prescribing practices, and strengthening infection-prevention strategies. Although several new antimicrobial agents are in clinical development, most of them belong to already existing antibiotic classes and do not represent true novel drug classes. Moreover, even newly developed compounds face the risk of rapid obsolescence due to microorganisms' remarkable capacity for adaptation. Therefore, novel treatment strategies are urgently needed to address established and emerging forms of AMR.

The development pipeline for new antimicrobial compounds continues to decline for complex reasons, despite increasing AMR rates and growing awareness of antibiotic persistence. A major factor is the pharmaceutical industry's focus on more profitable treatments for non-infectious chronic diseases, such as cancers, metabolic disorders, and cardiovascular conditions. Nevertheless, novel strategies and substances are being planned and discovered in basic research and preclinical studies, offering potential treatment options for AMR in the near future.

One promising example is the discovery of chimera peptidomimetic antibiotics, which have shown broad-spectrum antibacterial properties against Gram-negative ESKAPE pathogens and other Gram-negative bacteria.[85] Luther *et al.*[85] showed that the bactericidal activity of these chimeric antibiotics involves binding to both LPSs and BamA, the primary component of the β-barrel folding complex required for folding and inserting β-barrel proteins into the outer membrane. This unique targeting strategy allows activity against Gram-negative bacteria while sparing eukaryotic cell membranes. However, the exact mechanism remains an open question. Among these antibacterial candidates, POL7306 has advanced to preclinical trials, with current research focusing on optimizing peptide designs and broadening therapeutic margins before clinical testing.[86]

In addition, the widespread and often unregulated use of antimicrobials in agriculture has emerged as a major driver of AMR. In poultry, swine, and other livestock production, antibiotics such as tetracyclines, macrolides, and fluoroquinolones are commonly administered not only for therapeutic purposes but also for disease prevention and growth promotion. This prolonged and sub-therapeutic exposure creates selective pressure, fostering the emergence and proliferation of resistant bacterial strains, including *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. These pathogens can be transmitted to humans through multiple routes, including the consumption of contaminated food, direct contact with animals, and environmental dissemination through soil, water, and air contaminated with animal waste. Importantly, resistance genes harbored by these bacteria can also be transferred horizontally to human-associated pathogens, amplifying the risk of difficult-to-treat infections. Thus, the agricultural use of antimicrobials serves as a critical link in the broader ecology of resistance, necessitating urgent implementation of stewardship programs, regulatory oversight, and global One Health approaches to mitigate AMR at the human-animal-environment interface.[87,88]

A drug-drug interaction occurs when one drug affects the activity of another drug when administered together. Such interactions can either enhance or reduce the effectiveness or toxicity of one or both drugs. Drug-drug interactions occur through several mechanisms, including pharmacokinetic interactions, pharmacodynamic interactions, combined toxicity, and additive or synergistic effects. In pharmacokinetic interactions, the absorption, distribution, metabolism, or excretion of one or both drugs is involved. In pharmacodynamic interactions, two drugs with similar or opposing effects interact at the same receptor site or related physiological pathways. Combined toxicity arises when co-administered drugs produce similar toxic effects, thereby increasing the risk of adverse reactions. Additive or synergistic effects occur when two drugs with similar pharmacological actions produce a combined effect greater than the sum of their individual effects.

Drug-drug interactions are a major concern in pharmacotherapy, where the pharmacological effect of a victim drug is either exaggerated or suppressed by the perpetrator drug. Combinations of drugs are clinically used to treat or cure diseases. It has been proven that, in some cases, the combination of two drugs provides superior outcomes compared to monotherapy.[89] For example, anti-tuberculosis drug combinations enhance treatment efficacy and reduce the risk of resistance in *Mycobacterium tuberculosis* infections.[90] Beyond drug-drug interactions, interactions can also occur between drugs and metabolites, endogenous substances, food, or diagnostic agents.[91]

Although drug-drug interactions can sometimes enhance drug efficacy, they may also reduce therapeutic efficacy by altering the nature, intensity, duration, side effects, or toxicity of the drugs involved.[92] In some cases, such interactions lead to unexpected adverse effects, which are considered harmful events.[93] Adverse drug reactions may result from a single drug or a combination therapy, but the risk is particularly elevated when multiple drugs are administered simultaneously.[94] This complexity increases the likelihood of clinically relevant drug–drug interactions, contributing to higher healthcare costs, increased hospitalization rates, and longer hospitalization duration.[95]

7. Urgent public health strategies to break the cycle of co-infection and AMR

Various strategies can be considered from a public health point of view to implement integrated STD and HIV service delivery, particularly in high-risk populations. Delivering integrated screening, treatment, and prevention services—including PrEP and ART—through a single platform enhances outcomes while reducing redundancy in services.[96] The following strategies need to be considered:

- Integrated STI/HIV services to ensure routine testing, treatment, and follow-up
- Antimicrobial stewardship programs in HIV clinics and STD services to curb empirical and syndromic overuse of antibiotics
- Expanded PrEP coverage, especially among high-risk groups with recurrent STDs
- Use of point-of-care molecular diagnostics to reduce inappropriate antibiotic use
- Behavioral interventions, including condom promotion and chemsex harm reduction
- Real-time genomic surveillance to detect resistant STD strains and guide treatment policies

However, several barriers hinder the effective implementation of these strategies, particularly in resource-limited settings:[97]

- Vertical programming and separate funding streams
- Shortages in trained personnel
- Stigma and discrimination in care settings
- Fragmented surveillance systems.

8. Research gaps and future directions

HIV, STDs, and AMR are interconnected through biological, clinical, and public health mechanisms. STDs increase the risk of HIV acquisition and transmission by causing mucosal disruption, local inflammation, and immune activation that enhances the availability of HIV target cells. In turn, HIV-induced immunosuppression complicates the management of STDs, often leading to persistent or recurrent infections. The frequent and often syndromic use of antibiotics for bacterial STDs—particularly among people living with HIV—contributes to the emergence and spread of resistant strains, such as *N. gonorrhoeae*, *M. genitalium*, and *T. pallidum*. This exacerbates treatment challenges and limits therapeutic options.

Moving forward, the development of combination treatment strategies that address both HIV and co-occurring STDs while minimizing AMR risk is essential. Integrated care models, antimicrobial stewardship, and the development of prophylactic vaccines against STDs, such as gonorrhea, chlamydia, and syphilis, could reduce HIV transmission by addressing key cofactors. Strengthening global surveillance systems, expanding access to healthcare for vulnerable populations, and implementing targeted public health interventions—including condom promotion, regular screening, PrEP, and comprehensive sexual health education—are critical to disrupting the cycle of HIV, STDs, and AMR.

9. Conclusion

The interplay between HIV, STDs, and AMR in the cART and antibiotic era presents complex challenges that require comprehensive, multifaceted interventions. Effective management involves the integration of HIV and STD programs to ensure coordinated screening, treatment, and prevention efforts, particularly among high-risk populations. Prioritizing epidemiological surveillance for both established and emerging pathogens, such as *M. genitalium* and drug-resistant *N. gonorrhoeae*, is essential to inform evidence-based responses. Concurrently, the rational use of antimicrobials both in clinical and agricultural settings must be enforced through both stewardship programs and regulatory oversight to mitigate resistance development. Expanding access to preventive tools, such as PrEP, maternal ART, and vaccination (HPV, HBV), remains central to reducing transmission, especially in low-income and vulnerable communities. Furthermore, culturally sensitive health education, youth engagement, and social determinants such as early marriage, stigma, and inadequate sex education are vital for long-term behavioral change. To ensure sustained progress, investments in research and development are urgently needed to deliver novel therapeutics, vaccines, point-of-care diagnostics, and resistance-guided therapies. Ultimately, tackling these interlinked threats requires a unified strategy involving public health authorities, clinicians, researchers, policymakers, and communities working collaboratively to reduce the global burden of co-infections and drug resistance.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Microbiota–gut–brain axis: Modulation of gut microbiota in the management of Alzheimer's disease

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Abstract

Insights into the role of the gut microbiota are advancing our understanding of Alzheimer's disease (AD), which is the most common cause of dementia and a condition characterized by progressive cognitive decline and pathological hallmarks such as amyloid beta (Aβ) plaques and neurofibrillary tangles. The gut microbiota, composed of diverse microorganisms, influences brain health through the microbiota–gut–brain axis. This review outlines how alterations in gut microbial composition and metabolites occur across different stages of cognitive decline. The microbiota–gut–brain axis mediates interactions between the gut and brain, influencing neuroinflammation, Aβ accumulation, tau pathology, and oxidative stress. Therapeutic strategies targeting gut microbiota, including diet modulation, probiotics, prebiotics, synbiotics, microbial metabolites, and fecal microbiota transplantation, have shown potential in improving cognitive function in clinical and animal studies. Despite these advances, challenges remain in addressing individual variability, standardization, and long-term safety. Personalized microbiota-based interventions may provide promising tools for the diagnosis, prevention, and treatment of AD.

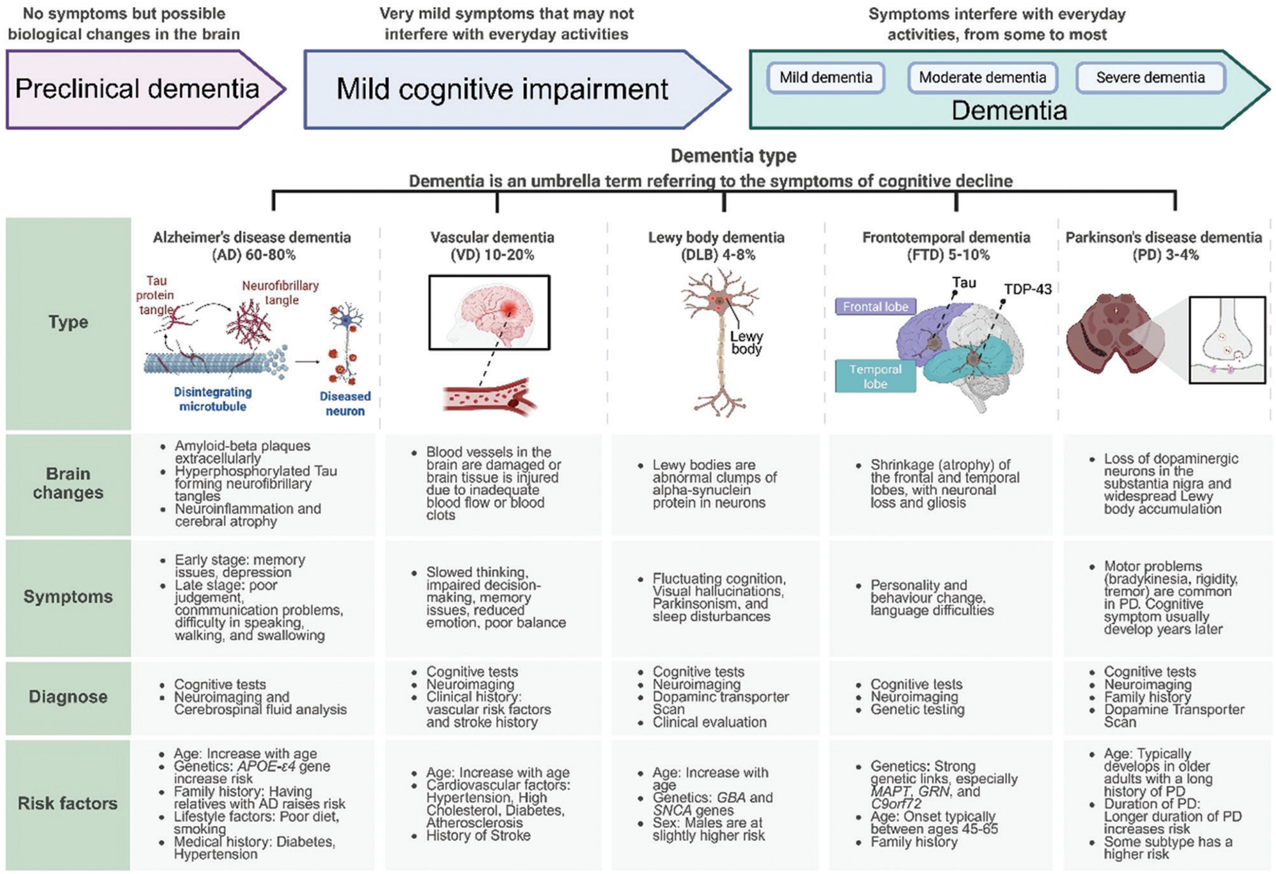
Keywords: Alzheimer's disease; Gut microbiota; Microbiota–gut–brain axis; Therapeutic strategies

1. Introduction

With the global population aging, dementia has become a major public health concern.[1] Dementia is a clinical syndrome characterized by progressive cognitive and memory decline that interferes with daily activities.[2,3] It has various underlying causes, depending on the brain changes involved, with major subtypes[2,3] including Alzheimer's disease (AD) dementia, vascular dementia, frontotemporal dementia, dementia with Lewy bodies, and Parkinson's disease dementia (Figure 1). AD is the most common subtype, accounting for about 60–80% of dementia cases in people over 65.[4] In the United States alone, more than 7 million people live with AD, imposing an estimated economic burden of 384 billion USD in 2024.[4] Mild cognitive impairment (MCI), a transitional stage before dementia, affects 8–11% of older adults.[5] These figures underscore the urgency of identifying modifiable factors, such as the gut microbiota, to develop novel strategies for delaying or preventing cognitive decline.

Amid the growing burden of AD, increasing attention has focused on the gut microbiota's role in human health.[6,7] The human gastrointestinal tract is densely populated by a diverse community of microorganisms, including bacteria, archaea, viruses, and fungi, collectively known as the gut microbiota. These microbes maintain gut barrier integrity, support immune defenses, and protect against pathogens. Beyond local effects, the gut microbiota communicates bidirectionally with the central nervous system (CNS) via the microbiota–gut–brain axis, influencing brain development, mood, and cognition. Recent studies demonstrate its significant impact on nervous system function, with growing evidence linking it to cognition and AD mechanisms.[8] Dysbiosis, involving changes in gut microbiota composition and function, is associated with systemic inflammation[9] and blood–brain barrier (BBB) disruption,[10] which may worsen neurodegeneration. In addition, microbial metabolites, such as short-chain fatty acids (SCFAs) and neurotransmitters, modulate immune responses and neuronal activity, affecting cognition through the microbiota–gut–brain axis.[11] These interactions present therapeutic potential in targeting gut microbiota to restore balance and slow cognitive decline in AD.

Although associations between gut dysbiosis and AD have been reported, most studies to date demonstrate correlations rather than direct causation, and the possibility of reverse causality or shared confounding factors (e.g., age, diet, and comorbidities) cannot be excluded. Nevertheless, understanding the mechanisms by which gut microbiota influence neuroinflammation and neurodegeneration remains essential. Building on this context, this review aims to deepen our insight into the alterations in gut microbial composition across different stages of cognitive decline and the roles of microbial metabolites. In addition, we explore emerging therapeutic strategies targeting the



Type	Alzheimer's disease dementia (AD) 60-80%	Vascular dementia (VD) 10-20%	Lewy body dementia (DLB) 4-8%	Frontotemporal dementia (FTD) 5-10%	Parkinson's disease dementia (PD) 3-4%
Brain changes	• Amyloid-beta plaques extracellularly • Hyperphosphorylated Tau forming neurofibrillary tangles • Neuroinflammation and cerebral atrophy	• Blood vessels in the brain are damaged or brain tissue is injured due to inadequate blood flow or blood clots	• Lewy bodies are abnormal clumps of alpha-synuclein protein in neurons	• Shrinkage (atrophy) of the frontal and temporal lobes, with neuronal loss and gliosis	• Loss of dopaminergic neurons in the substantia nigra and widespread Lewy body accumulation
Symptoms	• Early stage: memory issues, depression • Late stage: poor judgement, conmmunication problems, difficulty in speaking, walking, and swallowing	• Slowed thinking, impaired decision-making, memory issues, reduced emotion, poor balance	• Fluctuating cognition, Visual hallucinations, Parkinsonism, and sleep disturbances	• Personality and behaviour change, language difficulties	• Motor problems (bradykinesia, rigidity, tremor) are common in PD. Cognitive symptom usually develop years later
Diagnose	• Cognitive tests • Neuroimaging and Cerebrospinal fluid analysis	• Cognitive tests • Neuroimaging • Clinical history: vascular risk factors and stroke history	• Cognitive tests • Neuroimaging • Dopaminc transporter Scan • Clinical evaluation	• Cognitive tests • Neuroimaging • Genetic testing	• Cognitive tests • Neuroimaging • Family history • Dopamine Transporter Scan
Risk factors	• Age: Increase with age • Genetics: *APOE-ε4* gene increase risk • Family history: Having relatives with AD raises risk • Lifestyle factors: Poor diet, smoking • Medical history: Diabetes, Hypertension	• Age: Increase with age • Cardiovascular factors: Hypertension, High Cholesterol, Diabetes, Atherosclerosis • History of Stroke	• Age: Increase with age • Genetics: *GBA* and *SNCA* genes • Sex: Males are at slightly higher risk	• Genetics: Strong genetic links, especially *MAPT*, *GRN*, and *C9orf72* • Age: Onset typically between ages 45-65 • Family history	• Age: Typically develops in older adults with a long history of PD • Duration of PD: Longer duration of PD increases risk • Some sublype has a higher risk

Figure 1. Overview of the dementia continuum and different types of dementia. Created with BioRender.com. JIANG, H. (2025) https://BioRender.com/dw03crz.

gut microbiota, including probiotics, prebiotics, synbiotics, dietary modifications, and fecal microbiota transplantation (FMT), highlighting their potential to slow or prevent cognitive decline.

2. Gut microbiota alterations in AD

Clinical studies have increasingly shown that individuals with AD have altered gut microbiota, with reduced diversity and shifts in specific bacterial taxa (Table 1).[12-32] For instance, a study of 97 individuals, comprising patients with AD, MCI, and healthy controls (HC), revealed notable differences in gut microbiota profiles. AD patients showed reduced microbial diversity compared to MCI and HC. Compositionally, the population of Firmicutes was markedly decreased, while Proteobacteria was enriched in AD patients. These changes extended to higher taxonomic levels, with Gammaproteobacteria, Enterobacteriales, and Enterobacteriaceae progressively increasing from HC to MCI to AD. Functional analysis based on Kyoto Encyclopedia of Genes and Genomes pathways revealed increased modules related to glycan biosynthesis and metabolism in both MCI and AD groups, while immune-related pathways were decreased in AD. Importantly, discriminative models based on predominant microbiota effectively distinguished MCI and AD from HC (area under the curve [AUC] = 0.890 and 0.940, respectively), and also separated AD from MCI (AUC = 0.925).[17] Emerging evidence also suggests that gut microbiota alterations may appear during the early and even preclinical stages of AD. In one study of 164 cognitively normal individuals, which included 49 with early AD biomarkers, distinct microbial patterns were found in those with preclinical AD. These differences were linked to amyloid beta (Aβ) and tau pathology, as well as a reduced Firmicutes/Bacteroidetes ratio. When combined with clinical features, these microbial profiles improved the accuracy of machine learning models in predicting preclinical AD.[29] Another study proposed that gut microbiota, when combined with plasma Aβ, could serve as a non-invasive, cost-effective tool for early AD screening, with an accuracy of 87%.[27] With the advancement of next-generation sequencing technologies, especially shotgun metagenomics, more comprehensive microbial profiling has become possible. Most notably, a recent large-scale metagenomic study involving 476 Chinese participants across five AD stages provided the most detailed characterization to date.[31] The study identified over 70 microbial species with stage-dependent alterations. Notably, *Faecalibacterium* spp., known for producing SCFAs, were elevated in early AD, potentially modulating neuroinflammation and neurodegeneration. In contrast, *Alistipes*, *Bacteroides*, and *Prevotella* spp., associated with inflammation and neurotransmitter disruption, increased in MCI and AD stages. Reductions were also found in lactate-producing *Streptococcus*, possibly weakening anti-inflammatory effects. Functionally, over 10% of microbial gene families were altered, especially those involved in metabolism. These microbial signatures showed strong diagnostic value (AUC = 0.80 cross-validation; 0.75 external validation). Moreover, enrichment of certain opportunistic pathogens, such as *Erysipelatoclostridium ramosum* (formerly *Clostridium ramosum*), has been associated with poorer cognitive function,[33] suggesting a potential pathogenic role in cognitive decline. In addition, microbial metabolites also exhibit alterations in AD and hold promise as potential biomarkers. For example, levels of trimethylamine N-oxide (TMAO), a gut-derived metabolite, are elevated in the cerebrospinal fluid (CSF) of AD patients and correlate with CSF markers of amyloid, tau pathology, and neurodegeneration.[34] Lipopolysaccharides (LPS) and amyloid-like microbial proteins have also been implicated in AD pathogenesis.[35] Moreover, SCFAs, including propionate, butyrate, acetate, valerate, and hexanoate, may influence disease progression by modulating immune-inflammatory responses through prostaglandin and T cell pathways.[36] Altogether, these findings underscore the intricate relationship between gut microbiota and brain health in AD, enabling the advancement of novel diagnostic and treatment strategies directed toward the microbiota–gut–brain axis. Given the strong influence of geography, diet, and ethnicity on gut microbiota composition, cross-population comparisons are essential to identify universal versus population-specific microbial biomarkers for AD. Most existing studies focus on single ethnic groups, such as Chinese or Western cohorts, limiting generalizability. Dietary habits and environmental exposures shape microbial profiles differently, which may affect the reliability of microbiota-based diagnostics across populations. Therefore, multi-ethnic studies are crucial for validating consistent microbial signatures and enhancing the global applicability of gut microbiota in AD prediction and intervention.

Comparable results have also been observed in animal models of AD, which generally show reduced microbial diversity and shifts toward proinflammatory taxa, alongside a decline in beneficial bacteria, such as *Faecalibacterium*, *Bifidobacterium*, and *Lactobacillus* (Table 2).[37-46] In the study by Gu *et al.*,[45] a significant increase in the relative abundance of Firmicutes was observed in amyloid precursor protein (APP)/presenilin-1 (PS1) mice (41% compared to 35% in wild type [WT]), while the Bacteroidetes was reduced (54% compared to 60% in WT). These consistent findings across animal models and human studies strengthen the evidence that gut microbiota dysbiosis is an early event in

Table 1. Overview of human gut microbiota alterations associated with healthy controls, mild cognitive impairment, and Alzheimer's disease

Author	Year	Subjects	Method	Main findings
Cattaneo *et al.*[12]	2017	Aβ^+ CI (*n*=40) Aβ^- CI (*n*=40) HC (*n*=10)	Quantitative polymerase chain reaction	Lower *Eubacterium rectale* and higher *Escherichia/Shigella* in Aβ^+ CI subjects compared with HC and Aβ^- CI groups.
Vogt *et al.*[13]	2017	AD (*n*=25) HC (*n*=10)	16S rRNA sequencing	Lower Firmicutes and *Bifidobacterium* and higher Bacteroidetes in AD patients.
Zhuang *et al.*[14]	2018	AD (*n*=43) HC (*n*=43)	16S rRNA sequencing	Lower Bacteroidetes and higher Actinobacteria in AD patients.
Haran *et al.*[15]	2019	AD (*n*=24) HC (*n*=51)	Metagenomics sequencing	Higher Bacteroides, Alistipes, Odoribacter, and Barnesiella, and lower Lachnoclostridium in AD patients.
Li *et al.*[16]	2019	AD (*n*=30) MCI (*n*=30) HC (*n*=30)	16S rRNA sequencing	Similar changes in gut bacterial populations in MCI and AD; AD and MCI subjects exhibited higher *Escherichia* in fecal and blood samples.
Liu *et al.*[17]	2019	AD (*n*=33) aMCI (*n*=32) HC (*n*=32)	16S rRNA sequencing	Lower Firmicutes and higher Proteobacteria in AD patients compared with HC controls.
Saji *et al.*[18]	2019	MCI (*n*=61) HC (*n*=21)	16S rRNA sequencing	Higher *Bacteroides* in MCI individuals.
Ling *et al.*[19]	2020	AD (*n*=100) HC (*n*=71)	16S rRNA sequencing	Lower *Faecalibacterium* and higher Bifidobacterium in AD patients.
Zhou *et al.*[20]	2021	AD (*n*=60) HC (*n*=32)	16S rRNA sequencing	Higher Bifidobacterium, Sphingomonas, Lactobacillus, and Blautia, and lower Odoribacter, Anaerobacterium, and Papillibacter in AD patients.
Zhang *et al.*[21]	2021	MCI (*n*=75) HC (*n*=52)	16S rRNA sequencing	Lower microbial diversity and reduced Faecalibacterium, Ruminococcaceae, and *Alistipes* in MCI individuals.
Guo *et al.*[22]	2021	AD (*n*=18) MCI (*n*=20) HC (*n*=18)	16S rRNA sequencing	Lower *Bacteroides*, *Lachnospira*, and *Ruminiclostridium_9* and higher *Prevotella* in AD patients compared with HC controls; *Lachnospira* was also reduced in MCI individuals relative to HC controls.
Pan *et al.*[23]	2021	MCI (*n*=22) HC (*n*=26)	16S rRNA sequencing	Lower Bacteroidetes and higher Fusobacteria in MCI individuals.
Liu *et al.*[24]	2021	aMCI (*n*=20) HC (*n*=22)	16S rRNA sequencing	Higher Bacteroidetes in aMCI individuals.
Jung *et al.*[25]	2022	Aβ^+ CN (*n*=18) Aβ^- CN (*n*=60)	16S rRNA sequencing	Higher Megamonas, Serratia, Leptotrichia, and Clostridium, and lower CF231, Victivallis, Enterococcus, and Mitsuokella in Aβ^+ CN individuals.
Zhu *et al.*[26]	2022	AD (*n*=83) MCI (*n*=125) HC (*n*=94)	16S rRNA sequencing	Higher Erysipelatoclostridiaceae, Erysipelotrichales, Patescibacteria, Saccharimonadales, and Saccharimonadia in AD and MCI subjects.
Sheng *et al.*[27]	2022	AD (*n*=11) MCI (*n*=11) CN$^+$ (*n*=32) CN$^-$ (*n*=34)	16S rRNA sequencing	Higher Bacteroidetes and lower Firmicutes and Deltaproteobacteria in CN$^+$individuals compared with CN$^-$ individuals.
Kaiyrlykyzy *et al.*[28]	2022	AD (*n*=41) HC (*n*=43)	16S rRNA sequencing	Higher Acidobacteriota, Verrucomicrobiota, Planctomycetota, Synergistota, *Prevotella*, *Akkermansia*, and *Ruminococcus*, and lower *Bifidobacterium*, *Roseburia*, Lactobacillaceae, and *Faecalibacterium* in AD patients.
Ferreiro *et al.*[29]	2023	Preclinical AD (*n*=49) HC (*n*=115)	Metagenomic sequencing	Dorea formicigenerans, *Faecalibacterium prausnitzii*, Coprococcus catus, and Anaerostipes hadrus were most strongly related to preclinical AD individuals.

(Cont'd)

Table 1. ***(Continued)***

Author	Year	Subjects	Method	Main findings
He *et al.*[30]	2023	CI (*n*=30) SCD (*n*=62) HC (*n*=35)	16S rRNA sequencing	Lower Lachnospiraceae, *Fusicatenibacter*, *Lachnospiracea_ incertae_sedis*, and *Anaerobutyricum* with decreasing cognitive ability; higher Rikenellaceae, *Alistipes*, and Odoribacteraceae in the CI individuals.
Jia *et al.*[31]	2025	NC (*n*=63) SCS (*n*=82) SCD (*n*=90) MCI (*n*=119) AD (*n*=122)	Metagenomic sequencing	Higher *Faecalibacterium* spp. in early-stage AD; increased *Alistipes*, *Bacteroides*, and *Prevotella* spp. in MCI and AD with more severe cognitive impairment.
Fan *et al.*[32]	2025	HC (*n*=320) MCI (*n*=119)	Metagenomic sequencing	A total of 59 microbial taxa were related to AD and MCI biomarkers; species from the same genus, such as *Bacteroides* and *Ruminococcus*, had contrasting effects; *Akkermansia muciniphila* was associated with lower amyloid burden.

Abbreviations: AD: Alzheimer's disease; aMCI: Amnestic mild cognitive impairment; Aβ: Amyloid beta; CI: Cognitive impairment; CN: Cognitive normal; HC: Healthy control; MCI: Mild cognitive impairment; SCD: Subjective cognitive decline; SCS: Subjective cognitive symptoms.

Table 2. Overview of gut microbiota dysbiosis in Alzheimer's disease animal models

Author	Year	Animals	Method	Main findings
Shen *et al.*[37]	2017	APP/PS1 mice WT mice	16S rRNA sequencing	Higher Helicobacteraceae and Desulfovibrionaceae (family level) and *Odoribacter* and *Helicobacter* (genus level) in APP/PS1 mice; *Prevotella* was higher in WT mice.
Zhang *et al.*[38]	2017	APP/PS1 mice WT mice	16S rRNA sequencing	Higher Proteobacteria and Verrucomicrobia in APP/PS1 mice.
Carolin *et al.*[39]	2017	5×FAD mice WT mice	16S rRNA sequencing	Higher Firmicutes and *Clostridium leptum* group and lower Bacteroidetes in 5×FAD mice.
Bäuerl *et al.*[40]	2018	APP/PS1 mice WT mice	16S rRNA sequencing	Higher Proteobacteria and Erysipelotrichaceae in APP/PS1 mice.
Sun *et al.*[41]	2019	P301L mice WT mice	16S rRNA sequencing	Higher Bacteroidetes and lower Firmicutes at the phylum level in P301L mice compared with WT mice after 3 months of age.
Wang *et al.*[42]	2019	5×FAD mice WT mice	16S rRNA sequencing	Higher Bacillota/Bacteroidota ratio in 5×FAD mice.
Chen *et al.*[43]	2020	APP/PS1 mice WT mice	16S rRNA sequencing	Higher Proteobacteriaceae, Verrucomicrobiaceae, Bifidobacteriaceae, Erysipelotrichaceae, and Prevotellaceae, and lower Bacteroidaceae and Rikenellaceae in APP/PS1 mice.
Tan *et al.*[44]	2020	Drosophila melanogaster WT controls	Gut microbiota composition analysis	Higher *Wolbachia* and lower gut microbiota diversity in AD flies.
Gu *et al.*[45]	2021	APP/PS1 mice WT mice	16S rRNA sequencing	Lower SCFA-producer bacteria (*Parasutterella* and *Blautia*) and higher Bacillota/ Bacteroidota ratio in APP/PS1 mice.
Medina *et al.*[46]	2021	3×Tg-AD mice WT mice	16S rRNA sequencing	Lower Actinobacteria in 3×Tg-AD mice at 3 months of age.

Abbreviations: AD: Alzheimer's disease; APP: Amyloid precursor protein; PS1: Presenilin-1; WT: Wild-type.

AD development and may actively contribute to disease progression. It is critical to interpret these findings with caution, as the majority of evidence, both across human studies and preclinical models, demonstrates observational associations and correlations rather than definitive causation. The temporal link between alterations in gut microbiota and the emergence of AD is complex, and the potential for reverse causality (e.g., AD-related pathological and lifestyle changes influencing the microbiome) or confounding by factors, such as diet, medication, and comorbidities, cannot be excluded. Despite consensus, both clinical and preclinical studies have shown conflicting trends. For instance, some studies have noted a reduction in Bacteroidetes abundance in AD patients,[14,22] while others have observed an increase.[15,24] These inconsistencies likely stem from differences in population characteristics, diet, environmental conditions, and sequencing methods, highlighting the need for standardized, multicenter studies. Moreover, while reduced microbial diversity is generally linked to poor health, this is not always the case. Studies have found that individuals with major depressive disorder and autism may exhibit greater gut microbial diversity than HC,[47] indicating that diversity alone may not reflect health status. Instead, specific microbial taxa or functional profiles may play a more decisive role in influencing brain health and AD risk. In view of this, research should perhaps focus less on the overall diversity of the gut microbiota and more on the key microbial signatures that might be protective or harmful. As evidenced by the growing body of research summarized in Table 1, multiple microbial taxa have demonstrated consistent associations with AD across various studies. For instance, genera such as *Bacteroides* (often increased) and *Faecalibacterium* (often decreased) are frequently reported. With the advancement of metagenomic sequencing, species-level signatures are increasingly being identified. However, it is worth noting that the establishment of universal microbial biomarkers remains challenging. The lack of a single consistently replicated signature across all cohorts chiefly stems from substantial heterogeneity in study populations (e.g., genetics, diet, and geography), the functional redundancy of the gut ecosystem, and methodological differences in sequencing and bioinformatic analysis. Therefore, large-scale, multicenter prospective studies with standardized methodologies are urgently needed to overcome these limitations, to definitively elucidate which bacterial species or community structures are causally linked to cognitive trajectories, and to translate these findings into targeted preventive or therapeutic strategies.

3. Microbiota–gut–brain axis and its dysregulation in AD

3.1. Microbiota–gut–brain axis and bidirectional communication

The microbiota–gut–brain axis enables complex bidirectional communication between the gut microbiota, enteric nervous system, and CNS, involving three interconnected routes: local neuronal modulation, systemic circulation, and multiple host-microbe interactions. Locally, microbial signals, such as gamma-aminobutyric acid (GABA), primarily produced by *Lactobacillus*, could modulate the vagus nerve or act directly on enteric neurons to influence brain function and cognition.[48] At the systemic level, microbiota-derived metabolites (e.g., SCFAs and tryptophan derivatives) and host molecules (e.g., cytokines and hormones) might enter the bloodstream and reach the brain via the BBB.[49] Indole derivatives, such as indolepropionic acid and kynurenines, have neuroactive and antioxidative properties, modulating neuronal function and inflammation.[50] At the interface level, microbiota interact with gut enteroendocrine cells, enterochromaffin cells, and mucosal immune cells to influence the release of neuromodulators, such as serotonin (5-HT), which activate receptors on neurons and immune cells, ultimately affecting brain activity via the autonomic nervous system and the hypothalamic–pituitary–adrenal axis.[51] Together, the local, systemic, and immune–endocrine pathways of the microbiota–gut–brain axis illustrate how gut microbiota dynamically influence brain function and homeostasis. These communication routes are tightly regulated by the intestinal barriers and BBBs, which protect the CNS from harmful insults. Disruption of these barriers or alterations in microbial signaling may impair neuroimmune balance and neuronal regulation.

3.2. Dysregulation of the microbiota–gut–brain axis in AD

Growing evidence indicates that gut microbiota dysbiosis contributes to AD pathogenesis through multiple interconnected mechanisms, including Aβ accumulation, neuroinflammation, oxidative stress, and tau pathology (Figure 2).

3.2.1. Aβ accumulation

According to the well-established Amyloid–Tau–Neurodegeneration framework, extracellular deposition of Aβ is a core pathological marker of amyloid pathology in AD. Aβ peptides are generated from the sequential proteolytic cleavage of the APP, first by β-secretase and subsequently by γ-secretase, yielding aggregation-prone species, such as Aβ40 and Aβ42. These peptides can oligomerize and form extracellular plaques, contributing to neurotoxicity and synaptic dysfunction.[52,53] Microbial metabolites, notably SCFAs, such as butyrate, and tryptophan derivatives, influence cerebral Aβ dynamics. In AD mouse models, oral administration of butyrate has been shown to reduce brain Aβ levels and enhance cognitive memory performance, with a negative correlation observed between brain Aβ deposition and the levels of butyrate and IL-10.[54,55] Furthermore, SCFAs, such as butyrate, propionate, and pentanoate, can inhibit the formation of Aβ dimers and trimers, with pentanoate also impeding the conversion of Aβ monomers into fibrils.[56] This effect may be partly mediated through 5-HT signaling pathways, as serotonin availability depends on tryptophan metabolism and serotonin transporter function.[57] Dysbiosis of the gut microbiota can impair SCFAs' function and disrupt tryptophan availability, exacerbating brain Aβ deposition due to insufficient 5-HT synthesis in the brain.

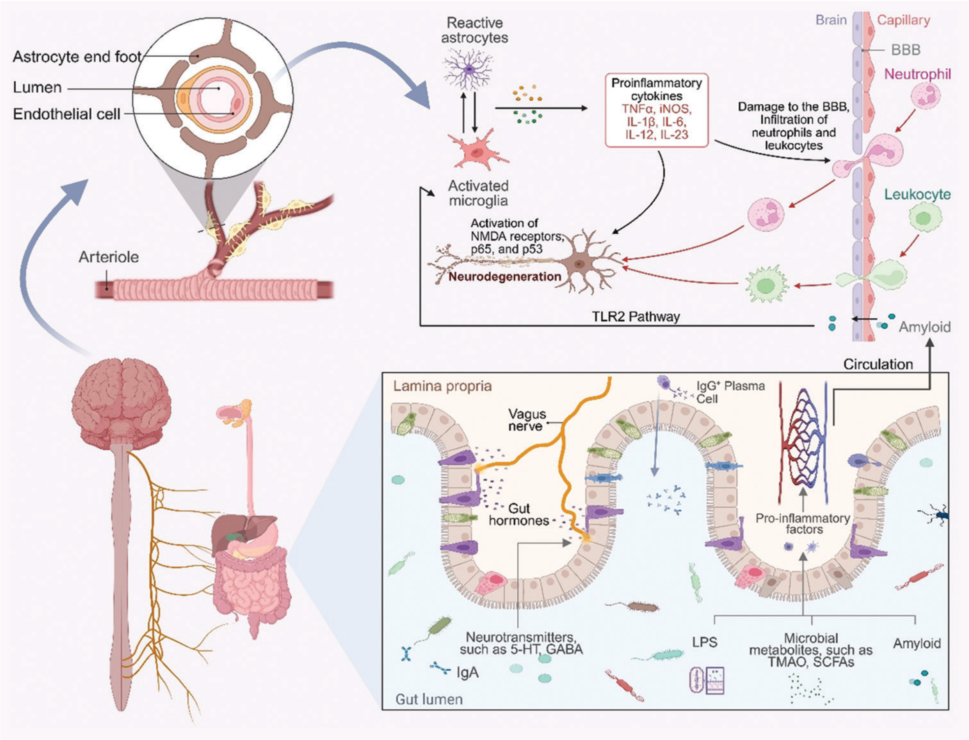


Figure 2. Interplay between gut dysfunction, neuroinflammation, and the blood–brain barrier in the pathogenesis of Alzheimer's disease. Created with BioRender.com. JIANG, H. (2025) https://BioRender.com/izd3ej5.

Abbreviations: 5-HT: Serotonin; BBB: Blood–brain barrier; GABA: Gamma-aminobutyric acid; IL: Interleukin; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; NMDA: N-methyl-D-aspartate; SCFAs: Short-chain fatty acids; TLR: Toll-like receptor; TMAO: Trimethylamine N-oxide; TNF: Tumor necrosis factor.

3.2.2. Neuroinflammation and barrier dysfunction

Recent research shows that gut microbiota imbalance plays a critical role in the complex mechanisms of AD, including chronic neuroinflammation, barrier damage, and immune responses. A compromised intestinal barrier ("leaky gut") allows LPS, TMAO, and other proinflammatory molecules to enter circulation, triggering systemic inflammation and BBB dysfunction.[58,59] This facilitates microglial and astrocytic activation and sustained cytokine release (e.g., interleukin [IL]-1β, IL-6, and tumor necrosis factor [TNF]-α), which initially promote Aβ clearance but prolonged activation of microglia leads to the sustained release of proinflammatory cytokines, initiating an inflammatory cascade that damages the BBB barrier, ultimately resulting in neuronal deterioration and death. TNF-α and IL-1β can directly cause synaptic loss through caspase activation, glutamate toxicity, and complement-mediated pruning.[60] In addition, microbial amyloids from *Escherichia coli* and *Staphylococcus aureus* can activate microglia through the toll-like receptor 2 pathway, leading to elevated levels of cytokines IL-17A and IL-22, triggering nuclear factor-κB (NF-κB) signaling and cyclooxygenase-2 activation, inducing inflammatory responses and phagocytosis, thereby exacerbating brain damage.[61] It is well-established that with age, the permeability of the gastrointestinal mucosa and the BBB increases.[10] It has been hypothesized that in this context of increased barrier permeability, microbial components (such as amyloids) could potentially translocate more easily. Crucially, upon entering the brain, these bacterial amyloids are proposed to act as potent seeds that directly catalyze the misfolding and aggregation of endogenous human Aβ through a process of cross-seeding, thereby accelerating the formation of toxic oligomers and fibrils.[62,63] Furthermore, the presence of bacterial amyloids intensifies neuroinflammation, establishing a self-perpetuating loop that destabilizes proteostasis and contributes to AD pathogenesis. While experimental models support this mechanism, the extent of its contribution to human AD requires further robust evidence. However, SCFAs, such as butyrate from the gut microbiota, could help maintain barrier integrity and suppress inflammation, supporting their potential therapeutic role.[55]

3.2.3. Oxidative stress and mitochondrial dysfunction

There is a reciprocal relationship between oxidative stress and Aβ aggregation in AD, with each reinforcing the other.[64] The gut microbiota significantly influences oxidative stress, mitochondrial dysfunction, and neurodegenerative diseases by producing SCFAs, tryptophan derivatives, and antioxidants, which protect mitochondrial functions. Butyrate, an SCFA, activates Nuclear factor erythroid 2-related factor 2 (Nrf2) by inhibiting histone deacetylases (HDAC) and induces epigenetic modifications of Nrf2 promoters associated with antioxidant responses, thereby mitigating oxidative stress. Hoyles *et al.*[65,66] further demonstrated, using an *in vitro* BBB model with hCMEC/D3 cells, that propionate activates Nrf2, regulates redox homeostasis, and reduces the release of reactive oxygen species, thereby protecting the BBB.[65,66]

3.2.4. Tau pathology and neurofibrillary tangles

Two pathological markers, Tau protein and insoluble neurofibrillary tangles, are primarily found in the neuronal axons of the brain. Proinflammatory cytokines elevated by gut dysbiosis can activate kinases, such as glycogen synthase kinase-3β and cyclin-dependent kinase 5, promoting tau phosphorylation.[67,68] Tau hyperphosphorylation and aggregation are also linked to gut dysbiosis, particularly through microbiota-induced inflammation in apolipoprotein E (APOE)-sensitive models.[69] Animal studies using germ-free or antibiotic-treated models show that modulation of gut microbiota reduces tau pathology, suggesting a causal role.[70] Therapeutic interventions targeting the microbiota have shown promise in attenuating tau-related neurodegeneration in preclinical studies.[69] While mechanisms remain to be fully elucidated, these findings highlight the microbiota–gut–brain axis as a novel target in tau pathology.

In summary, dysregulation of the microbiota–gut–brain axis contributes to AD by promoting neuroinflammation, barrier dysfunction, and oxidative stress. Gut microbiota and their metabolites also influence Aβ deposition and tau pathology, key features of AD. This complex bidirectional communication highlights the important role of gut microbes in disease progression. Understanding these interactions may offer new opportunities for microbiota-targeted therapies to slow or prevent AD.

4. Therapeutics targeted at the gut microbiota in AD

Given the accumulating evidence that gut microbiota dysbiosis contributes to AD pathogenesis through neuroinflammation, amyloid deposition, and barrier dysfunction, therapeutic strategies targeting the gut microbiome have emerged as promising avenues to slow or prevent cognitive decline. These interventions include dietary modification, prebiotics, probiotics, synbiotics, FMT, and other approaches (Figure 3).

4.1. Diet

Recent mechanistic evidence further emphasizes the fundamental role of diet in shaping gut microbiota resilience. Kennedy *et al.*[71] demonstrated that mice treated with antibiotics recovered their gut microbial diversity and function only when fed a balanced, fiber-rich chow diet. In contrast, a Western-style diet, characterized by high fat and low fiber, impaired microbiota recovery and increased vulnerability to *Salmonella* infection. The study revealed that an appropriate dietary environment supports beneficial microbial interactions and metabolic functions, highlighting diet as a necessary condition for restoring gut homeostasis and maintaining host health. Through its influence on gut composition and function, diet plays a central role in regulating the microbiota–gut–brain axis, thereby contributing to cognitive health and potentially mitigating the progression of neurodegenerative diseases.

Several dietary patterns have demonstrated neuroprotective effects partly through microbiota modulation. The Mediterranean diet (MD), rich in fruits, vegetables, whole grains, legumes, nuts, olive oil, and moderate fish intake, is the most extensively studied. Meta-analyses report that adherence to MD correlates with approximately 20% reduced risk of dementia and improvements in global cognition and memory domains.[72] The MD fosters an increase in SCFAs-producing bacteria, such as *Faecalibacterium*, which contribute to anti-inflammatory effects and maintain intestinal barrier integrity.[73] In addition, MD components, such as olive oil, contain polyphenols that attenuate Aβ and tau pathology, reduce oxidative stress, and strengthen BBB function.[74] The Dietary Approaches to Stop Hypertension (DASH) diet emphasizes vegetables, fruits, low-fat dairy, whole grains, and lean proteins. It reduces vascular risk factors for dementia and modulates microbiota toward beneficial taxa, resulting in improved anti-inflammatory profiles and cognitive function.[75] The Mediterranean-DASH intervention for neurodegenerative delay diet combines elements of MD and DASH, focusing on brain-healthy foods. Emerging data indicate its effectiveness in slowing cognitive decline by enriching beneficial gut microbiota and enhancing SCFAs production.[76] The ketogenic diet (KD), high in fat and low in carbohydrates, shifts metabolism to ketone body utilization. A modified Mediterranean-KD was shown to increase beneficial SCFAs and alter gut microbiota composition, such as increasing *Akkermansia*, in association with improved AD biomarkers in individuals with MCI.[77] However, there are also studies presenting opposing viewpoints. Wang *et al.*[78] reported that KD-related dyslipidemia may negatively impact AD neuropathology by initiating the formation

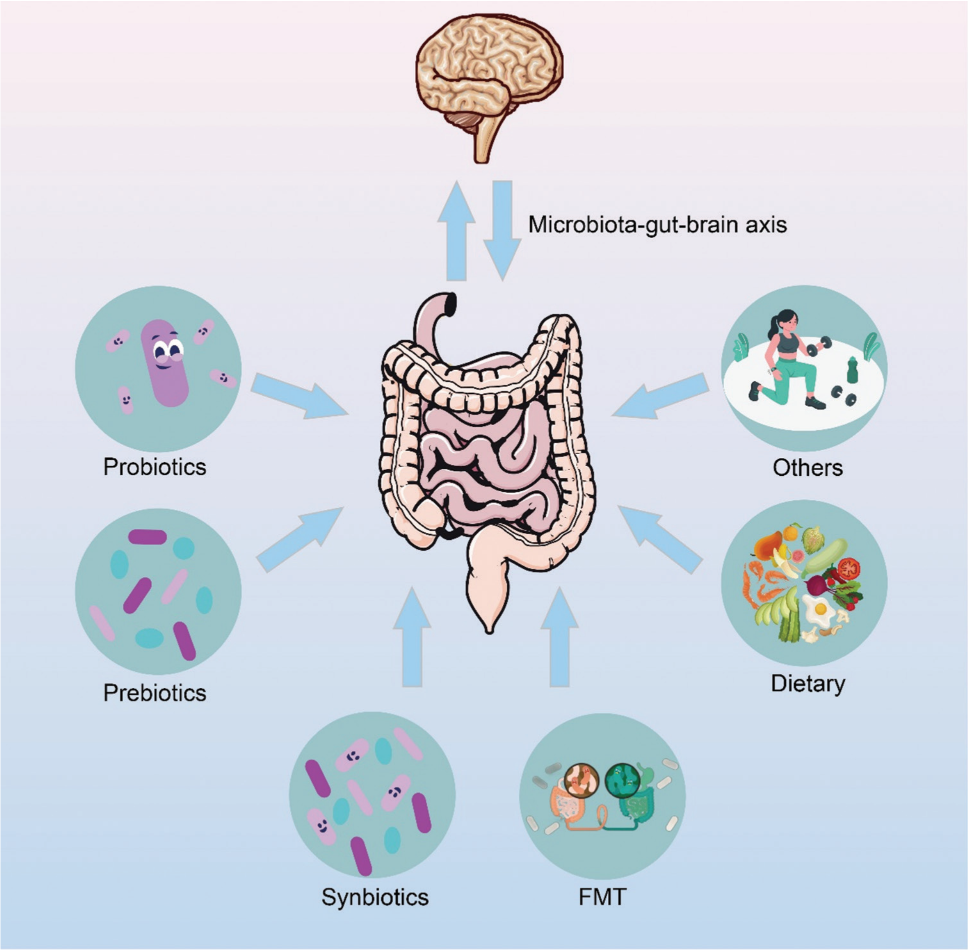


Figure 3. Overview of gut microbiota-targeted interventions in Alzheimer's disease. Image created by the authors.
Abbreviation: FMT: Fecal microbiota transplantation.

of abnormal neural connectivity in individuals with AD. Therefore, the use of the KD diet in AD patients should be approached with caution. Among the various dietary components, dietary fiber plays a particularly important role in modulating the gut microbiota and supporting cognitive health. A diet rich in fiber is associated with increased gut microbial diversity, promoting microbial balance and inhibiting pathogenic bacteria.[79] Fiber in the diet functions as a nutrient source for beneficial microbes, stimulating the production of SCFAs and supporting overall host well-being.[80] Through fiber fermentation, gut acidity increases, supporting the proliferation of beneficial anaerobes and inhibiting microbes linked to inflammation.[81] In contrast, low-fiber, refined-grain-rich diets may diminish these benefits, promoting proinflammatory bacteria, such as Enterobacteriaceae, and elevating circulating LPS. Increased LPS levels can induce systemic and neuroinflammation, leading to BBB impairment and cognitive deterioration.[82]

In summary, these studies mentioned above primarily emphasize the advantages of a diet abundant in vegetables and fruits for individuals with AD. However, the optimal intake levels of these foods have not yet been established in the literature. A previous study conducted by our team examined the basic requirements for fruits and vegetables and found that consuming a minimum of three servings of vegetables and two servings of fruits every day may aid in preventing dementia among older adults.[83] In conclusion, diet significantly affects the composition of the gut microbiota, potentially improving neuroprotection. Overall, a comprehensive mechanistic understanding is needed to elucidate the complex interactions between various diet patterns, gut microbiota, microbial metabolites, and their effects on cognitive function and the progression of AD. Moreover, addressing the need for personalized diets to accommodate significant individual variations and maximize therapeutic efficacy is a critical issue.

4.2. Prebiotics

Prebiotics are currently described as ingredients that are selectively fermented by gut microbiota, not absorbed in the digestive tract, and that positively influence the gut microbiota, thereby benefiting the host's health.[84] Recently, the effect of prebiotic and protein on muscle in older twins randomized controlled trial investigated the effects of a prebiotic supplement on cognition in older adults.[85] In this double-blind, placebo-controlled study, 72 participants aged above 60 received daily prebiotics or a placebo for 12 weeks alongside resistance exercise and branched-chain amino acid supplementation. Prebiotic supplementation significantly improved cognitive performance compared to placebo ($\beta = -0.482$; $p=0.014$), accompanied by increased abundance of beneficial *Bifidobacterium* species. This trial highlights the potential of prebiotics to enhance cognitive function through modulating gut microbiota in aging populations. Supporting the clinical finding, animal studies have demonstrated that prebiotics function as substrates for microbial fermentation, resulting in the generation of SCFAs, which are essential for modulating the host's immune response by inducing T-cell differentiation. Several prebiotics have shown direct benefits on brain function. For example, fructo-oligosaccharides supplementation in transgenic AD mice increased synapsin-1 expression and activated the glucagon-like peptide-1 signaling pathway, leading to improved brain insulin sensitivity and cognitive function.[86] Similarly, xylo-oligosaccharides helped restore BBB integrity, reduced inflammation, and improved cognition by upregulating tight junction proteins, such as zonula ocludens-1, and reshaping gut microbiota.[87] Ferulic acid, a plant-derived compound with prebiotic-like effects, also attenuated neuroinflammation and Aβ burden in AD mice, possibly via improvement of synaptic plasticity.[88] In addition, advances in synthetic chemistry and bioengineering have enabled the development of designed prebiotics.[89] These structures allow targeted fermentation by beneficial microbes closely linked to human health, such as *Faecalibacterium prausnitzii* and *Roseburia* spp. Designed prebiotics offer enhanced specificity, structural consistency, and controllable metabolic outcomes, such as boosting SCFAs production. These precision tools hold promise for personalized nutrition or therapeutic adjuncts in neurodegenerative disease management.

Together, these clinical and preclinical findings underscore the promising role of prebiotics in modulating the gut microbiota to improve cognitive function and counteract neuroinflammation in aging and AD. However, further research is needed to optimize prebiotic formulations, dosages, and long-term efficacy in diverse human populations before widespread clinical application.

4.3. Probiotics and synbiotics

Probiotics are beneficial microorganisms that reside in the human intestines, which could prevent harmful bacteria from establishing themselves and boost the body's immune response.[90] Probiotics improve gut microbiota diversity, maintain intestinal barrier and BBB integrity, reduce Aβ, decrease neuroinflammation, increase butyrate-producing bacteria, such as *F. prausnitzii*, *Ruminococcus*, and *Clostridium*, and enhance cognition.[91,92] They also modulate oxidative stress in AD neuropathology. In Aβ-treated rats, *Lactobacillus* and *Bacillus* strains boosted antioxidant defenses.[93] Neuroinflammation also plays a significant role in this process. Zhu *et al.*[94] showed that *Bifidobacterium breve HNXY26M4* improved cognition, reduced neuroinflammation, restored gut microbiota, increased SCFAs, and enhanced intestinal barrier in APP/PS1 mice. Clinical studies also support the benefits: 24-week *B. breve A1* supplementation improved mini-mental state examination scores (+ 1.7, $p<0.01$), as well as profile of mood states 2nd edition and gastrointestinal symptom rating scale scores, which suggested that *B. breve A1* may enhance cognitive performance and life quality in elderly populations with MCI.[95] In a randomized controlled trial involving 130 patients aged 65–88 years, daily supplementation with *B. breve MCC1274* for 24 weeks resulted in significant improvements in cognitive function compared to the placebo group. While overall gut microbiota composition did not change markedly, probiotic supplementation appeared to suppress brain atrophy progression, particularly in those with advanced brain atrophy. These results suggest that *B. breve MCC1274* may be beneficial in preventing cognitive decline in MCI patients.[96]

Synbiotics are combinations of prebiotics and probiotics that synergistically promote beneficial gut microbes.[84] Arora *et al.*[97] reported that a synbiotic formula containing *Acetobacter*, *Lactobacillus fermentum*, *Bifidobacterium*, *Kluyveromyces*, and others in a 4% kefir matrix improved memory, executive function, and language in humans, while reducing pro-inflammatory cytokines (IL-12, IL-8, TNF-α) and enhancing anti-inflammatory responses. In APP/PS1 mice, Deng *et al.*[98] found that a synbiotic combining inulin with multiple probiotic strains (e.g., *Bacillus coagulans*, *Bifidobacterium longum*) improved cognition, reduced Aβ42 levels, and alleviated inflammation more effectively than inulin alone. Similarly, Westfall showed that synbiotics combining *Trifolium pratense* extract with probiotics improved survival in *Drosophila melanogaster*, outperforming either component alone.[99] Studies in APP/PS1 mice also showed that synbiotics corrected gut dysbiosis, reduced neuroinflammation, and slowed disease progression through peroxisome proliferator-activated receptor pathway activation.[100,101] These findings suggest synbiotics may offer a more comprehensive strategy than single-agent approaches in AD prevention and management. However, clinical evidence remains scarce. Further research is needed to validate their effects and clarify whether synbiotics outperform probiotics or prebiotics alone.

4.4. Microbiota-derived metabolites

Gut microbiota-derived metabolites include amino acid derivatives (such as tryptophan metabolites, spermidine, and nicotinamide N-oxide), fatty acid metabolites (such as butyrate, propionate, and acetate), and other compounds, such as secondary bile acids, GABA, TMAO, and urolithin A.[102-104] Among these metabolite groups, SCFAs, particularly acetate, propionate, and butyrate, represent one of the most extensively investigated categories. Current evidence suggests these compounds mediate their physiological effects through several distinct molecular mechanisms: (i) Epigenetic regulation: SCFAs act as broad-spectrum HDAC inhibitors, with butyrate exhibiting the highest inhibitory efficiency (~80%) and propionate ~60%.[105,106] HDAC inhibition increases histone acetylation, loosening chromatin structure and enhancing transcription factor access to promoters. This leads to upregulation of genes involved in neuronal survival, synaptic plasticity, oxidative stress defense, and cellular homeostasis, which collectively support cognitive function;[107-109] (ii) Immunomodulation: SCFAs bind to G-protein-coupled receptors (GPR41/FFAR3, GPR43/FFAR2, GPR109A/HCAR2) on immune cells, including neutrophils, monocytes, and lymphocytes.[110,111] Receptor activation triggers downstream pathways (Gαi, Gαq, mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2, β-arrestin2), inhibits nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing protein 3 inflammasome activation, promotes differentiation of regulatory T cells, and reduces proinflammatory cytokines while increasing IL-10.[112] These immunomodulatory effects help maintain systemic and neuroimmune homeostasis, reducing microglial overactivation and neuroinflammation that contribute to AD pathology; (iii) Microglial modulation: Butyrate suppresses microglial overactivation, enhances synaptic plasticity, and improves cognition in 5×FAD mice.[113] Some studies report SCFAs may also increase Aβ deposition through altered microglial phagocytosis, potentially APOE-dependent;[114,115] (iv) Neurotransmitter regulation: SCFAs influence neurotransmitter synthesis through enteroendocrine signaling, enhancing serotonin (via tryptophan hydroxylase), GABA, dopamine, and norepinephrine metabolism. These changes impact cognitive function, supported by SCFA-mediated improvements in AD models.[116,117] Through these mechanisms, SCFAs play critical roles in neuroprotection and cognitive function, suggesting that modulating their levels may represent a targeted therapeutic strategy in AD. In animal models, oral administration of sodium butyrate and sodium propionate has been shown to significantly reduce Aβ accumulation and improve cognitive function in AD mice.[65,118] Supplementation with tauroursodeoxycholic acid, a secondary bile acid, also improved spatial and recognition memory while reducing Aβ deposits in the brain.[119] Conversely, elevated levels of acetate and TMAO have been associated with greater Aβ burden and cognitive decline, suggesting that lowering these metabolites may be beneficial.[120,121]

Compared with administering live bacteria, modulating microbiota-derived metabolites may avoid risks, such as antibiotic resistance, virulence factors, or bloodstream infections. Nonetheless, while several metabolites have shown neuroprotective effects in animal studies, robust clinical trials in humans are still lacking. Further research is needed to validate their therapeutic potential in AD.

4.5. FMT

FMT is a treatment method designed to restore the balance and diversity of the gut microbiota.[122] Today, FMT is used in conditions like *Clostridium difficile* infection and inflammatory bowel diseases, and is gaining interest in neurodegenerative disorders, such as AD,[123] based on the role of gut microbiota in AD pathogenesis.

Animal studies suggest FMT may benefit AD by restoring microbial balance and regulating SCFAs production. For example, D'Amato *et al.*[124] found that FMT from aged to young mice impaired spatial memory, likely due to loss of SCFAs-producing bacteria, such as *Faecalibaculum*, Lachnospiraceae, and Ruminococcaceae. In contrast, FMT in AD mouse models improved cognition, reduced Aβ accumulation, and increased butyrate production.[125,126] Recent evidence also reveals a dose-dependent effect: in 5×FAD mice, FMT given every other day for 8 weeks improved memory and reduced Aβ load, whereas weekly administration showed minimal benefit. These effects were linked to inhibition of the toll-like receptor 4/IκB kinase β/NF-κB pathway and lower LPS levels in the colon and hippocampus.[127] However, cognitive improvements declined over time, suggesting a time-sensitive response. Clinical evidence is limited to two case reports. One described cognitive and mood improvements in an 82-year-old man after receiving FMT from his wife; another reported enhanced cognition, microbial diversity, and SCFAs in a 90-year-old woman post-FMT from a younger donor.[128,129] Overall, FMT shows promise as a novel AD intervention through gut microbiota modulation. However, the translational challenges are substantial, including interspecies differences in microbiota composition and the difficulty in selecting optimal donors. Therefore, while FMT holds promise as a novel intervention for AD, large-scale, well-controlled clinical trials are urgently needed to evaluate its efficacy, safety, dose-response relationships, and long-term outcomes.

4.6. Others

In addition to the aforementioned interventions, recent studies highlight the potential of exercise and lifestyle changes in modulating the microbiota–gut–brain axis and improving cognitive outcomes in AD. Nicolas *et al.*[130] reported that chronic disruption of gut microbiota using an antibiotic cocktail impaired hippocampal neurogenesis-dependent behaviors in rats. Notably, voluntary exercise attenuated these behavioral deficits and restored adult hippocampal neurogenesis. While changes in the hippocampal metabolome were not observed, improvements were linked to alterations in cecal metabolites, highlighting the critical role of gut microbiota in brain function and the potential of lifestyle factors, such as exercise, to counteract dysbiosis-induced impairments.

5. Considerations and implications

The global rise in the prevalence of AD, alongside challenges in current diagnostics and uncertain long-term efficacy of new amyloid-targeting antibodies,[131] demands alternative strategies. Gut microbiota-based targets show promise but face key obstacles: Microbiota variability influenced by diet, age, medication, and geography complicates biomarker standardization; most studies are cross-sectional, so longitudinal research is needed to confirm causal links with AD. Developing reliable, non-invasive, and affordable diagnostic tools requires standardized sample handling. The complex bidirectional host-microbiota interactions remain underexplored, limiting precise interventions. Comorbidities and medications further confound results. Variability in sampling and analysis hinders reproducibility and clinical translation. Although microbial metabolites hold therapeutic potential, their systemic effects and delivery require in-depth study. Ethical and regulatory considerations are crucial, particularly in protecting vulnerable populations with cognitive impairment during trials, ensuring informed consent and autonomy. The long-term safety of interventions, such as FMT, must be rigorously assessed. Access inequities may worsen disparities in AD care; collaborative efforts should promote fair distribution. Safe, effective microbiota-targeted therapies could slow or reverse cognitive decline, reduce caregiver and societal burdens, and complement existing treatments by targeting neurodegeneration mechanisms. Moreover, microbiota modulation may aid AD prevention, delaying onset and benefiting public health. Realizing this requires interdisciplinary collaboration among neuroscientists, microbiologists, clinicians, regulators, and patient advocates to design rigorous, safe, and effective clinical trials and responsibly translate findings.

6. Conclusion

This review underscores the pivotal role of the microbiota–gut–brain axis in AD, linking gut dysbiosis to key pathological processes. Therapeutic strategies targeting the gut microbiota show promising potential in managing and possibly preventing AD. Future efforts should focus on precise microbial profiling, personalized interventions, and well-designed clinical trials to translate these findings into effective clinical applications. Advancing this field offers hope for improved diagnosis, treatment, and prevention of AD.

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Conflict of interest

Zhilu Xu is Scientist (Diagnostics) of GenieBiome Ltd. Siew C. Ng has served as an advisory board member for Pfizer, Ferring, Janssen and Abbvie and received honoraria as a speaker for Ferring, Tillotts, Menarini, Janssen, Abbvie and Takeda; has received research grants through her affiliated institutions from Olympus, Ferring and Abbvie; is a founder member, non-executive director, non-executive scientific advisor and shareholder of GenieBiome Ltd which is non-remunerative; is a shareholder of MicroSigX Diagnostic Holding Limited; is a founder member, non-executive Board Director, and non-executive scientific advisor of MicroSigX Biotech Diagnostic Limited, which is non-remunerative; and receives patent royalties through her affiliated institutions; is a named inventor of patent applications held by The Chinese University of Hong Kong and MagIC that cover the therapeutic and diagnostic use of the microbiome. All other authors declare that they have no conflicts of interest.

Author contributions

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ACCSCIENCE PUBLISHING

Microbes & Immunity

PERSPECTIVE ARTICLE

Unraveling complex interactions between microbiota and immune system

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Abstract

The intricate relationship between the gut microbiota and the immune system has garnered significant attention in recent years, revealing a complex interplay essential for maintaining health and preventing disease. This perspective article delves into the dynamic interactions between the gut microbiota and the immune system, exploring how microbial communities influence immune development, function, and homeostasis. Emerging research highlights the impact of microbial metabolites, signaling pathways, and host genetics on immune responses. We also address the implications of microbiota-immune interactions in various diseases, including autoimmune disorders, infections, and cancer. Unraveling these complex interactions may provide a comprehensive understanding of the microbiota-immune system axis and its potential for guiding new therapeutic interventions. This article emphasizes the need for interdisciplinary approaches and advanced technologies to further elucidate the mechanisms underpinning this critical biological partnership.

Keywords: Gut microbiota; Immune system; Diet; Signaling pathways

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1. Introduction

The human gut microbiota is a vast and complex community of microorganisms, including bacteria, viruses, fungi, and archaea, that live in the gastrointestinal tract.[1-3] These microorganisms outnumber human cells and play an integral role in maintaining health and homeostasis. The gut microbiota is established early in life and continues to evolve throughout an individual's lifetime, influenced by factors such as diet, environment, and lifestyle.[4-10] This dynamic microbial ecosystem is crucial for various physiological processes including digestion, nutrient absorption, and the synthesis of essential vitamins and bioactive compounds (Figure 1).

Along with these primary functions, the gut microbiota also heavily acts as a key modulator of the host's immune system responses.[11-14] It also promotes immune tolerance to beneficial microbes and food antigens to facilitate immune system development and maturation while maintaining vigilance against pathogens (Figure 1).[15,16] In addition, the gut microbiota constitutes a protective barrier against infections by competing with pathogenic microorganisms for resources and space.

Recent research has highlighted that the gut microbiota has a profound impact on overall health and is associated with different conditions through various molecular mechanisms (Table 1).[17-20] An imbalance in the microbial community, called dysbiosis,

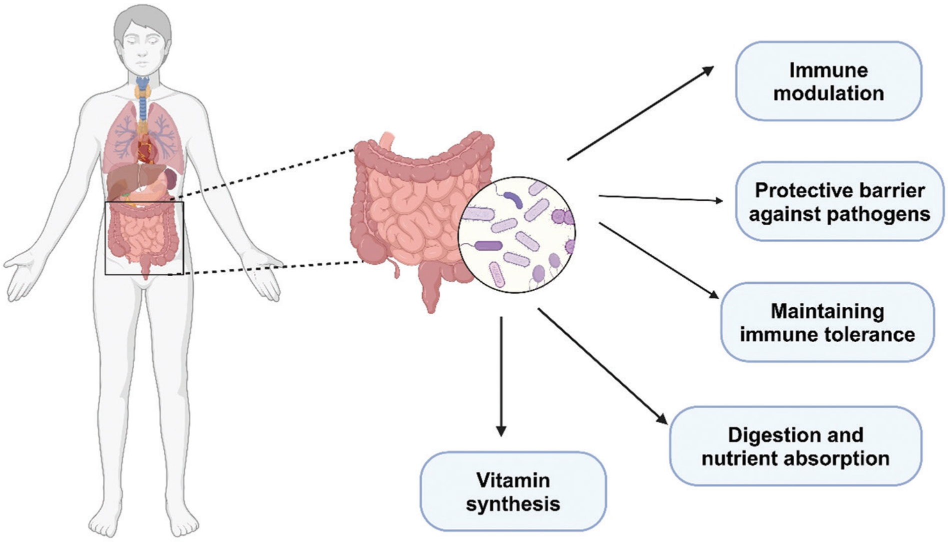

Figure 1. A schematic representation of the key functions of gut microbiota and their impact on health. Created with Biorender.com.

has been linked to many disorders,[21-24] while alterations in the gut microbiota have been implicated in the development of autoimmune diseases, allergies, and even neurological conditions.[25-30] Understanding the intricate relationship between the gut microbiota and health is opening new avenues for therapeutic interventions. Strategies such as dietary modifications, probiotics, prebiotics, and fecal microbiota transplantation are being explored to restore and maintain a healthy microbiota balance,[31-35] offering potential benefits for disease prevention and treatment.

At the same time, host immunity also plays a crucial role in shaping the gut microbiome by influencing which microbes can survive and thrive in the gut environment (Table 2).[36] Immune responses, including the production of antimicrobial peptides (AMPs), secretory immunoglobulin A (IgA), and pro-inflammatory cytokines, regulate microbial populations and contribute to maintaining a balanced and diverse gut ecosystem.[37] This bidirectional relationship highlights the importance of both the gut microbiota and the immune system in maintaining overall health. By continuously unraveling the complexities of these interactions, we will be able to clarify the role of gut microbiota in human health, establishing it as a target for novel therapeutic strategies.

2. Development of immune tolerance following microbial exposure

2.1. Microbial colonization and immune education

Microbial exposure begins at birth when newborns are first colonized by microorganisms from their mothers and the surrounding environment.[38] The mode of delivery and breastfeeding can influence the infant microbiome.[39-43] The initial infant gut colonization is crucial for training the immune system. In particular, the gut microbiota plays a significant role in shaping the immune landscape. Microbial antigens and metabolites interact with epithelial cells and immune cells in the gut, leading to the activation and differentiation of immune cells such as dendritic cells, macrophages, and T cells.[44,45] Through these interactions, the immune system acquires the ability to distinguish between harmless commensal microbes and potential pathogens, which is essential for maintaining immune tolerance.

2.2. Regulatory T cells and immune homeostasis

Regulatory T cells (Tregs) are a subset of T cells that are vital for maintaining immune tolerance.[44,45] They suppress immune responses that could otherwise lead to autoimmunity and chronic inflammation. The gut microbiota significantly influences the development and function of Tregs. Specific microbial species, such as certain strains of *Bacteroides* and *Clostridia*, promote the differentiation and expansion of Tregs.[46,47] By producing metabolites such as short-chain fatty acids (SCFAs), particularly butyrate, these gut microbes enhance the function of Tregs by modulating their gene expression and metabolic activity.[48,49] This ensures that the immune system remains tolerant to commensal microbes and dietary antigens while still mounting effective responses against pathogens.

2.3. Microbial metabolites and immune modulation

The metabolites produced by the gut microbiota, such as SCFAs, play a crucial role in immune regulation.

Table 1. A summary of the diverse mechanisms through which the gut microbiome influences host immunity

Mechanism	Description	Impact on host immunity
PRRs	The gut microbiota releases MAMPs that are recognized by PRRs such as TLRs and NLRs on innate immune cells such as macrophages and dendritic cells.	Activation of PRRs triggers immune responses, including cytokine release and AMP production, which regulate microbial balance and defense.
Modulation of immune cell function	Gut microbes interact with innate immune cells such as macrophages, dendritic cells, and neutrophils, influencing their functions and promoting either tolerance or immune activation depending on microbial composition.	In homeostasis, gut macrophages promote tolerance to commensal bacteria. In dysbiosis, immune activation can lead to inflammatory diseases such as IBD.
Production of SCFAs	Gut bacteria ferment dietary fiber into SCFAs such as butyrate, acetate, and propionate. These metabolites influence both innate and adaptive immune responses.	SCFAs have anti-inflammatory effects, promoting Tregs and enhancing barrier function while also modulating dendritic cells and macrophages.
Production of AMP	Microbial signals can stimulate the gut epithelium to produce AMPs, such as defensins and cathelicidins, that help maintain the integrity of the intestinal barrier.	AMPs limit the growth of pathogenic bacteria and help maintain a balanced microbiome, thus protecting the host from infections and promoting immune tolerance.
Production of IgA	The gut microbiota induces the production of secretory IgA, which coats commensal bacteria and pathogens, preventing their overgrowth and invasion into host tissues.	IgA helps maintain microbial diversity and balance, preventing immune overreaction and fostering tolerance to beneficial microbes.
T cell differentiation	The gut microbiota influences the differentiation of T cells, including Tregs, Th17 cells, and other adaptive immune cells, by producing metabolites and interacting with dendritic cells.	A balanced microbiome promotes Treg differentiation, which prevents excessive inflammation, while dysbiosis can enhance Th17 responses, leading to autoimmunity.

Abbreviations: AMP: Antimicrobial peptide; IBD: Inflammatory bowel disease; IgA: Immunoglobulin A; MAMPs: Microbial-associated molecular patterns; NLRs: NOD-like receptors; PRRs: Pattern recognition receptors; SCFAs: Short-chain fatty acids; Th17 cells: Th17 cells; TLRs: Toll-like receptors; Tregs: Regulatory T cells.

Table 2. A summary of the key mechanisms by which host immunity influences the gut microbiome

Immune mechanism	Description	Impact on gut microbiome
AMPs	The host immune system produces AMPs such as defensins and cathelicidins, which target and kill specific microbes in the gut.	AMPs shape the composition of the microbiome by selectively eliminating harmful bacteria while allowing beneficial microbes to thrive.
IgA	IgA, secreted by B cells in the gut, binds to commensal and pathogenic bacteria, preventing their adherence to the intestinal lining and controlling microbial load.	IgA helps maintain a balanced microbiota by coating commensal bacteria, reducing pathogen overgrowth, and promoting microbial diversity.
T cell responses	T cells, especially Tregs and Th17 cells, regulate immune tolerance and inflammation in the gut through cytokine secretion.	Tregs promote tolerance to commensal microbes, reducing inflammation, whereas Th17 cells, if overactive, can induce dysbiosis and contribute to inflammatory diseases.
Inflammation (Cytokine release)	Pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α are released during immune responses, driving inflammation in response to infection or injury.	Chronic inflammation can disrupt the gut microbial balance, leading to dysbiosis, favoring pro-inflammatory microbes, and reducing beneficial bacterial populations.
Innate immune recognition (PRRs)	PRRs detect microbial components (e.g., LPS and flagellin), triggering immune responses to control pathogenic bacteria.	Activation of PRRs can modulate microbial populations by eliminating pathogens while promoting the survival of symbiotic microbes that are not recognized as threats.
Neutrophil and macrophage activity	Neutrophils and macrophages engulf and destroy harmful bacteria through phagocytosis, especially during infection or inflammation.	Excessive neutrophil activity or inflammation can disrupt the balance of the microbiome, leading to the loss of beneficial microbes and overgrowth of harmful ones.
Gut barrier function	The immune system maintains the integrity of the intestinal barrier, preventing microbial translocation into the systemic circulation.	A well-maintained barrier limits microbial translocation and keeps the microbiome confined to the gut, protecting the host from systemic infections.
Secretory factors (mucus, bile acids)	Immune cells promote the production of mucus and bile acids, which modulate the microbial environment by influencing nutrient availability and habitat structure.	Changes in mucus and bile acid production can alter microbial niches in the gut, impacting microbial diversity and function.

Abbreviations: AMP: Antimicrobial peptide; IgA: Immunoglobulin A; IL: Interleukin; LPS: Lipopolysaccharide; PRRs: Pattern recognition receptors; Th17 cells: Th17 cells; TNF-α: Tumor necrosis factor alpha; Tregs: Regulatory T cells.

SCFAs, including acetate, propionate, and butyrate, have significant effects on immune cells.[50-52] For instance, butyrate has been shown to enhance the production of anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor-beta, which are vital for Treg function.[48,49] In addition, SCFAs can inhibit the production of pro-inflammatory cytokines by immune cells, thereby promoting a balanced immune response that favors tolerance over inflammation.[50-52] This metabolic crosstalk between gut microbes and the host immune system is a fundamental mechanism for immune tolerance.

2.4. Microbiota and mucosal immunity

The gut-associated lymphoid tissue (GALT) is a critical site for inducing immune tolerance,[53,54] where gut microbiota influences the development and function of various immune cells. For example, the presence of certain commensal bacteria stimulates the production of IgA by B cells.[55] IgA is the most abundant antibody in mucosal surfaces and plays a key role in neutralizing pathogens without causing inflammation. In addition, gut microbes can induce the formation of isolated lymphoid follicles and Peyer's patches, which are essential for generating Tregs and maintaining mucosal immunity.[56,57] This localized immune environment ensures that the immune system remains tolerant to beneficial microbes and dietary antigens while being vigilant against harmful pathogens.

2.5. Impact of early-life microbial exposure

The timing of microbial exposure is also critical for the development of immune tolerance. Early-life exposure to a diverse microbiota is essential for proper training of the immune system. Studies have shown that reduced microbial diversity in early life, due to factors such as cesarean section delivery, formula feeding, and antibiotic use, is associated with an increased risk of allergic and autoimmune diseases.[39-43] This is because a diverse microbiota provides a broader range of antigens and metabolites needed to fully train the immune system. Therefore, early microbial exposure plays a pivotal role in establishing a balanced immune system that is capable of distinguishing between harmless and harmful antigens.

In summary, microbial exposure leads to the development of immune tolerance through a complex interplay of microbial antigens, metabolites, and host immune cells.[44,45] This process is essential for maintaining immune homeostasis and preventing autoimmune and inflammatory diseases. Thus, understanding these mechanisms provides valuable insight into how we can manipulate the microbiota to promote immune health and prevent disease.

3. Key microbial species involved in promoting regulatory T-cell function

Several key microbial species have been identified as crucial players in promoting Treg function. Among these, certain strains of *Bacteroides* and *Clostridia* stand out for their significant impact on Treg induction and function. *Bacteroides fragilis*, for instance, is known for producing polysaccharide A, a molecule that can directly interact with the host's immune system.[58-60] Polysaccharide A has been shown to promote the differentiation of Tregs by modulating dendritic cell function, leading to the production of anti-inflammatory cytokines such as IL-10.[61] This cytokine environment is conducive to the expansion of Tregs, which play a crucial role in suppressing excessive immune responses and maintaining tolerance.

Similarly, certain clusters of *Clostridia*, particularly Clostridium clusters IV and XIVa, have a profound effect on Treg development.[46] These bacteria are abundant producers of SCFAs such as butyrate, which have potent anti-inflammatory properties. Butyrate acts on Tregs by enhancing their proliferation and increasing the expression of Foxp3, a transcription factor essential for Treg development and function. Furthermore, butyrate modulates the epigenetic landscape of Tregs, leading to the upregulation of genes associated with their suppressive functions. This interaction not only promotes the expansion of Tregs but also enhances their ability to control immune responses and maintain intestinal homeostasis.

Another significant group of bacteria involved in Treg modulation is Bifidobacteria.[62,63] These bacteria, prevalent in the gut microbiota of breastfed infants, contribute to the development of a healthy immune system.[62,63] Bifidobacteria produce metabolites that support the maturation of Tregs and the establishment of a balanced immune response.[64] In addition, they help maintain the integrity of the gut barrier, preventing the translocation of pathogens and inflammatory molecules that could otherwise trigger immune responses.[65-67]

Faecalibacterium prausnitzii, another beneficial gut microbe, is also associated with Treg induction. This bacterium produces anti-inflammatory compounds that can stimulate the production of IL-10 and other regulatory cytokines.[68] Its presence in the gut microbiota is often linked with a healthy inflammatory profile and a reduced risk of inflammatory diseases.[69,70]

In summary, key microbial species such as *B. fragilis*, *Clostridia* clusters IV and XIVa, Bifidobacteria, and *F. prausnitzii* play critical roles in promoting Treg function. They achieve this through the production of specific metabolites and molecules that modulate the immune environment, enhance Treg proliferation, and support Tregs' suppressive functions. Understanding the interactions between these microbes and the immune system offers valuable insights into the mechanisms of immune tolerance that can lead to potential therapeutic strategies for autoimmune and inflammatory diseases.

4. Effect of diet on microbiome

4.1. Dietary fibers and plant-based diets

Dietary fibers are a key component of plant-based diets and have a profound impact on gut microbiota composition and immune response.[10] Fibers, which are indigestible by human enzymes, are fermented by gut bacteria, leading to the production of SCFAs such as acetate, propionate, and butyrate. These serve as signaling molecules that modulate immune responses. For example, butyrate has anti-inflammatory properties and enhances Treg function, which is crucial for maintaining immune tolerance and preventing autoimmune diseases as discussed previously.[71,72] A diet rich in fruits, vegetables, legumes, and whole grains promotes a diverse and stable microbiota, contributing to a balanced immune system and reducing the risk of inflammatory diseases [73,74]

4.2. Western diet and high-fat, high-sugar foods

The Western diet, characterized by a high intake of fats, sugars, and processed foods, has detrimental effects on the gut microbiota and immune health. High-fat diets tend to reduce microbial diversity and promote the growth of potentially harmful bacteria, such as *Bilophila wadsworthia*, which is associated with inflammatory conditions.[75] High sugar intake can also disrupt the balance of the gut microbiota, favoring the proliferation of pathogenic bacteria over beneficial strains. This dysbiosis can lead to increased intestinal permeability, also known as "leaky gut," that allows microbial products to enter the bloodstream and trigger systemic inflammation.[76,77] Chronic inflammation induced by a Western diet is linked to a higher risk of developing metabolic disorders, such as obesity, type 2 diabetes, and cardiovascular diseases, as well as inflammatory bowel diseases (IBDs).

4.3. Mediterranean diet and healthy fats

The Mediterranean diet, rich in healthy fats from olive oil, nuts, and fatty fish, along with abundant fruits, vegetables, and whole grains, positively influences gut microbiota composition and immune function. This diet is associated with the growth of more beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, which contribute to gut health and immune regulation.[78-80] The healthy fats in the Mediterranean diet, particularly omega-3 fatty acids, have anti-inflammatory effects and can modulate the immune response by influencing the production of cytokines.[81] The polyphenols found in fruits and vegetables also act as prebiotics, promoting the growth of beneficial gut bacteria and enhancing the production of SCFAs, which support immune tolerance and anti-inflammatory pathways.[82]

4.4. Impact of protein sources

Different sources and types of dietary protein can significantly affect the gut microbiota and immune responses. Animal-based proteins, especially red and processed meats, can increase the abundance of bile-tolerant bacteria, such as *Bacteroides*, and decrease beneficial species, resulting in an inflammatory microbiota profile.[83] High intake of these proteins is associated with an increased risk of colorectal cancer and inflammatory diseases.[84,85] Conversely, plant-based proteins from legumes, nuts, and seeds are associated with a more favorable microbiota composition, promoting the growth of beneficial bacteria and reducing inflammation.[73] Plant proteins are also rich in fibers and other bioactive compounds that support a healthy immune response.

4.5. Probiotics and fermented foods

Probiotics and fermented foods, such as yogurt, kefir, sauerkraut, and kimchi, contain live beneficial bacteria that can enhance gut microbiota composition and function.[86-88] Regular consumption of these foods can increase the abundance of beneficial microbes, such as *Lactobacillus* and *Bifidobacterium*, which have immunomodulatory effects.[89] These bacteria can enhance the barrier function of the gut, produce SCFAs, and stimulate the production of anti-inflammatory cytokines. Fermented foods also contain bioactive compounds that can further support immune health by reducing oxidative stress and inflammation.

4.6. Impact of caloric intake and fasting

Caloric intake and fasting regimens can also influence the gut microbiota and immune responses.[6,8] Overconsumption of calories, particularly from nutrient-poor, high-fat, and high-sugar foods, can lead to obesity and metabolic dysregulation, which are associated with a pro-inflammatory microbiota profile. Conversely, caloric restriction and intermittent fasting have been shown to promote microbial diversity and reduce inflammation.[8] These dietary patterns can enhance the production of SCFAs, improve gut barrier function, and modulate immune responses, contributing to better overall health and resilience against diseases.

In summary, different dietary patterns significantly affect gut microbiota composition and immune responses.[4,5] Diets rich in dietary fibers, healthy fats, and plant-based proteins promote a diverse and beneficial microbiota, supporting immune tolerance and reducing inflammation. Conversely, high-fat, high-sugar, and animal-based diets can lead to dysbiosis, increased intestinal permeability, and chronic inflammation. Understanding the impact of diet on the microbiota-immune axis provides valuable insight for developing dietary strategies to improve immune health and prevent inflammatory diseases.

5. Other factors impacting gut microbiome

Besides diet, other factors such as genetics, epigenetics, comorbidities, lifestyle, and environmental exposure impact gut microbiota and immunity. Genetic factors significantly shape gut microbiota composition and influence immune responses.[90] Host genetic variations, especially those linked to immune function such as polymorphisms in genes such as *NOD2* and *TLRs*, affect microbial community structure and increase susceptibility to immune-mediated diseases. These genetic differences can create an environment that selectively promotes or inhibits specific microbial taxa, thus influencing the host's immune interactions. In addition, host genetics affect the expression of mucosal barrier proteins and AMPs, which are crucial for maintaining microbial balance and immune tolerance.

Epigenetic modifications, including DNA methylation and histone modifications, further regulate gut microbiota and immune interactions.[91] These changes, often induced by environmental factors, can influence gene expression in ways that impact immune cell activity and gut barrier integrity. For instance, epigenetic alterations in immune-regulatory genes can lead to changes in cytokine production, modulating immune tolerance or activation in response to microbial signals.[92] Epigenetic mechanisms also contribute to transgenerational effects, wherein exposures and conditions early in life can affect gut microbiota composition and immune responses across generations.[93]

Comorbidities such as obesity, metabolic syndrome, and type 2 diabetes profoundly affect both gut microbiota and immune function.[94] For instance, obesity is often associated with a dysbiotic microbiota profile that promotes low-grade inflammation and alters immune cell behavior, which in turn exacerbates metabolic complications.[95] Autoimmune diseases can create an inflammatory environment that leads to gut dysbiosis and compromised intestinal barrier function, further aggravating immune dysregulation.[96] These comorbidities contribute to a feedback loop where the gut microbiota continuously interacts with the immune system, potentially accelerating disease progression and increasing inflammatory responses.

Lifestyle factors such as exercise and stress also play critical roles in modulating gut microbiota and immunity.[97] Regular physical activity has been shown to increase microbiota diversity and support a balanced immune response, whereas chronic stress and irregular sleep disrupt the microbiome and lead to immune activation.[98,99] These lifestyle factors influence both gut microbiota composition and immune homeostasis, potentially affecting resilience to diseases linked to chronic inflammation.

Environmental exposures, including pollutants, pesticides, and pharmaceuticals, induce changes in both microbiota composition and immune function.[100-102] For example, exposure to antibiotics disrupts microbial diversity, reducing resilience and promoting dysbiosis. Other environmental factors, such as air pollution and chemical contaminants, have been associated with increased inflammation and immune dysregulation due to changes in gut microbial composition.[103] Early-life exposure to a variety of microbial environments, such as rural settings or household pets, can support immune tolerance and promote microbiota diversity, which may be protective against immune-mediated diseases.[104] Environmental exposures play a critical role in gut barrier function and immune priming, and disruptions in these exposures can contribute to chronic inflammatory conditions.

These factors interact within a complex system, where genetic predispositions may be influenced by lifestyle choices, environmental exposures, and comorbidities. Individuals with genetic susceptibilities to immune dysregulation may experience greater microbiota instability when exposed to lifestyle and environmental factors that promote inflammation. Epigenetic modifications serve as molecular intermediaries, linking environmental exposures to long-term immune outcomes by altering responses to microbial signaling. This integrated view highlights the feedback loops between microbiota and immunity, where initial disruptions in microbial composition can propagate through genetic and environmental pathways, ultimately affecting health and disease susceptibility.

6. Interaction of gut microbiome with innate and adaptive immunity

The gut microbiome plays a critical role in regulating both innate and adaptive immunity, influencing the host's immune system in complex and dynamic ways.

6.1. Influence on innate immunity

The gut microbiota influences the function of various innate immune cells.[57] Macrophages and dendritic cells are essential in sampling microbial antigens and orchestrating the initial immune response. In a homeostatic state, gut-resident macrophages have an anti-inflammatory phenotype, promoting tolerance to commensal bacteria.[105,106] However, changes in the microbiota can shift macrophage function toward a more pro-inflammatory state, contributing to inflammatory conditions such as IBD.[107] Similarly, dendritic cells in the gut are involved in maintaining immune tolerance and preventing unnecessary immune activation against harmless commensals.

Microbial metabolites play a significant role in modulating innate immune responses. SCFAs, including butyrate, acetate, and propionate, are produced by the fermentation of dietary fiber by gut bacteria. These metabolites have anti-inflammatory effects and influence the activity of innate immune cells such as macrophages and dendritic cells.[108] SCFAs help to reinforce the intestinal barrier and reduce excessive inflammation, which is crucial for preventing chronic inflammatory diseases.[109-111]

The gut microbiome is essential for maintaining the integrity of the intestinal barrier, a key component of innate immunity.[112] A healthy microbiome helps stimulate the production of mucus by goblet cells, tight junction proteins, and AMPs that fortify the gut barrier.[76,113] A compromised microbiome, often resulting from dysbiosis, can lead to increased intestinal permeability or "leaky gut," allowing pathogens and toxins to enter the bloodstream and triggering systemic immune responses.[114] This can result in chronic inflammation and increase susceptibility to diseases such as autoimmune disorders and metabolic syndrome.

6.2. Influence on adaptive immunity

The gut microbiome plays a crucial role in shaping adaptive immunity by interacting with immune cells, influencing their differentiation, and modulating immune responses. This interaction is largely driven by microbial metabolites, cell wall components, and antigens that signal through host immune receptors, leading to a complex interplay between commensal microorganisms and adaptive immune cells.

One key mechanism through which the gut microbiome influences adaptive immunity is through the production of SCFAs, such as butyrate and acetate. SCFAs are metabolic byproducts of microbial fermentation of dietary fibers, and they have been shown to modulate the differentiation of B cells and T-helper cells into specific immune subtypes. For example, SCFAs can promote the production of IgA, a critical immunoglobulin that helps maintain gut homeostasis by binding to and neutralizing pathogens while also ensuring that beneficial microbes remain in the gut.[115]

In addition, microbial antigens and metabolites can interact with antigen-presenting cells such as dendritic cells, which present microbial-derived antigens to naïve T cells. This interaction plays a pivotal role in directing T-cell responses. Depending on the type of microbial signals, the adaptive immune system can mount different types of responses, such as Th1, Th2, or Th17 responses. For instance, Th17 cells are particularly important in defending mucosal barriers against bacterial and fungal pathogens, and their activation is often influenced by signals from specific commensal bacteria.[116] Dysregulation of these pathways, often caused by an altered gut microbiome, has been linked to autoimmune and inflammatory diseases.

7. Impact of the host immune system on the microbiome and its consequences

There is a bidirectional interaction between the host immune system and the gut microbiome that plays a critical role in maintaining both health and disease states. The immune system influences the composition and function of the microbiome, promoting symbiosis with commensal microbes while defending against pathogenic organisms. This balance is maintained through mechanisms such as the secretion of AMPs and IgA, which help regulate microbial populations. However, when this balance is disrupted, it can lead to dysbiosis, an imbalance in the microbial community. Dysbiosis has been linked to a variety of diseases, including IBD, autoimmune disorders, and metabolic conditions. Thus, the relationship between the immune system and the microbiome is a delicate one, as disturbances on either side can significantly influence overall health.

The immune system modulates the microbiome through several mechanisms, including the secretion of AMPs and IgA, and the regulation of inflammatory responses.[117] These factors help maintain microbial diversity and prevent overgrowth of harmful microbes. In turn, the microbiome influences the maturation and function of the immune system by producing metabolites such as SCFAs that have immunomodulatory effects.

Dysbiosis, induced by either a hyperactive or a weakened immune response, can have significant consequences.[118] For instance, an overactive immune system may eliminate beneficial bacteria, promoting chronic inflammation and contributing to diseases such as IBD. Conversely, an underactive immune response may allow the proliferation of pathogenic microbes, further exacerbating disease states.

Recent studies have highlighted the dynamic interaction between the host immune system and the microbiome, underscoring the importance of immune regulation in maintaining microbial homeostasis and its far-reaching implications for human health.[119] These findings suggest that therapies targeting both the immune system and the microbiome may provide new avenues for treating diseases characterized by immune dysregulation and microbial imbalance.

8. Future prospects and research directions

Future research into personalized nutrition and microbiome-based therapies is highly promising. Scientists are working to create personalized dietary interventions tailored to individual microbiome profiles by understanding the complex relationship between diet, microbiota, and the immune system. This approach has the potential to optimize gut health, enhance immune function, and prevent or manage chronic diseases by fostering beneficial microbial communities and modulating immune responses effectively. Another promising area of research is developing microbiota-targeted therapies for autoimmune disease. This involved using specific microbial species and their metabolites to restore immune tolerance and mitigate autoimmune responses in conditions such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes. Furthermore, the interaction between microbiome and cancer immunotherapy offers significant potential as a therapeutic target. The gut microbiota can impact the effectiveness of immune checkpoint inhibitors and other cancer treatments, suggesting that prebiotic and probiotic interventions or fecal microbiota transplants could optimize the microbiome of cancer patients, improving treatment responses and reducing adverse effects. Finally, microbial metabolites such as SCFAs are vital for immune modulation, affecting immune cell function and inflammation, and future studies could explore their therapeutic potential further, leading to the discovery of novel bioactive compounds with immunomodulatory effects.

In addition, there is a need to understand how gut-immune interactions may influence other organ systems beyond the gastrointestinal tract. For example, the gut-liver axis is a critical pathway where immune responses triggered in the gut can affect liver function.[120,121] This is particularly evident in conditions such as non-alcoholic fatty liver disease, where microbial products and metabolites from the gut can cross into the liver, triggering inflammation through immune cell activation.[122] Dysbiosis, or microbial imbalance in the gut, has been linked to increased intestinal permeability (also known as “leaky gut”), which allows microbial antigens to reach the liver and induce a cascade of immune-mediated liver inflammation. This connection demonstrates the systemic influence that gut microbiome-immune interactions can have beyond the intestines.

Another critical area of research is the gut-brain axis, which highlights the bidirectional communication between the gut microbiota and the central nervous system.[22,123-125] Immune cells in the gut produce cytokines and other signaling molecules that can influence brain function, potentially affecting neurodevelopment and behavior.[19,126,127] Emerging research suggests that microbial metabolites such as SCFAs and tryptophan metabolites can modulate the immune system in ways that impact brain inflammation and neurodegenerative diseases such as Alzheimer’s disease and autism spectrum disorder. Understanding how the immune system mediates this gut-brain communication is key to uncovering new therapeutic targets for neuroinflammatory and neurological conditions.

To study these complex interactions, researchers use a range of *in vitro*, *in vivo*, and clinical models. *In vitro* models, including organoids and intestinal epithelial cell cultures, allow for controlled studies of specific immune-microbial interactions.[128,129] These models provide insights into the molecular mechanisms governing immune responses to gut microbiota.[130] *In vivo* models, such as germ-free mice or specific pathogen-free mice, enable researchers to study the broader systemic effects of gut microbiota in the context of a whole organism, allowing for exploration of gut-liver, gut-brain, and gut-immune interactions in a physiological setting.[131,132]

In addition, omics technologies, such as metagenomics, proteomics, and metabolomics, are revolutionizing our understanding of the gut microbiome by providing high-resolution data on microbial diversity, metabolic pathways, and immune responses.[133] These techniques allow for comprehensive mapping of the microbial ecosystem and its interactions with the host immune system.[134] In parallel, clinical studies continue to validate these findings in human populations, helping to identify biomarkers for diseases associated with gut dysbiosis and immune dysfunction. These methods, when integrated, provide a powerful toolkit for exploring how immune system interactions with the gut microbiome influence various organ systems.

9. Conclusion

The future microbiota research will have profound health implications and impact strategies of disease prevention. Understanding the complex interactions between microbiota and the immune system can revolutionize personalized medicine, enabling tailored interventions that optimize individual health outcomes. This can transform how we approach a wide range of conditions, from autoimmune and metabolic disorders to cancer and infectious diseases, using therapies that restore and maintain a healthy microbial balance by targeting diet, probiotics, and the microbiome. As we contemplate this exciting frontier, the need for continued exploration and innovation in this field is crucial. Interdisciplinary collaboration, large-scale longitudinal studies, and advanced analytical tools will be essential to unravel the complexities of the microbiota-immune system axis. Therefore, a call to action is necessary for researchers, health-care providers, and policymakers to prioritize and support microbiota research, fostering an environment that encourages groundbreaking discoveries and the translation of those discoveries into practical, life-changing health interventions.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Healthcare-associated infections in a resource-limited setting: Microbial contamination and antimicrobial resistance in a Cameroonian referral hospital

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Abstract

Antimicrobial resistance is a silent pandemic threatening the lives of millions on the African continent. This exploratory study investigates microbial contamination of surfaces and medical devices, evaluates antibiotic resistance profiles, and identifies high-risk pathogens within a referral hospital in Yaoundé, Cameroon. A descriptive cross-sectional study was conducted from May to July 2024 in the obstetrics–gynecology department. Thirty samples were collected using sterile swabs from high-touch surfaces and from medical devices. Bacterial isolates were identified using standard culture and biochemical methods, and antibiotic susceptibility testing was performed through disk diffusion, with methicillin-resistant *Staphylococcus aureus* confirmed using cefoxitin discs. Data were analyzed using R Statistics version 4.4.2. The findings revealed that all samples were contaminated, yielding 55 bacterial isolates. Gram-positive bacteria were predominant (60%), primarily *S. aureus* (36%), and other *Staphylococcus* spp. (24%). Gram-negative pathogens included *Proteus mirabilis* (13%) and *Klebsiella* spp. (7%). Taps (14 isolates) and trolleys (100% contamination rate) were identified as the most contaminated sites. Antibiotic resistance was high: 70% of *Staphylococcus* species were methicillin-resistant, and 100% of Gram-negative isolates exhibited multidrug resistance (MDR), including resistance to penicillin (≥70%), cephalosporins (≥80%), and fosfomycin (≥75%). Carbapenems and quinolones remained effective against Gram-negative strains. These findings highlight widespread contamination of hospital environments with MDR pathogens, posing significant risks to maternal and neonatal health. The predominance of methicillin-resistant *S. aureus* and MDR Gram-negative bacteria underscores the urgent need to strengthen infection protocols, antimicrobial stewardship, and national antimicrobial resistance surveillance in Cameroon. Implementation of the World Health Organization infection prevention and control guidelines and targeted staff training is essential to reduce preventable healthcare-associated infections in resource-limited settings.

Keywords: Healthcare-associated infections; Antimicrobial resistance; *Staphylococcus aureus*; Methicillin-resistant *Staphylococcus aureus*; Infection prevention and control; Obstetrics–gynecology; Cameroon

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1. Introduction

Maternal mortality remains critically high, with an estimated 260,000 women dying during or after pregnancy and childbirth in 2023. Most of these deaths (92%) occurred in low- and lower-middle-income countries, where the vast majority were preventable.[1] The postpartum stage is the most vulnerable for child survival, with 2.3 million newborn deaths reported in 2022.[2] Scientific evidence indicates that the leading causes of maternal death globally are hemorrhage, followed by hypertensive disorders of pregnancy.[3] In addition, sepsis and other complications often occur during the postpartum period, contributing to maternal deaths.[4]

Sepsis, which can prolong hospital stay or cause maternal death, results from untreated, poorly treated, or complicated puerperal infections, such as urinary tract infections, endometritis, pneumonia, and perineal or cesarean wound infections.[5] These puerperal infections occurring during hospital stay are often attributed to healthcare-associated infections (HAIs).[6] The risk of such infections increases with poor compliance with infection prevention and control (IPC) measures, which are protocols derived from guidelines designed to minimize pathogen transmission within healthcare settings.[7,8]

Adherence to IPC guidelines among healthcare workers is critical, particularly since Cameroon lacks a fully effective national IPC program. Only certain components of the World Health Organization (WHO)'s IPC core components have been implemented.[9] As a result, inconsistent adherence to standard precautions, including poor hand hygiene and inadequate use of personal protective equipment, can facilitate the transfer of pathogens from the healthcare environment to mothers and newborns, causing early neonatal infections.[10,11]

Studies indicate that biofilms are commonly found on the surfaces of hospital instruments and body tissues and have become significant contributors to global health problems due to antibiotic resistance.[12] A bacterial biofilm comprises a structured community of microorganisms, including bacteria, fungi, archaea, protozoa, and yeasts, encased in an extracellular matrix with channel structures that regulate the movement of gases, nutrients, and antimicrobials.[13]

These biofilms are often rich in multidrug-resistant (MDR) bacteria, resulting from frequent exposure to antibiotics that are present in non-bactericidal doses within healthcare environments. They can also originate from residual body fluids of severely ill patients who have either irrationally used antibiotics or poorly adhered to prescribed regimens. In gynecological and obstetrical departments, infections caused by these MDR bacteria can lead to prolonged hospital stays and even maternal and neonatal death, largely due to limited treatment options.[14]

In Cameroon, suboptimal adherence to standard precautions and a lack of comprehensive antimicrobial resistance surveillance are further complicated by limited evidence, with only two prior studies assessing bacterial flora in healthcare settings.[15,16] Therefore, this study aims to identify high-risk microbial pathogens in gynecological and obstetric departments, evaluate their antibiotic resistance profiles, and generate actionable data to advocate for full adoption of the WHO IPC guidelines. By bridging this critical evidence gap, our findings may inform targeted IPC interventions and empirical treatment protocols, ultimately reducing preventable maternal and neonatal deaths in resource-limited contexts.

2. Methods

2.1. Study design and period

This descriptive cross-sectional study, conducted from May to July 2024, focused on healthcare workers and the healthcare environment to characterize the microbial flora and assess the risk of pathogen exposure.

2.2. Settings

The study was conducted in Yaoundé, the capital city of Cameroon, which has a population of approximately 3.2 million and is served by a tiered healthcare system comprising first-, second-, third-, and fourth-level facilities. The healthcare setting studied is a fourth-level referral center within the healthcare pyramid. It provides both short- and long-term specialized care and plays a key role in the training of healthcare workers.[17,18] The hospital has a capacity of 138 beds and receives between 500 and 1,000 patients/week. In 2023, the hospital recorded 35,945 outpatient consultations and 4,329 admissions, totaling 21,518 hospital days, with an average hospital stay of 5 days/inpatient.[17,19] In 2023, the obstetrics–gynecology department reported approximately 300 deliveries, 8 deaths, 625 prenatal consultations, and 77 cesarean sections. In addition, the department managed 101 obstetric complications and 33 post-abortion complications.[19]

2.3. Sampling

In this study, a total of 30 samples were collected from medical equipment (e.g., delivery table, trolley, drip stand, nursing table) and high-touch surfaces (e.g., taps, door handles). A purposive sampling design was employed. Ten samples were collected weekly for 3 weeks, divided into two phases of five samples, each collected 1 day apart.

Surface and medical device samples were collected in the early morning using sterile swabs premoistened with 0.9% sodium chloride solution (Missionpharma, Denmark), which were streaked across the surface in parallel lines and then cross-streaked perpendicularly. Swabs were placed in labeled cryotubes containing brain–heart infusion broth (Jiangsu Kangjie Medical Devices Co., Ltd., China) and immediately transported to the laboratory for bacteriological examination.[16,20]

2.4. Microbial analysis

Upon arrival at the bacteriology laboratory, each cotton swab was aseptically transferred to a tube containing 2 mL of sterile physiological solution, from which 0.1 to 0.4 mL aliquots were pipetted into selective culture media (eosin methylene blue agar for Gram-negative bacteria and mannitol salt agar for *Staphylococcus* spp.). The inoculated media were incubated at 37°C for 24–48 h to isolate specific pathogens. After incubation, bacterial isolates were identified using standard biochemical methods. Gram-negative bacilli were characterized based on their reactions to glucose, lactose, citrate, hydrogen sulfide, urea, indole, and gas production.

Antibiotic susceptibility testing was performed using the Müller–Hinton agar disk diffusion technique.[16] Bacterial suspensions were prepared according to the 0.5 McFarland standard. Discs were placed on inoculated agar and incubated at 37°C ± 2°C for 18–24 h. Selection and interpretation of the antibiotic discs were performed according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing 2023.[21] Detected *Staphylococcus aureus* strains were tested for methicillin resistance using a cefoxitin disc, with strains exhibiting an inhibition diameter of <27 mm considered methicillin-resistant *S. aureus* (MRSA).[20,22] MDR bacteria were defined as microorganisms resistant to at least one antibiotic from three or more different antimicrobial classes.[23] To minimize potential bias, rigorous quality control was ensured through culture conditions, media preparation, antibiotic authenticity, equipment calibration, and reagent validation to ensure the reliability and accuracy of the results.

2.5. Data analysis

Data were coded and analyzed using R Statistics version 4.4.2 (R Core Team, Austria). Fisher's exact test was used to compare proportions. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Microbiological analysis of environmental surfaces and medical devices

A total of 15 samples were collected from each subunit—the inpatient and maternity subunits. The findings revealed that all collected samples were bacterially contaminated. A total of 55 isolates were identified, most of which were Gram-positive (60%) and originated from the inpatient subunit (62%). Tap surfaces were identified as the most contaminated surfaces, with 14 isolates detected (Table 1).

The most prevalent microbial strains in the department included *S. aureus* (36%), other *Staphylococcus* species (24%), *Proteus mirabilis* (13%), and *Klebsiella* spp. (7%). Non-enterobacterial Gram-negative bacilli were the least represented (Figure 1).

In the maternity ward, most isolates were *Staphylococcus* spp. (70%), with *S. aureus* the most prevalent species (42%). However, a nearly equivalent distribution was observed between *Staphylococcus* spp. (52%) and Enterobacteria (45%) (Table 2).

Most microorganisms (74%) on door handles were *Staphylococcus* spp., with *S. aureus* the most prevalent species (57%). In contrast, the trolley surfaces were primarily contaminated with *Enterobacteriaceae* (66%), with *P. mirabilis* (33%) and *Klebsiella* (33%) spp. most frequently isolated (Table 3).

3.2. Antibiotic susceptibility of isolated staphylococcal strains

Most *Staphylococcus* isolates were found to be penicillinase producers (≥90%). Significant antibiotic resistance was also detected for fosfomycin, rifampicin, fusidic acid, and cotrimoxazole (≥70%). In contrast, the most effective antibiotics against the isolates were ciprofloxacin, erythromycin, and vancomycin, to which the bacteria exhibited low or no resistance (Table 4).

Table 1. Bacterial contamination of surfaces and medical devices by Gram category

Characteristic	Gram-negative (*n* [%])	Gram-positive (*n* [%])	Total	*p*-value[a]
Number of isolates	22 (40)	33 (60)	55 (100)	-
Subunit				
Inpatient	16 (47)	18 (53)	34	0.258
Maternity	6 (29)	15 (71)	21	
Surface and devices				
Trolley	4 (40)	6 (60)	10	0.723
Door handle	3 (25)	9 (75)	12	
Drip stand	4 (67)	2 (33)	6	
Tap	6 (43)	8 (57)	14	
Delivery table	2 (40)	3 (60)	5	
Nursing table	3 (37)	5 (63)	8	

Note: [a]Fisher's exact probability test.

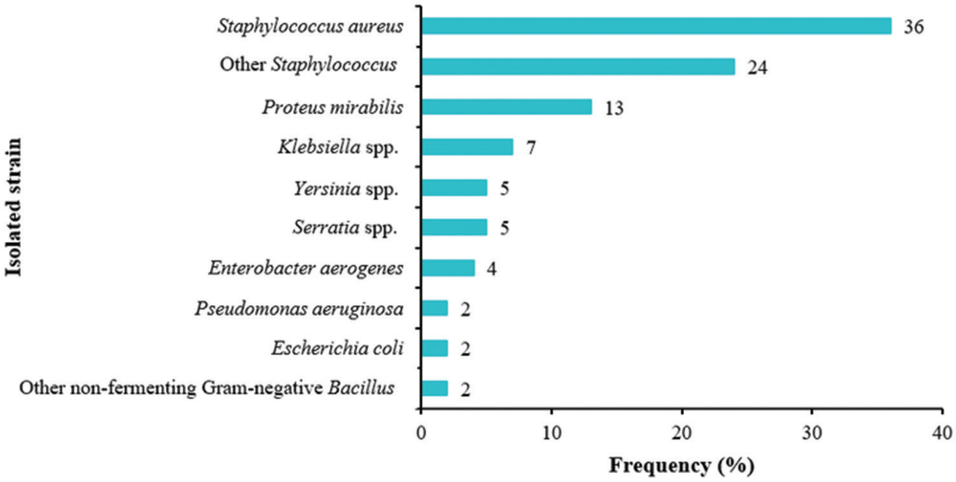

Figure 1. Bacterial profile on environmental surfaces and medical devices (*n* = 55)

Table 2. Bacterial strains isolated from the inpatient and maternity subunits

Isolated bacteria	Subunit (*n* [%])		Total	*p*-value[a]
	Inpatient	Maternity		
Number of isolates	34 (100)	21 (100)	55 (100)	0.291
Staphylococcus spp.				
Staphylococcus aureus	11 (31)	9 (42)	20	-
Others	7 (21)	6 (28)	13	
Total	18 (52)	15 (70)	33	
Enterobacteria				
Proteus mirabilis	7 (21)	0 (0)	7	-
Klebsiella spp.	3 (9)	1 (5)	4	
Serratia spp.	1 (3)	2 (10)	3	
Yersinia spp.	2 (6)	1 (5)	3	
Enterobacter aerogenes	1 (3)	1 (5)	2	
Escherichia coli	1 (3)	0 (0)	1	
Total	15 (45)	5 (25)	20	
Other Enterobacteria				
Pseudomonas aeruginosa	1 (3)	0 (0)	1	-
Other non-fermenting Gram-negative *Bacillus*	0 (0)	1 (5)	1	
Total	1 (3)	1 (5)	2	

Note: [a]Fisher's exact probability test.

Methicillin resistance was observed in 70% of the isolated *Staphylococcus* strains, with no significant difference between the two departmental subunits (*p*=0.722). The most frequently contaminated medical devices were the trolley surfaces (100%), delivery tables (100%), and door handles (78%) (Table 5).

3.3. Antimicrobial susceptibility of Gram-negative bacterial strains

Most of the identified Gram-negative bacteria were found to be penicillinase (≥70%) and cephalosporinase (≥80%) producers, particularly showing resistance to cefuroxime, cefepime, and cefoxitin. In contrast, cefotaxime and quinolone antibiotics (e.g., nalidixic acid, ciprofloxacin, and levofloxacin) were the most effective agents against these isolates.

High resistance (approximately 100%) was observed among Gram-negative bacteria to aztreonam, fosfomycin, and trimethoprim when used individually. However, their combination with sulfonamides (e.g., cotrimoxazole) was found to overcome this resistance. The bacteria also exhibited high susceptibility to carbapenems (e.g., imipenem and meropenem) and vancomycin (Table 6).

3.4. MDR assessment

MDR was observed in 100% of the bacteria isolated from the inpatient subunit. Nearly all environmental surfaces and medical devices were found to be contaminated with MDR bacteria (Table 7).

4. Discussion

HAIs represent a significant threat to patient safety, resulting in serious complications, additional healthcare costs, and increased mortality.[14] These infections affect a substantial number of hospitalized patients, underscoring the need for effective prevention and control measures.[24] The One Health approach emphasizes the role of the environment in the development and transmission of infectious agents, including emerging and re-emerging pathogens. In Cameroon, maternal and neonatal mortality

Table 3. Classification of bacterial strains isolated from environmental surfaces and medical devices

Isolated bacteria	Surface and medical device (*n* [%])						Total	*p*-value[a]
	Trolley	Door handle	Drip stand	Tap	Delivery table	Nursing table		
Number of isolates	10 (100)	12 (100)	6 (100)	14 (100)	5 (100)	8 (100)	55 (100)	-
Staphylococcus spp.								
Staphylococcus aureus	3 (30)	7 (57)	1 (17)	4 (29)	1 (20)	4 (51)	20	0.177
Others	3 (30)	2 (17)	1 (17)	4 (29)	2 (40)	1 (12)	13	
Total	6 (60)	9 (74)	2 (34)	8 (58)	3 (60)	5 (63)	33	
Enterobacteria								
Proteus mirabilis	0 (0)	1 (9)	2 (33)	2 (14)	0 (0)	2 (25)	7	-
Klebsiella spp.	0 (0)	0 (0)	2 (33)	2 (14)	0 (0)	0 (0)	4	
Serratia spp.	1 (10)	2 (17)	0 (0)	0 (0)	0 (0)	0 (0)	3	
Yersinia spp.	2 (20)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	3	
Enterobacter aerogenes	1 (10)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	2	
Escherichia coli	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (12)	1	
Total	4 (40)	2 (26)	4 (66)	5 (35)	1 (20)	3 (37)	20	
Non-Enterobacteria								
Pseudomonas aeruginosa	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	1	-
Other non-fermenting Gram-negative *Bacillus*	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	1	
Total	0 (0)	0 (0)	0 (0)	1 (7)	1 (20)	0 (0)	2	

Note: [a]Fisher's exact probability test.

rates remain alarmingly high.[25,26] To address this issue, the country has committed to implementing universal health coverage.[27-29]

4.1. Microbial flora profile of surfaces and equipment

Most of the identified isolates originated from the inpatient unit, likely reflecting greater interaction between healthcare workers, patients, and visitors, compared with the more restricted maternity unit, where access is restricted to healthcare workers and patients. Furthermore, the implementation of IPC measures, such as hygiene sessions and regular cleaning of medical equipment in the maternity unit, could also have contributed to these outcomes.

Tap surfaces were found to be the most contaminated sites, while the trolley surfaces exhibited the highest contamination rate relative to the number of samples analyzed. Hand-washing areas, frequently used by healthcare workers for hand and equipment hygiene, serve as primary sites for bacterial colonization, particularly in the absence of regular and appropriate cleaning by cleaning staff. Bacterial contamination of these areas can seriously compromise hand hygiene and facilitate the transmission of pathogens to patients. Similarly, trolleys used to transport drugs and nursing equipment can act as vehicles for the transmission of bacteria from one patient to another if hand hygiene precautions—such as hand washing, wearing gloves, and the use of hydroalcoholic gel—are not systematically followed. Our findings corroborate those observed at Treichville University Hospital in Côte d'Ivoire and the Jordan Medical Services in Cameroon, where wash stations and trolleys were also reported to be the most contaminated sites.[16,20]

The most common microbial strains identified were *S. aureus*, which has a remarkable capacity to adapt to both the healthcare environment and human hosts. *S. aureus* is part of the commensal microbiota of the nasal mucosa in approximately 20–40% of the general population, but is also responsible for transient colonization of the nasal mucosa in at least 60% of the remaining population. This may explain its predominance in this study.[30,31] It is the most clinically relevant staphylococcal species due to its involvement in HAIs and is the second most common pathogen responsible for this type of infection after *Escherichia coli.*[31] *S. aureus* infects humans when skin and mucosal barriers are disrupted—for example, as a result of chronic skin conditions, wounds, or surgical procedures—allowing access to underlying tissues or the bloodstream and causing infection. Individuals with invasive medical

Table 4. Antibiotic resistance profile of isolated *Staphylococcus* spp. (n=33)

Tested antibiotic	*Staphylococcus* spp. (n [%])	
	Staphylococcus aureus	*Others*
Number of isolates	20 (100)	13 (100)
Penicillin		
Penicillin G	20 (100)	13 (100)
Ampicillin	18 (90)	12 (92)
Oxacillin	18 (90)	13 (100)
Cephalosporin		
Cefoxitin	6 (30)	5 (38)
Aminoglycoside		
Gentamicin	3 (15)	4 (31)
Quinolone		
Ofloxacin	3 (15)	1 (8)
Ciprofloxacin	1 (5)	0 (0)
Norfloxacin	1 (5)	1 (8)
Cycline		
Tetracycline	8 (40)	4 (31)
Fusidanine		
Fusidic acid	15 (75)	9 (69)
Glycopeptide		
Vancomycin	1 (5)	0 (0)
Sulfonamide and Diaminopyrimidine		
Sulfamethoxazole+Trimethoprim	15 (75)	9 (69)
Macrolide		
Erythromycin	5 (25)	0 (0)
Streptogramin (synergistin)		
Pristinamycin	4 (20)	1 (8)
Phosphonic acid		
Fosfomycin	18 (90)	13 (100)
Rifamycin		
Rifampicin	17 (85)	13 (100)

devices or weakened immune systems, including pregnant women and premature newborns, are particularly susceptible to *S. aureus* infection.[30-32] Studies conducted at Yaoundé University Teaching Hospital have identified this bacterium as a common pathogen in skin and soft tissue HAIs.[17] Our findings were similar to those reported at the Douala General Hospital, where *S. aureus* was the most frequently isolated bacterium.[33]

P. mirabilis and *Klebsiella* spp. were the primary pathogenic Enterobacteria isolated in the obstetrics–gynecology department. These infectious agents are responsible for HAIs, especially in obstetrics–gynecology wards.[17,24,34] *P. mirabilis* is commonly found in abundance in soil and water. Although this bacterium is part of the normal human intestinal flora,[34-36] it is frequently responsible for infections of the human urinary tract, where it causes urinary tract infections and catheter-associated infections.[37] This is of concern given that the obstetrics–gynecology department regularly performs cesarean sections requiring the use of urinary catheters in parturient women. The circulation of this pathogen in the department increases the risk of women developing iatrogenic urinary tract infections after delivery.[36,38]

Table 5. Prevalence and distribution of methicillin-resistant *Staphylococcus* spp. (n=33)

Variable	Count (n)	Frequency (%)	Total	p-value[a]
Staphylococcus spp.				
Staphylococcus aureus	14	70	20	>0.999
Others	9	69	13	
Subunit				
Inpatient	12	67	18	0.722
Maternity	11	73	15	
Surface and medical device				
Trolley	3	50	6	0.667
Door handle	7	78	9	
Drip stand	2	100	2	
Tap	5	62	8	
Delivery table	3	100	3	
Nursing table	3	60	5	

Note: [a]Fisher's exact probability test.

Klebsiella spp. are responsible for infections characterized by high morbidity and mortality and by their potential to disseminate within the host and cause sepsis. Risk factors for this infection include local healthcare practices, antibiotic misuse, and compliance with infection control procedures.[39,40] Poor compliance with standard precautions in the obstetrics–gynecology department could lead to environmental contamination, as this bacterium has also been implicated in severe neonatal sepsis.[41] In addition, *Klebsiella pneumoniae* is one of the most commonly isolated pathogens in healthcare settings and is responsible for HAIs in Cameroon, Côte d'Ivoire, and Mali.[16,17,20,24,33]

4.2. Susceptibility profile of isolated staphylococcal strains

Antimicrobial resistance analysis revealed that most *Staphylococcus* spp. isolates were penicillinase producers (≥90%). In addition, significant resistance rates were observed for fosfomycin, rifampicin, fusidic acid, and cotrimoxazole (≥70%).

Table 6. Antibiotic resistance profile of Gram-negative bacilli in the inpatient and maternity subunit (*n*=22)

Tested antibiotic	Enterobacteria (*n* [%])						Non-Enterobacteria (*n* [%])	
	Proteus mirabilis	***Klebsiella* spp.**	***Yersinia* spp.**	***Serratia* spp.**	***Enterobacter aerogenes***	***Escherichia coli***	***Pseudomonas aeruginosa***	**Other Gram-negative non-fermenting *Bacillus***
Number of isolates	7 (100)	4 (100)	3 (100)	3 (100)	2 (100)	1 (100)	1 (100)	1 (100)
Penicillin								
Amoxicillin	5 (71)	3 (75)	2 (67)	3 (100)	2 (100)	0 (0)	1 (100)	1 (100)
Amoxicillin+clavulanate	5 (71)	3 (75)	2 (67)	3 (100)	2 (100)	0 (0)	1 (100)	1 (100)
Aminoglycoside								
Kanamycin	3 (43)	3 (75)	3 (100)	1 (33)	0 (0)	0 (0)	1 (100)	0 (0)
Cephalosporin								
Ceftazidime	4 (57)	1 (25)	2 (67)	2 (67)	1 (50)	0 (0)	1 (100)	0 (0)
Cefuroxime	7 (100)	4 (100)	3 (100)	3 (100)	2 (100)	1 (100)	1 (100)	1 (100)
Cefepime	6 (86)	4 (100)	3 (100)	3 (100)	2 (100)	0 (0)	1 (100)	1 (100)
Cefotaxime	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ceftriaxone	2 (29)	3 (75)	2 (67)	3 (100)	2 (100)	1 (100)	0 (0)	0 (0)
Cefoxitin	6 (86)	4 (100)	3 (100)	3 (100)	1 (50)	1 (100)	1 (100)	1 (100)
Quinolone								
Nalidixic acid	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Levofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Carbapenem								
Meropenem	0 (0)	0 (0)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	1 (100)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Glycopeptide								
Vancomycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phosphonic acid								
Fosfomycin	6 (86)	3 (75)	3 (100)	2 (67)	2 (100)	1 (100)	1 (100)	1 (100)
Diaminopyrimidine								
Trimethoprim	7 (100)	3 (75)	2 (67)	2 (67)	2 (100)	1 (100)	1 (100)	1 (100)
Sulfonamide and diaminopyrimidine								
Sulfamethoxazole+Trimethoprim	1 (14)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Monobactam								
Aztreonam	4 (57)	3 (75)	2 (67)	3 (100)	1 (50)	0 (0)	1 (100)	1 (100)

This proportion of penicillin-resistant strains was similar to that observed in studies conducted in Cameroon and higher than that reported at the University Hospital of Treichville, Côte d'Ivoire (28%).[16,20,42] This difference indicates more inappropriate antibiotic use in Cameroon, which may explain the high resistance to commonly used agents and limited effectiveness in the treatment of staphylococcal infections. Penicillin resistance is a major public health problem, as it complicates the treatment of infections and increases the risk of complications and mortality.[43]

Most *Staphylococcus* strains isolated (70%) were resistant to methicillin. MRSA is a major cause of morbidity and mortality in neonates admitted to neonatal intensive care units. Neonatal MRSA colonization is attributed to multiple sources, including mothers, healthcare workers, and environmental surfaces. It can lead to life-threatening infections, prolonged hospital stays, and significant economic costs.[32,44]

A meta-analysis of the worldwide distribution of MRSA revealed varying prevalence rates depending on

Table 7. Distribution of multidrug-resistant bacteria by subunit, surface/device, and bacterial species (n=55)

Variable	Characteristic	Count (n)	Frequency (%)	Total	p-value[a]
Location	Subunit				
	Inpatient	34	100	34	0.051
	Maternity	18	86	21	
	Surface and medical device				
	Tap	13	93	14	0.725
	Trolley	10	100	10	
	Door handle	10	83	12	
	Nursing table	8	100	8	
	Drip stand	6	100	6	
Bacterial isolates	*Staphylococcus* spp.				
	Staphylococcus aureus	17	85	20	0.726
	Others	13	100	13	
	Enterobacteria				
	Proteus mirabilis	7	100	7	-
	Klebsiella spp.	4	100	4	
	Yersinia spp.	3	100	3	
	Serratia spp.	3	100	3	
	Enterobacter aerogenes	2	100	2	
	Escherichia coli	1	100	1	
	Non-Enterobacteria				
	Pseudomonas aeruginosa	1	100	1	-
	Other non-fermenting Gram-negative *Bacillus*	1	100	1	

Note: [a]Fisher's exact probability test.

the populations studied, ranging from 0.3% to 5.1% for mothers of newborns, from 3.1% to 18.4% for healthcare workers, with the highest rates (3.5–36%) observed in environmental samples, thereby confirming the findings of our study.[44] In addition, MRSA is a common cause of HAIs in many countries, including Cameroon and Côte d'Ivoire, where methicillin-resistant strains have been identified in 71–81% of *Staphylococcus* spp. isolated from hospitals.[16,20,45] It is therefore essential to monitor antibiotic resistance and develop new strategies to combat penicillin-resistant staphylococcal infections.

4.3. Susceptibility profile of Gram-negative bacterial strains

Most of the Gram-negative bacteria identified were both penicillinase and cephalosporinase producers. A similar bacterial resistance profile was observed at Jordan Medical Services in Yaoundé.[16]

The majority of the bacteria identified (85–100%) exhibited MDR, which remains a significant concern for vulnerable patients in obstetrics–gynecology departments, particularly pregnant women, women in the immediate postpartum period, and their newborns. A meta-analysis conducted in Cameroon on human and environmental samples showed lower rates of MDR compared to those observed in our study.[46,47] The specific characteristics of the samples analyzed in our study may explain these differences.

4.4. MDR to antibiotics

Resistance to the most commonly prescribed antimicrobials leads to a significant increase in mortality, length of hospital stays, and healthcare costs. Faced with these MDR infections and delays in obtaining microbiological results, clinicians often resort to broad-spectrum antibiotics as empirical therapy. This practice can lead to the overuse and misuse of antibiotics, contributing to the development of resistance.[48]

The high prevalence of MDR bacterial strains underscores the existence of contributing factors, which include poor compliance with regulations and a lack of control over the sale and use of antibiotics by healthcare workers and the general public.[49,50] In addition, the scarcity of accurate epidemiological data on antibiotic use and bacterial resistance in Cameroon complicates the development of effective strategies.[51,52]

5. Limitations

It is important to recognize that the study was conducted in a specific hospital department. Consequently, the results obtained may not accurately reflect the situation in the entire health facility. The decision not to use automated systems, such as Vitek, for bacterial identification and susceptibility testing was primarily due to resource constraints and the need for a cost-effective surveillance model for resource-limited settings like Cameroon. These methods offer reliable results without the substantial financial and maintenance demands of automated systems, making our protocol directly applicable to the typical clinical laboratories where such interventions are most urgently needed. In addition, the sample size did not provide sufficient power to detect statistical significance for certain variables that were observed but not considered significant. These limitations highlight the need for a more comprehensive study with a larger sample size.

6. Conclusion

This study highlights the critical threat of HAIs in the obstetrics–gynecology department of a referral hospital in

Cameroon, revealing widespread bacterial contamination of surfaces and medical devices, with *S. aureus* and MDR Gram-negative pathogens, such as *P. mirabilis* and *Klebsiella* spp., predominating. Most of the staphylococcal isolates were methicillin-resistant, and all Gram-negative bacteria exhibited MDR, underscoring the urgent need for enhanced IPC measures. These findings demonstrate how suboptimal IPC compliance, along with inadequate antimicrobial stewardship, promotes resistance, disproportionately endangering vulnerable maternal and neonatal populations. As an exploratory study, these findings highlight the urgent need for evidence-based cleaning protocols within the unit. Therefore, this study calls for a multi-level response, including the full implementation of WHO IPC guidelines, targeted staff training within the department, and hospital-wide action, whereby the administration expands environmental surveillance to all departments to inform a comprehensive, institution-wide strategy. Establishing robust antimicrobial resistance surveillance systems across Cameroon's healthcare facilities is critical, as addressing these gaps is essential to reducing preventable morbidity and mortality.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Writing–review & editing: All authors

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Availability of data

All data generated or analyzed during this study are provided in the manuscript.

Further disclosure

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

In silico docking and pharmacokinetic evaluation of African ethnomedicinal phytochemicals as dual-target inhibitors of multidrug-resistant Gram-negative bacteria

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Abstract

The emergence of multidrug-resistant (MDR) Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*, poses a critical global health challenge, necessitating innovative antimicrobial strategies. Leveraging Africa's rich phytochemical diversity, this study integrates molecular docking and *in silico* pharmacokinetic profiling to identify natural compounds with dual-target potential. Ten bioactive phytochemicals from ethnomedicinal plants were screened against four validated bacterial targets: LasR (2UV0), AcrB efflux pump (5NC5), DNA gyrase B (6F86), and TEM-1 β-lactamase (1BTL) with AutoDock Vina implemented in PyRx, and the resulting protein–ligand interactions analyzed in Discovery Studio. Docking identified luteolin as exhibiting the highest binding affinity for LasR (−10.8 kcal/mol) and TEM-1, indicating dual inhibition of quorum sensing and β-lactamase activity. Rutin (−9.2 kcal/mol) exhibited high affinity for AcrB, indicating potential efflux pump inhibition, while ellagic acid (−8.3 kcal/mol) targeted DNA gyrase, potentially impairing bacterial replication. Absorption, distribution, metabolism, excretion, and toxicity analyses, along with ProTox-3 predictions, indicated that luteolin exhibited high gastrointestinal absorption and moderate systemic toxicity. Ellagic acid exhibited excellent biocompatibility, whereas rutin showed favorable drug-likeness but low permeability. Collectively, luteolin, rutin, and ellagic acid emerged as promising computational leads with complementary inhibitory mechanisms. This study highlights the translational potential of African phytochemical scaffolds in rational, computer-aided antimicrobial design and provides a foundation for subsequent *in vitro* and *in vivo* validation toward novel anti–MDR therapeutics.

Keywords: Luteolin; Rutin; Ellagic acid; Multidrug resistance; Gram-negative bacteria; Molecular docking; ADMET; African medicinal plants

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1. Introduction

The global emergence of antimicrobial-resistant microorganisms poses a severe threat to public health and food security, largely due to the proliferation of multidrug-resistant (MDR) Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.[1] These pathogens have evolved through diverse resistance mechanisms, including β-lactamase production, efflux pump overexpression, and target modification, enabling them to withstand multiple antibiotic classes, such as β-lactams, fluoroquinolones, and aminoglycosides.[2] The World Health Organization (WHO) has designated these species as "critical priority pathogens" requiring urgent discovery of new therapeutic agents.[3]

Despite continuous pharmaceutical research, the antibiotic development pipeline remains stagnant, with most new approvals in recent decades being structural modifications of existing scaffolds rather than truly novel compounds.[4] Plant-derived natural products offer promising potential as alternatives owing to their structural diversity, ecological abundance, and long history of therapeutic use.[5]

Medicinal plants are particularly rich in bioactive compounds, such as flavonoids, which have shown significant antimicrobial properties. Recent studies indicate that these phytochemicals can modulate microbial activity and contribute to host health.[6]

In particular, African medicinal plants widely applied in ethnomedicine for the treatment of infectious diseases remain underexplored for their antimicrobial potential in modern drug discovery.[7,8] Advances in computational biology now enable high-throughput *in silico* screening of natural products against validated microbial targets, integrating techniques such as molecular docking, pharmacokinetic (absorption, distribution, metabolism, excretion, and toxicity [ADMET]) profiling, and molecular dynamics (MD) simulations.[9,10] This approach is well-suited for identifying "dual-action" compounds capable of exerting direct antibacterial activity while simultaneously modulating bacterial virulence factors, including quorum sensing (QS) and biofilm formation.[11,12]

Targeting QS systems, such as *P. aeruginosa* LasR and the universal LuxS/autoinducer-2 signaling pathway, offers a potential strategy to attenuate pathogenicity without applying direct selective pressure, thereby potentially reducing resistance emergence.[13,14] Several classes of plant-derived compounds, including flavonoids, terpenoids, and coumarins, have demonstrated both QS inhibition and direct interference with bacterial enzymes such as DNA gyrase and β-lactamases.[15,16]

Given the rich yet underutilized phytochemical diversity of African flora, this study aims to computationally evaluate bioactive compounds from 10 ethnomedicinal plants for their dual-action potential against MDR Gram-negative pathogens.[17] Specifically, molecular docking was used to identify promising phytochemicals capable of inhibiting bacterial enzymes and QS targets, followed by ADMET prediction to assess their drug-likeness and potential safety profiles.

2. Materials and methods

2.1. Selection of medicinal plants and phytochemicals

Table 1 lists the scientific names, families, and selected phytochemicals of 10 African medicinal plants chosen for their ethnomedicinal relevance, documented antimicrobial activity, and/or QS inhibitory potential, as reported in previous ethnobotanical and phytochemical studies.[7,8] Major bioactive compounds from each plant were identified via literature mining and retrieved in Structure Data File format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed on August 5, 2025).

2.2. Protein structure retrieval and preparation

Crystal structures of bacterial protein targets associated with virulence and antimicrobial resistance were retrieved from the RCSB Protein Data Bank (PDB; https://www.rcsb.org/, accessed on August 5, 2025) in the Biological Assembly 1 format. The selected targets included:

(i) LasR QS regulator from *P. aeruginosa* (PDB ID: 2UV0)
(ii) AcrB efflux pump transporter from *E. coli* (PDB ID: 5NC5)
(iii) DNA gyrase subunit B from *E. coli* (PDB ID: 6F86)
(iv) TEM-1 β-lactamase from *E. coli* (PDB ID: 1BTL)

Protein preprocessing was performed using UCSF Chimera Classic version 1.16 (UCSF, USA)[18] to eliminate crystallographic artifacts (e.g., designed ankyrin repeat proteins, sulfate ion, hydrogen bonded moieties [OH···N], and cyclotryptophan-(D)-tryptophan fragments), detergents, and solvent molecules, followed by protonation at pH 7.0 using Dock Prep (Resource for Biocomputing, Visualization, and Informatics (RBVI), University of California San Francisco, USA) and geometry optimization using the Amber force field. Chain selection and correction of alternate conformations were carried out where applicable. Non-standard residues, such as selenomethionine (MSE), were converted to methionine (MET).

Protein structure preprocessing was carried out using UCSF Chimera Classic version 1.16 (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, USA) to eliminate

Table 1. African medicinal plants and their selected phytochemicals

Plant (scientific name)	Family	Selected phytochemical	PubChem CID	Class
Ocimum gratissimum	Lamiaceae	Eugenol	3314	Phenylpropanoid
Moringa oleifera	Moringaceae	Quercetin	5280343	Flavonoid
Vernonia amygdalina	Asteraceae	Kaempferol	5280863	Flavonoid
Tithonia diversifolia	Asteraceae	Luteolin	5280445	Flavonoid
Garcinia kola	Clusiaceae	Catechin	9064	Flavan-3-ol
Citrus aurantifolia	Rutaceae	Naringenin	932	Flavanone
Terminalia catappa	Combretaceae	Ellagic acid	5281855	Polyphenol
Ficus exasperata	Moraceae	Gallic acid	370	Phenolic acid
Newbouldia laevis	Bignoniaceae	Rutin	5280805	Flavonoid glycoside
Clerodendrum volubile	Lamiaceae	Apigenin	5280443	Flavonoid

Abbreviation: CID: Compound ID.

crystallographic artifacts, including designed ankyrin repeat proteins (DARPins), sulfate ions, hydrogen-bonded moieties (OH···N), and cyclotryptophan-(D)-tryptophan fragments.

Each prepared protein was imported into PyRx version 0.9.8 (Scripps Research Institute, USA)[19] as a macromolecule and converted to PDBQT format for docking. A blind docking protocol was applied to span the entire protein surface, ensuring that all potential binding sites were explored.

The stereochemical quality of all prepared proteins was validated using the Ramachandran plot (RamPlot) server (https://www.ramplot.in/),[20] which generated two- and three-dimensional Ramachandran plots. Residue distributions in favored and allowed regions were used to confirm the structural reliability before docking.

2.3. Ligand preparation

Ligands were retrieved from PubChem in Structure Data File format and processed using Open Babel version 3.1.1 (Open Babel Team, USA) integrated within PyRx version 0.9.8.[21] The workflow included conversion to three-dimensional structures, energy minimization using the universal force field, and conversion to PDBQT format for compatibility using AutoDock Vina (Scripps Research Institute, USA).

2.4. Molecular docking

Molecular docking was carried out using AutoDock Vina version 1.1.2 integrated within PyRx version 0.9.8.[22] Blind docking grids were set to span the entire protein structure. For each ligand–protein pair, multiple conformations were generated and ranked based on binding affinity (kcal/mol).

The top-ranked docking poses were visualized using BIOVIA Discovery Studio Visualizer version 21.1.0.20298 (Dassault Systèmes, USA) and LigPlot+ version 2.2 (European Bioinformatics Institute (EMBL-EBI), United Kingdom), in which hydrogen bonds, π–π stacking, hydrophobic interactions, and other non-covalent interactions were analyzed.

2.5. ADMET and drug-likeness prediction

The highest-affinity phytochemicals for each protein target were subjected to *in silico* pharmacokinetic and toxicity prediction using:

(i) SwissADME (http://www.swissadme.ch/, accessed on August 6, 2025) to evaluate Lipinski's rule of five, water solubility, gastrointestinal (GI) absorption, and blood–brain barrier permeability.[10]
(ii) pkCSM (http://biosig.unimelb.edu.au/pkcsm/, accessed on August 6, 2025) to predict metabolic and excretion profiling.
(iii) ProTox-II (https://tox-new.charite.de/protox_II, accessed on August 6, 2025) for oral toxicity classification and median lethal dose (LD_{50}) prediction.

No MD simulations were conducted in this study; instead, molecular docking and ADMET profiling were used as the primary computational screening approach.

3. Results and discussion

3.1. Molecular docking analysis

Molecular docking revealed strong binding affinities between the selected phytochemicals and key bacterial resistance and virulence proteins, as shown in Table 2. Protein–ligand interactions were visualized using BIOVIA Discovery Studio Visualizer.[23] Structural validation was performed using the RamPlot server. ADMET and drug-likeness predictions were carried out using SwissADME and ProTox-II, respectively.

To better understand the molecular basis of these binding affinities, the top-scoring complexes were subjected to detailed residue-level interaction analysis, highlighting hydrogen bonds, van der Waals interactions, and π-related interactions.

3.1.1. Interaction studies

Protein–ligand interactions of the top-scoring complexes were analyzed in BIOVIA Discovery Studio Visualizer version 21.1. The most favorable complexes were selected for detailed analysis based on docking affinities: CID 5280445 with 1BTL (−8.3 kcal/mol), CID 5280445 with 2UV0 (−10.8 kcal/mol), CID 5280805 with 5NC5 chain A (−9.5 kcal/mol), and CID 5281855 with 6F86 (−8.3 kcal/mol). These ligands were chosen as representative inhibitors, as they demonstrated the strongest binding to their respective targets. Table 3 provides residue-level interaction details for each protein–ligand complex, including specific residues involved in hydrogen bonding, van der Waals interactions, π interactions, and unfavorable interactions.

In the 1BTL-CID 5280445 complex, the ligand formed a hydrogen-bonding network with ASN132, SER235, and ARG244, anchoring it within the active site. Van der Waals interactions with SER70, SER130, PRO167, LYS234, GLY236, and ALA237 contributed to structural

Table 2. Summary of protein–ligand interactions for top-scoring complexes

Protein (PDB ID)	Ligand (CID)	Binding affinity (kcal/mol)	Key interacting residues	Interaction types
1BTL (*Escherichia coli*)	5280445	−8.3	ASN132, SER235, ARG244	Hydrogen bonds
			SER70, SER130, PRO167, LYS234, GLY236, ALA237	Van der Waals
			TYR105	π–π stacking
			ARG244	π–cation, donor–donor (unfavorable)
2UV0 (*Pseudomonas sp.*)	5280445	−10.8	SER80, ASP123, LYS173	Hydrogen bonds
			GLN81, PRO124, LEU172, GLY174, TYR176	Van der Waals
			PHE175, LEU178	π–alkyl
			HIS177	Donor–donor (unfavorable)
6F86 (*Escherichia coli*)	5281855	−8.3	GLU274, LYS305, TYR309	Hydrogen bonds
			THR271, ALA273, LEU308	Van der Waals
			PHE310	π–π stacking
			GLY272	amide–π
			GLU274	π–anion
			TYR309	π–donor, hydrogen bond
5NC5 chain A (*Escherichia coli AcrBZ*)	5280805	−9.5	ASN100, ASP153	Hydrogen bonds
			GLY72, ILE101, VAL155	Van der Waals
			PHE102, LEU150	π–alkyl
			-	Fewer stabilizing interactions in the pump-associated complex; donor–donor clashes present

Abbreviations: CID: Compound ID; PBD: Protein Data Bank.

Table 3. Summary of binding affinities and interaction types for selected protein–ligand complexes

Protein (PDB ID)	Ligand (CID)	Binding affinity (kcal/mol)	No. of hydrogen bonds	Key interaction types
1BTL (*Escherichia coli*)	5280445	−8.3	3	Hydrogen bonds, van der Waals, π–π stacking, π–cation
2UV0 (*Pseudomonas sp.*)	5280445	−10.8	3	Hydrogen bonds, van der Waals, π–alkyl
6F86 (*Escherichia coli*)	5281855	−8.3	3	Hydrogen bonds, van der Waals, π–π stacking, π–anion, amide–π
5NC5 chain A (*Escherichia coli* AcrBZ)	5280805	−9.5	2	Hydrogen bonds, van der Waals, π–alkyl (pump-associated form showed reduced affinity)

Abbreviations: CID: Compound ID; PBD: Protein Data Bank.

complementarity. TYR105 engaged in π–π stacking, while ARG244 contributed a π–cation interaction, partially offsetting one unfavorable donor–donor clash. This balanced network of hydrogen bonds, van der Waals forces, and π-related interactions resulted in moderate to strong binding stability.

The 2UV0–CID 5280445 (Figure 1) exhibited the strongest binding affinity among all proteins. Hydrogen bonds with SER80, ASP123, and LYS173 provided strong directional stabilization. Van der Waals interactions with GLN81, PRO124, LEU172, GLY174, and TYR176 promoted tight ligand accommodation. π–alkyl stabilization with PHE175 and LEU178 added hydrophobic reinforcement, while a donor–donor clash with HIS177 was present but outweighed by favorable interactions. This cooperative network of polar and hydrophobic interactions explains the exceptionally high affinity observed.

While hydrogen bonding and electrostatic contacts contribute to initial stabilization, hydrophobic interactions play a crucial role in ligand accommodation and binding pocket complementarity, highlighting the need for a dedicated analysis.

Binding was stabilized by hydrogen bonds with GLU274, LYS305, and TYR309, complemented by van der Waals interactions with THR271, ALA273, and LEU308. Aromatic residues made critical contributions: PHE310 formed π–π stacking, GLY272 participated in amide–π interactions, and GLU274 engaged in a π–anion contact. TYR309 contributed a π–donor hydrogen bond, reinforcing complex stability. The diverse range of polar and aromatic interactions highlights the chemical complementarity between the ligand and the binding pocket.

Hydrogen bonds with ASN100 and ASP153 anchored the ligand within the binding site, supported by van der Waals interactions with GLY72, ILE101, and VAL155. π–alkyl interactions with PHE102 and LEU150 provided additional stabilization. In contrast, when chain A was docked with the efflux pump, the affinity decreased and unfavorable donor–donor interactions emerged, indicating that pump association introduces steric hindrance and reduces effective ligand accommodation. Thus, the isolated chain A environment was more favorable for ligand binding.

Across all proteins, phytochemical inhibition was mediated by a synergistic balance of hydrogen bonding, van der Waals stabilization, and π interactions. The strongest inhibition was observed for 2UV0 with CID 5280445 (−10.8 kcal/mol), reflecting a highly complementary binding environment. 5NC5 chain A with CID 5280805 (−9.5 kcal/mol) also exhibited strong inhibition, although affinity decreased in pump-associated forms, highlighting the effect of accessory proteins on ligand accessibility. Both 1BTL and 6F86 complexes demonstrated moderate affinities (−8.3 kcal/mol each) but were stabilized by diverse polar and aromatic interactions, as shown in Figures 1-4. Together, these findings confirm that the selected phytochemicals formed stable binding networks within their target proteins, supporting their potential as effective inhibitors.

To further elucidate the role of non-polar stabilization, a dedicated hydrophobic interaction analysis was performed. These findings, including hydrophobic residue mapping, interaction profiles, and surface visualizations, are discussed in the following section.

3.2. Hydrophobic interaction analysis

Hydrophobic interactions were further examined to evaluate their contribution to ligand stabilization and

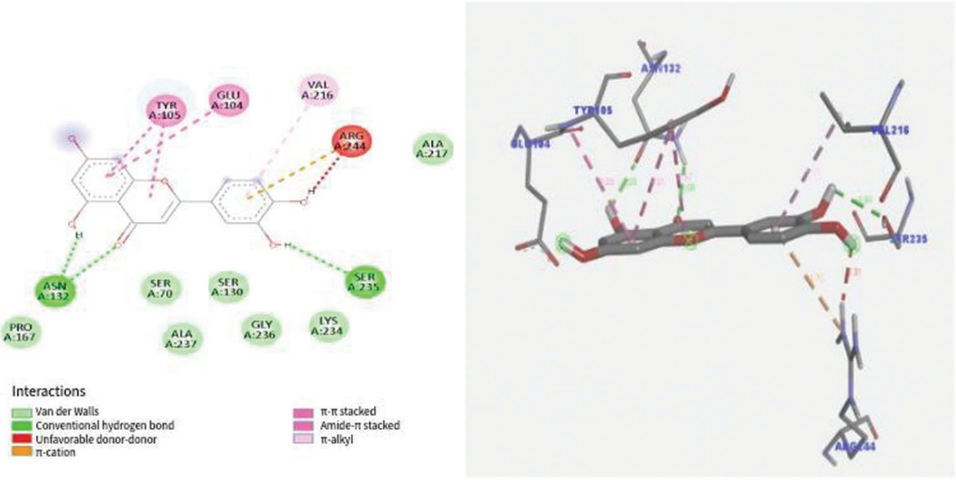

Figure 1. 1BTL (*Escherichia coli*)–CID 5280445 complex (−8.3 kcal/mol)

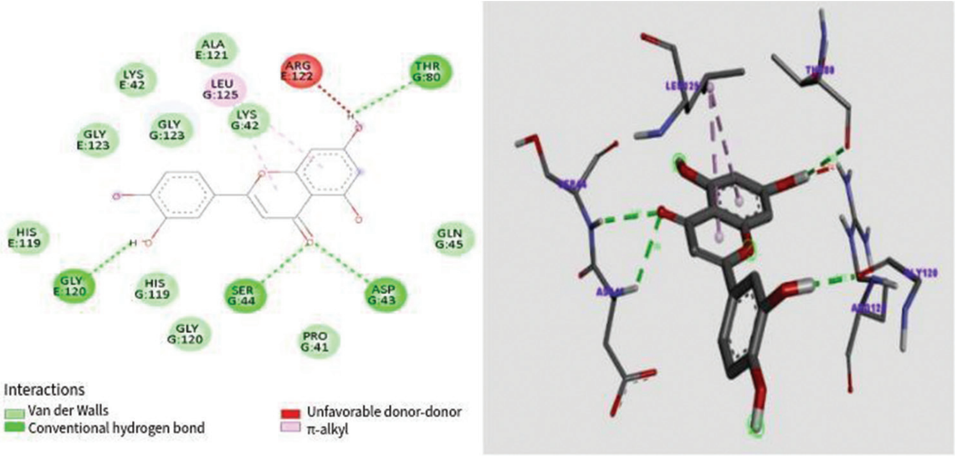

Figure 2. 2UV0 (*Pseudomonas sp.*)–CID 5280445 complex (−10.8 kcal/mol)

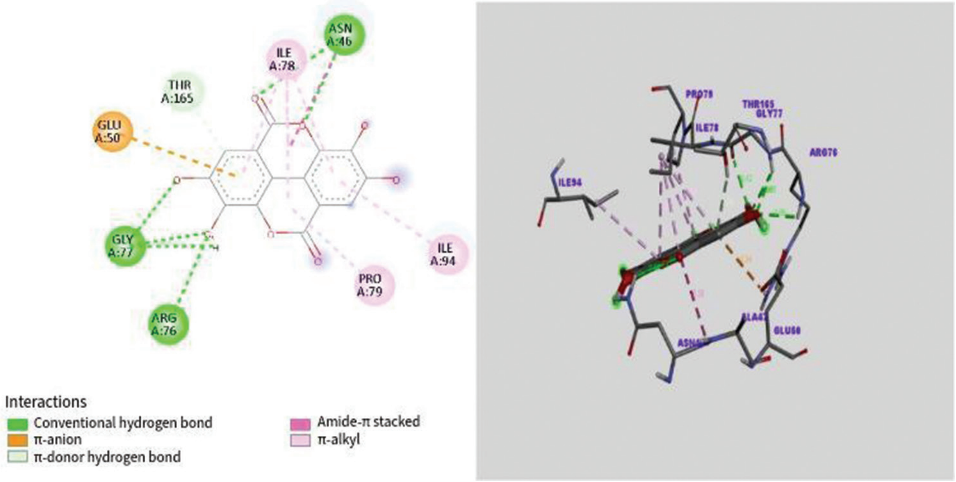

Figure 3. 6F86 (*Escherichia coli*)–CID 5281855 complex (−8.3 kcal/mol)

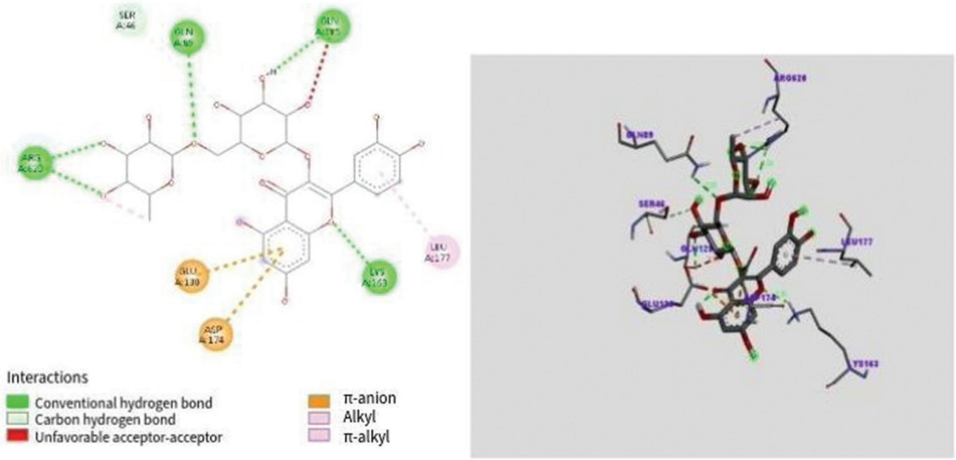

Figure 4. 5NC5 Chain A (*Escherichia coli* AcrBZ)–CID 5280805 complex (−9.5 kcal/mol)

solvent exclusion within the binding pockets, as shown in Table 4. Hydrophobic maps were generated using BIOVIA Discovery Studio Visualizer version 21.1, with particular attention to van der Waals interactions, π–alkyl interactions, and aromatic stacking residues. These interactions provide a non-polar environment that complements hydrogen bonding and electrostatic stabilization, and are often critical determinants of binding affinity.

Beyond non-polar interactions, charged and polar residues contributed additional stabilization through electrostatic complementarity, synergistically enhancing overall binding affinity.

3.3. Per-protein hydrophobic mapping

Figure 5A-D illustrates the hydrophobic mapping of ligand–protein complexes.

For the 1BTL–CID 5280445 complex (−8.3 kcal/mol; Figure 5A), hydrophobic stabilization was mediated by van der Waals interactions with SER70, SER130, PRO167, LYS234, GLY236, and ALA237, creating a compact non-polar microenvironment. TYR105 provided π–π stacking with the ligand's aromatic ring, while ARG244 engaged in π–cation stabilization.

For the 2UV0–CID 5280445 complex (−10.8 kcal/mol; Figure 5B), the most extensive hydrophobic network was observed, involving van der Waals interactions with GLN81, PRO124, LEU172, GLY174, and TYR176. π–alkyl interactions with PHE175 and LEU178 further shielded the ligand from solvent exposure, consistent with the highest observed affinity.

For the 6F86–CID 5281855 complex (−8.3 kcal/mol; Figure 5C), non-polar stabilization was mediated by THR271, ALA273, and LEU308. In addition, aromatic residues PHE310 and TYR309 contributed to π–π stacking

Table 4. Hydrophobic interactions observed in the selected protein–ligand complexes

Protein (PDB ID)	Ligand (CID)	Binding affinity (kcal/mol)	Key hydrophobic residues	Interaction types
1BTL (*Escherichia coli*)	5280445	−8.3	SER70, SER130, PRO167, LYS234, GLY236, ALA237, TYR105, ARG244	Van der Waals, π–π stacking, π–cation
2UV0 (*Pseudomonas sp.*)	5280445	−10.8	GLN81, PRO124, LEU172, GLY174, TYR176, PHE175, LEU178	Van der Waals, π–alkyl
6F86 (*Escherichia coli*)	5281855	−8.3	THR271, ALA273, LEU308, PHE310, TYR309	Van der Waals, π–π stacking, π–donor
5NC5 chain A (*Escherichia coli AcrBZ*)	5280805	−9.5	GLY72, ILE101, VAL155, PHE102, LEU150	Van der Waals, π–alkyl

Abbreviations: CID: Compound ID; PBD: Protein Data Bank.

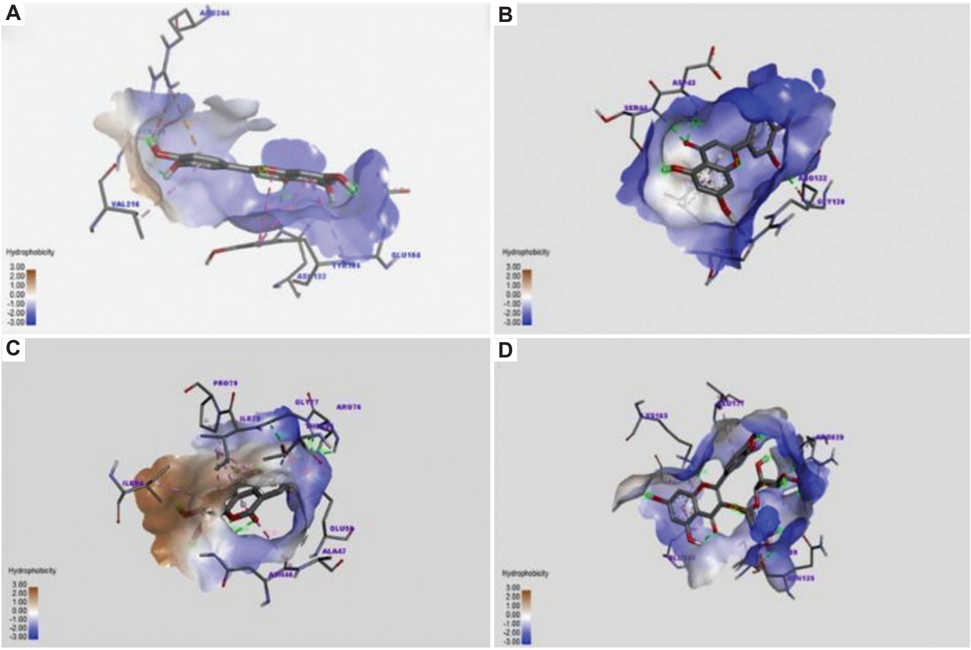

Figure 5. Hydrophobic surface mapping of ligand–protein complexes: (A) 1BTL–CID 5280445 (−8.3 kcal/mol), (B) 2UV0–CID 5280445 (−10.8 kcal/mol), (C) 6F86–CID 5281855 (−8.3 kcal/mol), and (D) 5NC5 chain A–CID 5280805 (−9.5 kcal/mol)
Abbreviation: CID: Compound ID.

and π–donor hydrogen bonding, respectively, effectively positioning the ligand within a hydrophobic cavity

For the 5NC5 chain A–CID 5280805 complex (−9.5 kcal/mol; Figure 5D), van der Waals interactions with GLY72, ILE101, and VAL155 provided a hydrophobic framework, supplemented by π–alkyl stabilization from PHE102 and LEU150. The loss of several of these hydrophobic interactions in the pump-associated complex correlated with reduced affinity.

Hydrophobic stabilization was found to be a consistent determinant of binding strength across all studied complexes. The 2UV0–CID 5280445 complex exhibited the densest hydrophobic packing, consistent with its strongest binding affinity (−10.8 kcal/mol). The 5NC5 chain A–CID 5280805 complex also showed significant hydrophobic reinforcement (−9.5 kcal/mol), although steric effects in the pump-associated form led to reduced binding stability. Both 1BTL and 6F86 demonstrated moderate hydrophobic contributions, with stabilization strongly dependent on key aromatic residues (TYR105 and PHE310/TYR309, respectively). These findings underscore the crucial role of hydrophobic complementarity in determining phytochemical-mediated inhibition of bacterial target proteins.

3.4. Ionizability and electrostatic interaction analysis

Electrostatic complementarity between ionizable residues in the protein and the polar or charged groups of the ligands was analyzed to evaluate their contribution to binding stability. Charged residues frequently stabilized the ligand through salt bridges, ionic interactions, or charge-assisted hydrogen bonds. These interactions helped orient the ligands within the binding pocket and synergistically enhanced affinity in cooperation with hydrophobic packing, as shown in Figure 6A-D.

For the 1BTL–CID 5280445 complex (−8.3 kcal/mol; Figure 6A), electrostatic stabilization was mediated by ARG244, which formed a charge-assisted hydrogen bond with the ligand's polar group while also engaging in π–cation interactions. The dual role of this residue highlights its importance in anchoring the ligand despite the presence of a donor–donor clash.

For the 2UV0–CID 5280445 complex (−10.8 kcal/mol; Figure 6B), strong ionic contributions were observed from ASP123 and LYS173, which formed polar hydrogen bonds with the ligand's functional groups. These residues generated a stabilizing electrostatic environment that complemented the extensive hydrophobic pocket, thereby explaining the exceptionally high affinity of this complex.

For the 6F86–CID 5281855 complex (−8.3 kcal/mol; Figure 6C), electrostatic interactions were primarily mediated by GLU274 and LYS305. GLU274 provided a π–anion interaction with the ligand's aromatic group, while LYS305 stabilized the binding through a conventional

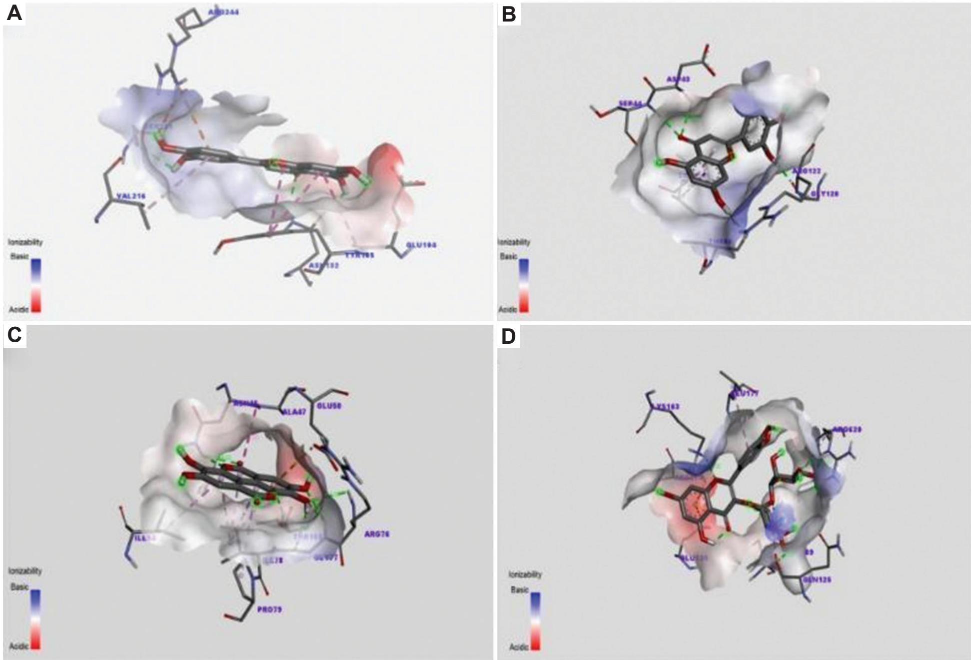

Figure 6. Electrostatic interaction mapping of ligand–protein complexes: (A) 1BTL–CID 5280445 (−8.3 kcal/mol), (B) 2UV0–CID 5280445 (−10.8 kcal/mol), (C) 6F86–CID 5281855 (−8.3 kcal/mol), and (D) 5NC5 chain A–CID 5280805 (−9.5 kcal/mol)
Abbreviation: CID: Compound ID.

hydrogen bond. Together, these ionizable residues balanced the polar–non-polar interactions within the binding cavity.

However, for the 5NC5 chain A–CID 5280805 complex (−9.5 kcal/mol; Figure 6D), electrostatic interactions were comparatively weaker, limited to polar interactions with ASN100 and ASP153. While not as extensive as those in the 2UV0–CID 5280445 complex, these residues nevertheless provided key anchoring points for the ligand within the AcrBZ chain A binding pocket. In the pump-associated form, steric interference reduced both hydrophobic and electrostatic contacts, resulting in decreased binding stability.

Electrostatic interactions played a supportive yet essential role in stabilizing the docked phytochemicals. Complexes with higher affinity (2UV0 and 5NC5 chain A) displayed well-distributed ionic interactions involving both acidic (ASP, GLU) and basic (ARG, LYS) residues, which complemented the dense hydrophobic packing. In contrast, complexes with moderate affinity (1BTL and 6F86) exhibited fewer electrostatic interactions, relying more heavily on aromatic and van der Waals interactions, as presented in Table 5. These findings indicate that ionizability and charge complementarity are critical determinants in fine-tuning phytochemical inhibition of bacterial targets.

To further assess how ligand positioning and burial within the binding pocket influence binding strength, solvent-accessible surface (SAS) analyses were conducted.

3.5. Solvent-accessible surface analysis

To assess the degree of ligand burial within the protein cavities, SAS areas were calculated and visualized using BIOVIA Discovery Studio Visualizer v21.1. This analysis provides insights into the extent of ligand exposure to solvent versus its encapsulation within the binding pocket. A reduced SAS typically indicates deeper ligand insertion and stronger hydrophobic stabilization, whereas higher SAS values suggest partial solvent exposure and weaker binding, as illustrated in Figure 7A-D.

For the 1BTL–CID 5280445 complex (−8.3 kcal/mol; Figure 7A), the ligand was moderately buried within the binding pocket, with aromatic moieties partially shielded by TYR105 and ARG244. SAS mapping revealed that while hydrophobic residues contributed to partial encapsulation, some solvent exposure remained, consistent with its intermediate binding affinity.

For the 2UV0–CID 5280445 complex (−10.8 kcal/mol; Figure 7B), the ligand displayed the deepest insertion among all complexes, with SAS mapping revealing near-complete encapsulation by hydrophobic residues GLN81, PRO124, LEU172, and PHE175. This low solvent accessibility correlated with the highest binding affinity, reflecting efficient desolvation and tight packing within the non-polar pocket.

For the 6F86–CID 5281855 complex (−8.3 kcal/mol; Figure 7C), the ligand was partially embedded, with SAS analysis showing moderate burial. While PHE310 and TYR309 shielded the aromatic ring system, other portions of the ligand remained solvent-exposed. This balance between burial and exposure likely contributed to the moderate binding affinity of this complex.

For the 5NC5 chain A–CID 5280805 complex (−9.5 kcal/mol; Figure 7D), the ligand exhibited strong burial within the chain A pocket, with van der Waals and π–alkyl interactions reducing solvent exposure. SAS visualization confirmed that the isolated chain A complex had greater encapsulation compared with the pump-associated form, where steric interference increased solvent accessibility and reduced affinity.

Solvent-accessible surface analysis confirmed that ligand burial strongly correlated with binding affinity. The 2UV0–CID 5280445 complex demonstrated the lowest solvent exposure, consistent with its highest affinity (−10.8 kcal/mol). The 5NC5 chain A–CID 5280805 complex also exhibited strong burial, although this effect was reduced when docked with the efflux pump. In contrast, the 1BTL and 6F86 complexes showed partial solvent exposure,

Table 5. Ionizable residues and electrostatic interactions in the selected protein–ligand complexes

Protein (PDB ID)	Ligand (CID)	Binding affinity (kcal/mol)	Key ionizable residues	Electrostatic interaction types
1BTL (*Escherichia coli*)	5280445	−8.3	ARG244	Charge-assisted hydrogen bond, π–cation, donor–donor clash
2UV0 (*Pseudomonas sp.*)	5280445	−10.8	ASP123, LYS173	Ionic hydrogen bonds, salt bridge stabilization
6F86 (*Escherichia coli*)	5281855	−8.3	GLU274, LYS305, TYR309	π–anion (GLU274), charge-assisted hydrogen bond (LYS305), polar hydrogen bond donor (TYR309)
5NC5 chain A (*Escherichia coli* AcrBZ)	5280805	−9.5	ASN100, ASP153	Polar hydrogen bonds (acidic/basic complementarity)

Abbreviations: CID: Compound ID; PBD: Protein Data Bank.

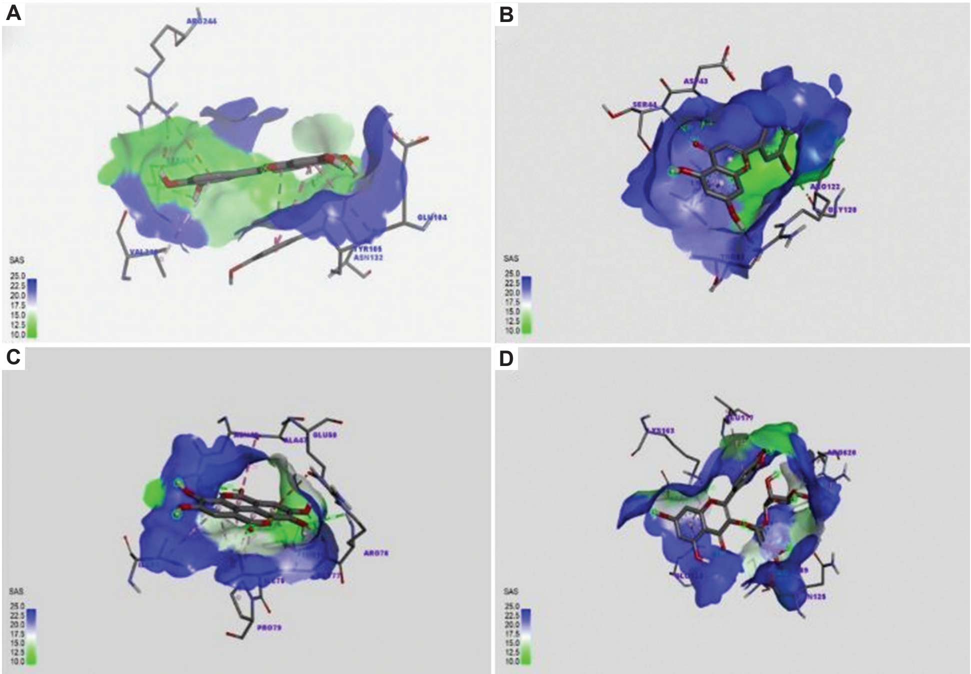

Figure 7. Solvent-accessible surface mapping of ligand–protein complexes: (A) 1BTL–CID 5280445 (−8.3 kcal/mol), (B) 2UV0–CID 5280445 (−10.8 kcal/mol), (C) 6F86–CID 5281855 (−8.3 kcal/mol), and (D) 5NC5 chain A–CID 5280805 (−9.5 kcal/mol)
Abbreviations: CID: Compound ID; SAS: Solvent-accessible surface analysis.

aligning with their moderate binding affinities. Together, these findings highlight that reduced SAS (greater ligand encapsulation) serves as a key determinant of phytochemical-mediated protein inhibition.

Complementing the solvent exposure data, hydrogen-bond length analysis provided insights into the precise geometries of hydrogen bonds and other stabilizing contacts that underpin complex stability.

3.6. Bond length analysis and its impact on interaction strength

3.6.1. 1BTL–ligand complex

The docking analysis of 1BTL with the ligand (CID 5280445) revealed hydrogen bonds with ASN132, SER235, and ARG244, with bond lengths ranging from 2.7 to 2.9 Å, consistent with strong stabilizing interactions. Van der Waals interactions with SER70, SER130, PRO167, LYS234, GLY236, and ALA237 supported close packing, while π–π stacking with TYR105 and π–cation interaction with ARG244 enhanced binding affinity. Despite a donor–donor repulsion observed with ARG244, the unfavorable contact occurred at a relatively longer bond length (>3.1 Å), thereby minimizing its destabilizing effect. Overall, the shorter hydrogen bond distances outweighed the penalty from unfavorable contacts.

3.6.2. 2UV0–ligand complex

In the 2UV0 complex, multiple stabilizing hydrogen bonds were observed with GLY120, GLY123, SER44, ASP43, and THR80, with bond lengths clustered around 2.8–3.0 Å, indicating stable anchoring. Van der Waals interactions with ALA121, HIS119, and PRO41, together with π–alkyl interactions (LEU125, LYS42), complemented the hydrogen bonding. An unfavorable donor–donor clash with ARG122 was detected, but its longer bond distance (approximately 3.2 Å) reduced its destabilizing contribution. Overall, the predominance of shorter hydrogen bonds and hydrophobic stabilization compensated for the repulsive contact.

3.6.3. 6F86–ligand complex

The ligand bound to 6F86 through hydrogen bonds with ASN46, GLY77, ARG76, and ILE78, exhibiting bond lengths of 2.6–2.9 Å, characteristic of strong interactions. π–anion interaction with GLU50 and amide–π stacking with PRO79 and ILE94 provided additional stabilization. A combination of π–donor hydrogen bonding and van der Waals forces further optimized the ligand's orientation. No significant unfavorable interactions were observed at short distances, making this complex one of the most stable based on its bond length profile.

3.6.4. 5NC5 (chain A, B, and C)–ligand complexes

In 5NC5, the effect of the pump protein was evident when comparing the isolated chains to chain–pump complexes.

For chain A, hydrogen bond lengths averaged 2.7–2.9 Å, but the presence of an additional donor–donor clash with ARG introduced local instability. Chain B displayed hydrogen bonds with bond lengths of approximately 2.85 Å, but simultaneous acceptor–acceptor repulsions at 2.95 Å weakened stability, resulting in slightly lower binding affinity. Chain C exhibited a comparable binding affinity to chain A, although unfavorable interactions were present at shorter distances, increasing repulsive effects. Overall, the isolated chains (without pump) demonstrated shorter, more stable hydrogen bonds and hence stronger affinities than their pump-associated counterparts. The results are provided in Table 6.

3.7. Ramachandran plot analysis

Ramachandran plots were generated for all proteins to assess stereochemical quality and backbone conformational preferences. In these plots, residues are distributed across favorable, allowed, and disallowed regions, with additional frequency maps providing insights into the dominant secondary structure motifs.

For 1BTL (Figure 8), the standard plot showed that the majority of residues were concentrated in favorable α-helical and β-sheet regions, with very few residues occupying disallowed zones (Figure 8A). The corresponding frequency plot revealed a high density in the helical region, confirming the structural dominance of α-helices (Figure 8B). Residue-specific maps further demonstrated the expected flexibility of glycine residues and the conformational restriction of proline residues (Figure 8C), while the extended map confirmed overall stereochemical stability with minimal strain (Figure 8D). With the protein structures validated, attention was then turned to the predicted toxicity profiles of the ligands to evaluate their potential systemic risks.

Similarly, the Ramachandran plots of 2UV0 demonstrated strong clustering of residues in favorable regions, particularly within β-strand conformations (Figure 9). The frequency distribution displayed a prominent density peak in the extended β-sheet region, supporting the prevalence of this secondary structure (Figure 9B). Glycine residues exhibited broader scattering across allowed regions, reflecting their inherent flexibility, whereas proline residues remained confined to restricted zones. The extended conformational overview suggested excellent stereochemical quality, with negligible outliers (Figure 9C).

For 5NC5 (chain A), the majority of residues were distributed within favorable regions, although a small proportion appeared in disallowed zones, likely due to loop conformations (Figure 10). The frequency map indicated a mixed distribution between α-helical and β-strand regions, consistent with its composite secondary structure (Figure 10B). Glycine residues displayed a wider conformational spread, whereas proline residues occupied the expected ϕ–ψ ranges. Despite a few deviations, the extended analysis confirmed that the structure retained acceptable stereochemical quality for docking (Figure 10C).

For 6F86, the plots confirmed the highest degree of stereochemical refinement among the analyzed proteins (Figure 11). The majority of residues clustered within favorable regions, with the frequency map showing a sharp density peak in the α-helical zone, indicating the structural dominance of α-helices (Figure 11B). Glycine residues displayed broader conformational variability, while proline residues remained appropriately confined. The extended conformational analysis reinforced the overall stability of this protein, with only rare outlier residues observed (Figure 11D).

3.8. Toxicity prediction (ProTox analysis)

The toxicity of ligands CID 5280445, CID 5280805, and CID 5281855 was predicted using the ProTox-3.0 platform. The

Table 6. Bond length analysis and stability assessment

Protein–ligand complex	Key hydrogen-bonding residues	Favorable bond lengths (Å)	Unfavorable interactions (type and length)	Stability assessment
1BTL–ligand	ASN132, SER235, ARG244	2.7–2.9 (hydrogen bonds)	Donor–donor with ARG244 (>3.1 Å)	Stable; short hydrogen bonds outweigh weak repulsion
2UV0–ligand	GLY120, GLY123, SER44, ASP43, THR80	2.8–3.0 (hydrogen bonds)	Donor–donor with ARG122 (~3.2 Å)	Stable; strong hydrogen-bond network compensates for minor repulsion
6F86–ligand	ASN46, GLY77, ARG76, ILE78	2.6–2.9 (hydrogen bonds); π–anion, π–π stacking	None significant	Very stable; multiple favorable short interactions
5NC5 chain A–ligand	Multiple hydrogen bonds	2.7–2.9 (hydrogen bonds)	Donor–donor with ARG (~3.0 Å)	Stable overall, slight perturbation from clash
5NC5 chain B–ligand	SER–ligand (hydrogen bond)	2.85 (hydrogen bonds)	Acceptor–acceptor (~2.95 Å)	Less stable; repulsion weakens bond
5NC5 chain C–ligand	Multiple hydrogen bonds	2.8–3.0 (hydrogen bonds)	Short unfavorable contacts (<3.0 Å)	Moderately stable; repulsion slightly reduces affinity

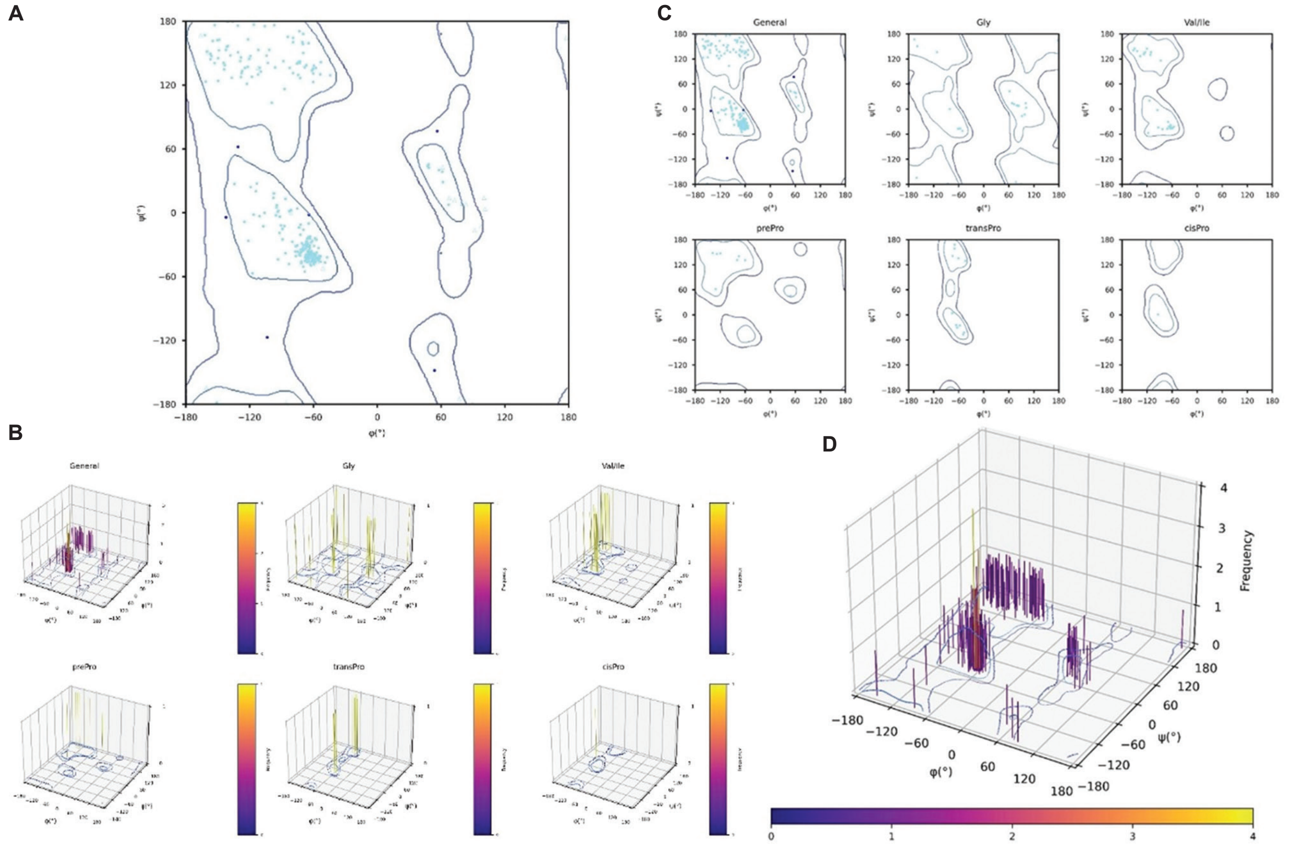

Figure 8. Ramachandran plot analysis. (A) Standard 2D-Ramachandran Map; (B) 3D Ramachandran bar plot frequency of torsion angles; (C) 2D Ramachandran plot (Alanine,15 amino acids; Gly; Val/Ile; pre-Pro; trans-Pro; cis-Pro); (D) Standard 3D Ramachandran bar plot frequency of torsion angles; favored (cyan), allowed (blue), disallowed regions (red).

predicted toxicological profiles encompass organ-specific toxicities, toxicity endpoints, nuclear receptor activation, stress responses, metabolic liabilities, dose distributions, and bond-level structural contributions to toxicity.

3.8.1. Organ toxicity and endpoints

Radar plots of organ toxicity revealed that all ligands were consistently predicted to be inactive for hepatotoxicity, neurotoxicity, and cardiotoxicity (Figure 12). However, nephrotoxicity and respiratory toxicity were predicted as active in all three ligands, with CID 5280805 showing the strongest nephrotoxic signal (probability: 0.77). Bar graphs of toxicity endpoints revealed compound-specific differences: CID 5280445 exhibited risks for carcinogenicity (0.68), mutagenicity (0.51), and nutritional toxicity (0.63), whereas CID 5280805 was predicted to be immunotoxic (0.98) and also showed nutritional toxicity (0.54). CID 5281855 showed an intermediate pattern with moderate immunotoxic and mutagenic predictions.

In addition to toxicity assessment, ADMET profiling was performed to evaluate pharmacokinetic behavior and overall drug-likeness properties of each phytochemical candidate.

3.9. Nuclear receptor and stress pathways

Analysis of nuclear receptor signaling revealed that CID 5280445 shows strong interactions with the aryl hydrocarbon receptor (0.91) and estrogen receptor (ER) pathways (Erα: 0.87; ER–ligand-binding domain: 0.95), suggesting possible endocrine-disrupting potential. In contrast, CID 5280805 and CID 5281855 displayed minimal receptor activation. Stress response predictions further identified CID 5280445 as a mitochondrial-stress liability, with strong activation of the mitochondrial membrane potential (probability: 1.0). No significant stress pathway activation was observed for CID 5280805 or CID 5281855.

3.9.1. Metabolic liabilities

Cytochrome P450 (CYP) enzyme interactions were predicted to be strongest for CID 5280445, which activated CYP1A2, CYP2C9, and CYP2C19, suggesting higher

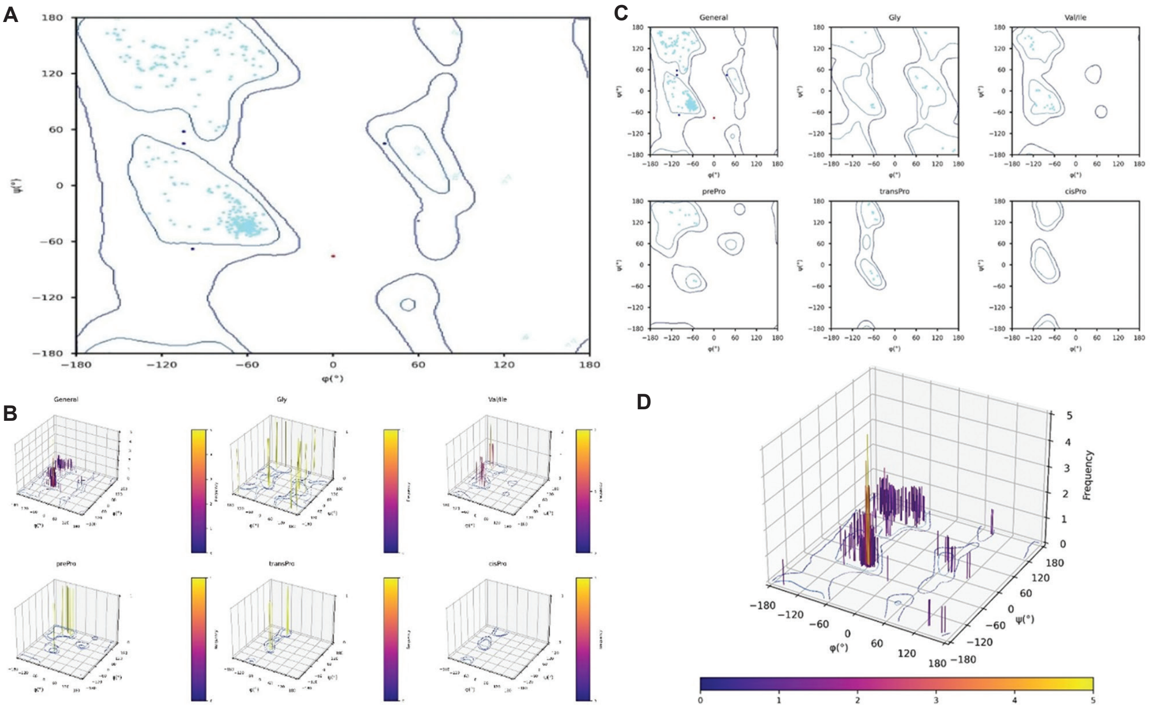

Figure 9. Ramachandran plot analysis. (A) Standard 2D-Ramachandran Map; (B) 3D Ramachandran bar plot frequency of torsion angles; (C) 2D Ramachandran plot (Alanine,15 amino acids; Gly; Val/Ile; pre-Pro; trans-Pro; cis-Pro); (D) Standard 3D Ramachandran bar plot frequency of torsion angles; favored (cyan), allowed (blue), disallowed regions (red).

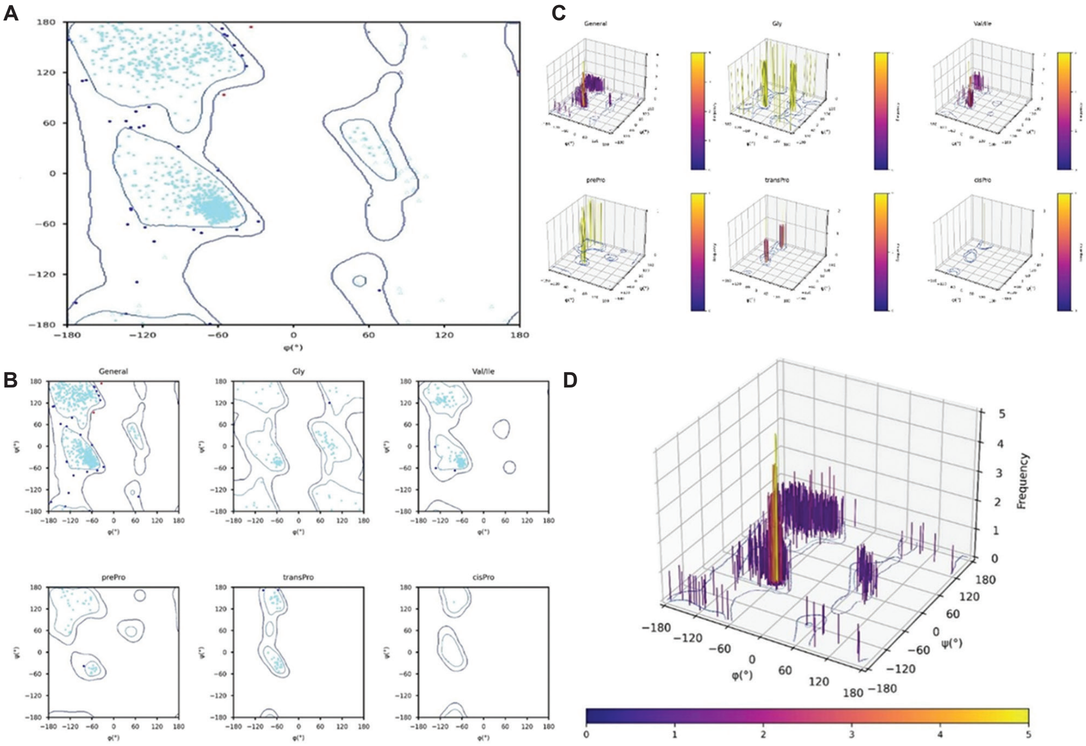

Figure 10. Ramachandran plot analysis. (A) Standard 2D-Ramachandran Map; (B) 3D Ramachandran bar plot frequency of torsion angles; (C) 2D Ramachandran plot (Alanine,15 amino acids; Gly; Val/Ile; pre-Pro; trans-Pro; cis-Pro); (D) Standard 3D Ramachandran bar plot frequency of torsion angles; favored (cyan), allowed (blue), disallowed regions (red).

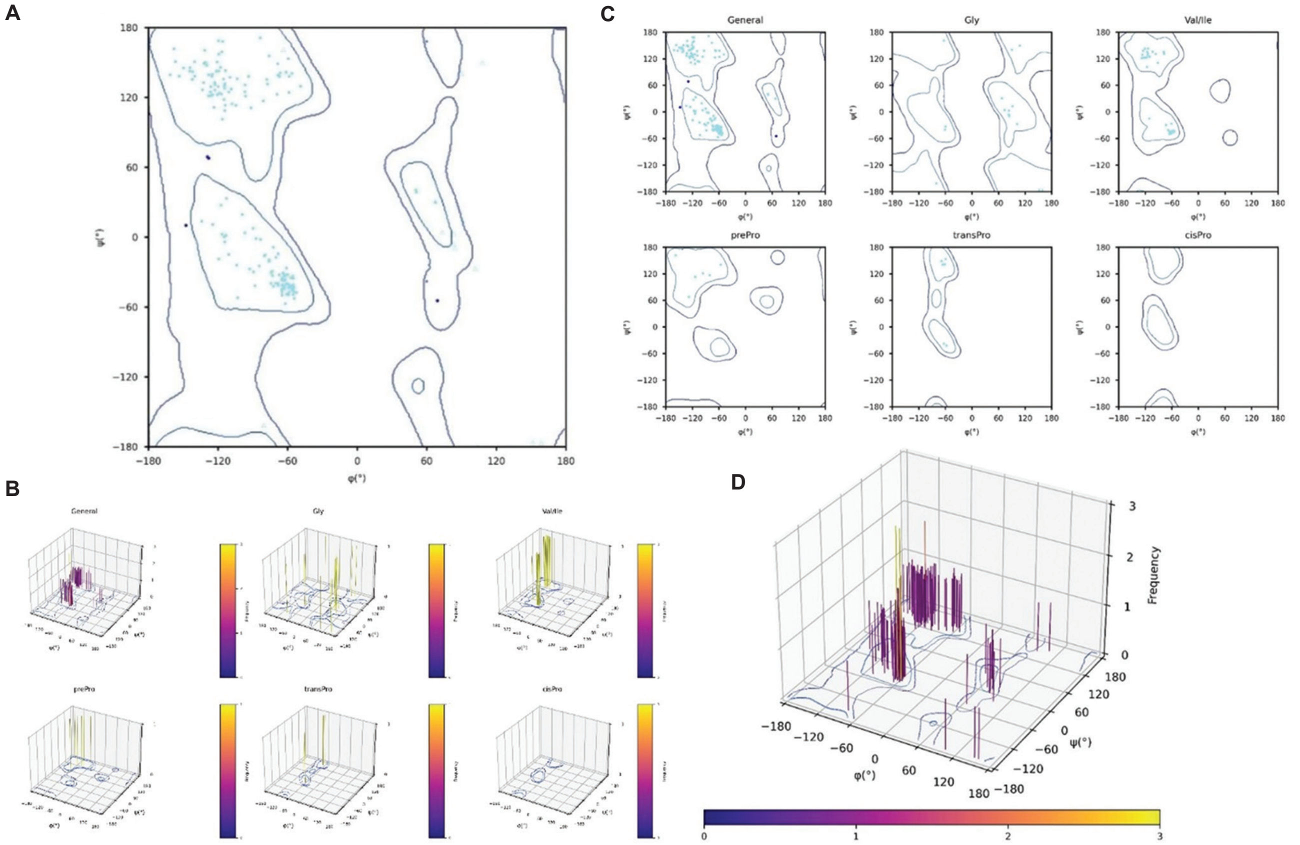

Figure 11. Ramachandran plot analysis. (A) Standard 2D-Ramachandran Map; (B) 3D Ramachandran bar plot frequency of torsion angles; (C) 2D Ramachandran plot (Alanine,15 amino acids; Gly; Val/Ile; pre-Pro; trans-Pro; cis-Pro); (D) Standard 3D Ramachandran bar plot frequency of torsion angles; favored (cyan), allowed (blue), disallowed regions (red).

metabolic turnover and a possible risk of drug–drug interactions. Both CID 5280805 and CID 5281855 exhibited minimal CYP450 activation, indicating comparatively safer metabolic profiles.

3.9.2. Dose distribution

The dose distribution plots (Figure 13) illustrate the predicted LD_{50} values and corresponding toxicity classes. CID 5280445 showed a distribution toward lower LD_{50} values, indicating higher acute toxicity and reduced dosage safety margins. In contrast, CID 5280805 and CID 5281855 were associated with distributions at higher LD_{50} values, reflecting comparatively lower predicted acute toxicity.

3.9.3. Bond distribution

As shown in Figure 14, bond distribution graphs revealed the structural determinants underlying toxicity predictions. For CID 5280445, aromatic and heteroatomic bonds contributed prominently to toxicity endpoints, consistent with its observed receptor pathway activations and mutagenicity. In contrast, CID 5280805 and CID 5281855 displayed more balanced bond distributions, with fewer structural alerts, supporting their comparatively safer toxicity predictions. These findings highlight how molecular structure directly influences toxicity risk.

Among the three ligands, CID 5280805 demonstrated the most favorable toxicity profile, with limited active predictions beyond immunotoxicity and nutritional toxicity. CID 5281855 exhibited an intermediate profile, whereas CID 5280445 exhibited the greatest toxicological risk, with strong carcinogenic, mutagenic, metabolic, and mitochondrial stress predictions. These findings provide an early safety evaluation and support the prioritization of ligands for further *in vitro* validation.

3.10. ADMET analysis

The pharmacokinetic and drug-likeness properties of luteolin (CID 5280445), rutin (CID 5280805), and ellagic acid (CID 5281855) were evaluated using *in silico* ADMET profiling tools. The assessment covered absorption, distribution, metabolism, excretion, and medicinal chemistry filters, which are essential for determining drug suitability.

Figure 12. Radar plots showing comparative toxicity profiles of ligands (A) CID 5280445, (B) CID 5280805, and (C) CID 5281855 against the average reference molecule, highlighting variations in nephrotoxic and immunotoxic tendencies
Abbreviations: CID: Compound ID.

3.10.1. Ligand 1: Luteolin (CID 5280445)

Luteolin ($C_{15}H_{10}O_6$; molecular weight [MW]: 286.24 g/mol) exhibited favorable physicochemical properties, including a topological polar surface area (TPSA) of 111.13 Å^2 and a consensus LogP of 1.73. The compound was predicted to have high GI absorption but not to cross the blood–brain barrier (BBB) and not to act as a P-glycoprotein substrate. It was predicted to inhibit CYP1A2, CYP2D6, and CYP3A4, suggesting moderate metabolic liabilities. Skin permeation was acceptable (log Kp = −6.25 cm/s).

Luteolin satisfied major drug-likeness filters (e.g., Lipinski, Ghose, Veber, Egan, and Muegge) without violations and had a bioavailability score of 0.55. Medicinal chemistry analysis flagged one pan-assay interference compound (PAINS) alert (catechol A) and one Brenk alert (catechol). Synthetic accessibility was favorable (score: 3.02). Overall, luteolin demonstrated good oral bioavailability and compliance with drug-likeness rules, although CYP inhibition and PAINS alerts should be considered.

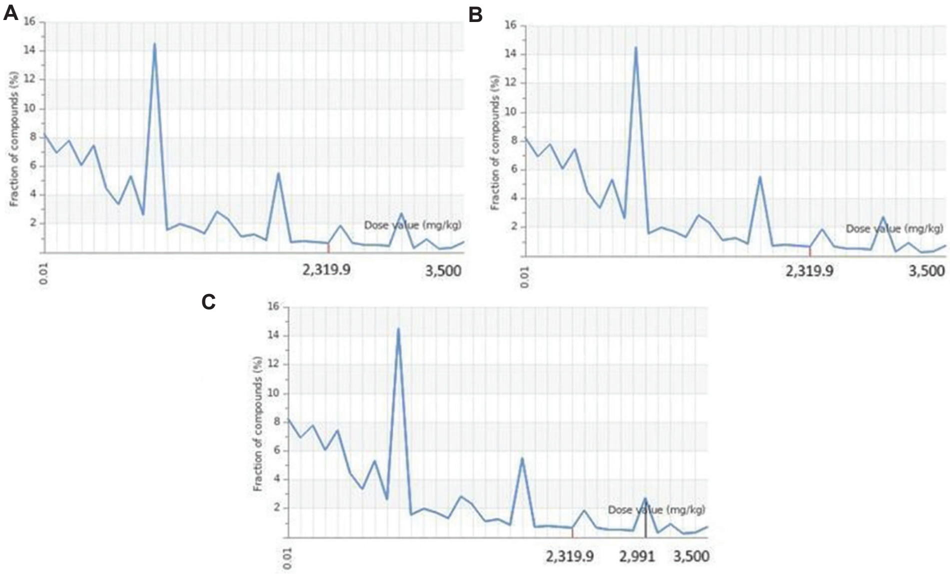

Figure 13. Dose distribution and LD_{50} profiles of ligands (A) CID 5280445, (B) CID 5280805, and (C) CID 5281855. CID 5280445 exhibited lower LD_{50}, indicating higher acute toxicity, whereas CID 5280805 and CID 5281855 exhibited higher LD_{50} values, suggesting lower toxicity.
Abbreviations: CID: Compound ID; LD_{50}: Median lethal dose.

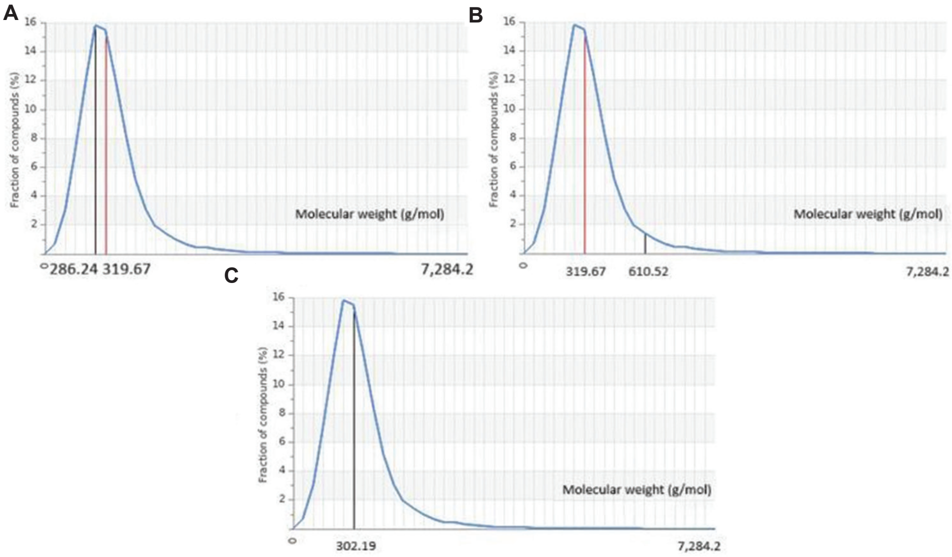

Figure 14. Bond distribution and structural determinants of ligands (A) CID 5280445, (B) CID 5280805, and (C) CID 5281855 toxicity. CID 5280445 exhibited higher aromatic and heteroatomic bond contributions associated with greater toxicity, whereas CID 5280805 and CID 5281855 displayed more balanced bond distributions, indicating comparatively lower toxicity risks.
Abbreviation: CID: Compound ID.

3.10.2. Ligand 2: Rutin (CID 5280805)

Rutin ($C_{27}H_{30}O_{16}$; MW: 610.52 g/mol) exhibited less favorable ADMET characteristics, with a TPSA of 269.43 Å^2 and a consensus LogP of −1.51, indicating high polarity and low lipophilicity. It was predicted to have low GI absorption, not to cross the BBB, and to act as a P-glycoprotein substrate, suggesting efflux limitations. Unlike luteolin, this compound was not predicted to inhibit major CYP450 isoforms, thereby minimizing risks of metabolic interactions. Skin permeation was very poor (log Kp = −10.26 cm/s).

Drug-likeness evaluation revealed multiple violations: Lipinski (MW >500, excess hydrogen-bond donors/acceptors), Ghose, Egan, and Muegge. The bioavailability score was low (0.17). Medicinal chemistry filters flagged catechol A and catechol alerts. Synthetic accessibility was poor (6.52), reflecting structural complexity. Overall, rutin demonstrated poor oral absorption and limited drug-likeness despite its favorable predicted CYP interaction profile.

3.10.3. Ligand 3: Ellagic acid (CID 5281855)

Ellagic acid ($C_{14}H_6O_8$; MW: 302.19 g/mol) exhibited moderate physicochemical properties, with a TPSA of 141.34 Å^2 and a consensus LogP of 1.00, reflecting balanced polarity. It was predicted to have high GI absorption but not to cross the BBB and not to act as a P-glycoprotein substrate. Importantly, it was not predicted to inhibit major CYP450 isoforms (e.g., CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4), indicating low metabolic liabilities. Skin permeation was low (log Kp = −7.36 cm/s).

Drug-likeness profiling showed compliance with Lipinski, Ghose, and Muegge filters, while Veber and Egan filters flagged a single violation due to its high TPSA (>131 Å^2). The bioavailability score was 0.55. Medicinal chemistry analysis identified one PAINS alert (catechol A) and three Brenk alerts (catechol, coumarin, polycyclic aromatic hydrocarbon). Nevertheless, ellagic acid's synthetic accessibility was favorable (3.17), suggesting that it is easier to synthesize than other polyphenolic scaffolds.

Overall, luteolin and ellagic acid demonstrated better ADMET profiles, with acceptable absorption, bioavailability scores of 0.55, and manageable synthetic accessibility. However, both were flagged for PAINS alerts and potential assay interference. Ellagic acid's high TPSA may limit permeability, whereas luteolin showed moderate metabolic liabilities via CYP inhibition. In contrast, rutin exhibited poor absorption, multiple rule violations, and low predicted bioavailability, making it the least favorable candidate.

This comparative assessment of ADMET properties across the top three ligands provided an integrated perspective on efficacy, safety, and drug-likeness.

3.11. Comparative summary of ADMET properties

Table 7 summarizes the pharmacokinetic and drug-likeness parameters of the top three phytochemicals—luteolin (CID 5280445), rutin (CID 5280805), and ellagic acid (CID 5281855)—that exhibited the most favorable docking affinities and stability across the selected bacterial targets.

Luteolin demonstrated the strongest binding affinity, particularly with *P. aeruginosa* LasR (2UV0; −10.8 kcal/mol), and also showed moderate interaction with *E. coli* β-lactamase (1BTL; −8.3 kcal/mol), indicating dual inhibitory potential against both QS and resistance enzymes. Rutin exhibited strong binding to the AcrBZ efflux pump chain A (−9.5 kcal/mol), but its stability decreased in pump-associated complexes, suggesting steric hindrance in intact systems. Ellagic acid displayed moderate affinity toward DNA gyrase (6F86; −8.3 kcal/mol), stabilized by hydrogen bonding and aromatic π–π stacking interactions.

Table 7. Summary of absorption, distribution, metabolism, excretion, and toxicity properties of selected ligands

Parameter	Luteolin (CID 5280445)	Rutin (CID 5280805)	Ellagic acid (CID 5281855)
Formula (molecular weight)	$C_{15}H_{10}O_6$ (286.24 g/mol)	$C_{27}H_{30}O_{16}$ (610.52 g/mol)	$C_{14}H_6O_8$ (302.19 g/mol)
TPSA (Å^2)	111.13	269.43	141.34
Consensus LogP	1.73	− 1.51	1.00
Gastrointestinal absorption	High	Low	Moderate
Blood–brain barrier permeability	No	No	No
P-glycoprotein substrate	No	Yes	No
CYP inhibition	CYP1A2, 2D6, 3A4	None	None
Skin permeation (log Kp)	−6.25 cm/s	−10.26 cm/s	−7.85 cm/s
Drug-likeness (rule violations)	None	Multiple (e.g., Lipinski, Ghose, Egan, Muegge)	Single (TPSA>131 Å^2)
Bioavailability score	0.55	0.17	0.55
PAINS/Brenk alerts	1/1	1/1	1/2
Synthetic accessibility	3.02 (favorable)	6.52 (poor)	3.25 (favorable)
Overall assessment	Good oral absorption; moderate CYP liabilities	Poor absorption; low drug-likeness	Moderate absorption; excellent safety profile

Abbreviations: CID: Compound ID; CYP: Cytochrome P450; TPSA: Topological polar surface area.

As presented in Table 7, ADMET profiling revealed that luteolin possessed high GI absorption and favorable drug-likeness but showed moderate CYP-related liabilities. Rutin exhibited poor absorption and multiple rule violations, while ellagic acid displayed a balanced pharmacokinetic and safety profile with low predicted toxicity. Overall, luteolin emerged as the most potent dual-target inhibitor, whereas ellagic acid offered superior safety, highlighting both compounds as promising candidates for further *in vitro* and *in vivo* validation.

Collectively, these computational analyses offer a comprehensive view of binding affinities, stability, and pharmacokinetic profiles, forming the basis for a detailed discussion of dual-action inhibitory potential.

4. Discussion

The present study leveraged computational approaches to evaluate 10 African medicinal plant phytochemicals for their potential as dual-action inhibitors against MDR Gram-negative bacteria. Molecular docking, hydrophobic mapping, electrostatic analysis, solvent accessibility, bond length evaluation, and *in silico* ADMET profiling collectively provided insights into ligand–protein complementarity and potential pharmacological behavior.[24,25]

Among the screened phytochemicals, luteolin (CID 5280445) consistently exhibited the highest binding affinities, particularly against LasR (−10.8 kcal/mol) and TEM-1 β-lactamase (−8.3 kcal/mol). Its strong inhibitory potential is underpinned by a synergistic network of hydrogen bonds (e.g., SER80, ASP123, LYS173), van der Waals interactions, and π–alkyl interactions, complemented by deep ligand burial within the hydrophobic pocket.[23] The donor–donor clash observed with HIS177 was minimal and did not significantly destabilize the complex.[26] The dense hydrophobic packing and extensive electrostatic complementarity of luteolin likely account for its dual activity in attenuating QS and β-lactamase function.[27,28]

Rutin (CID 5280805) demonstrated notable affinity for the AcrB efflux pump (−9.5 kcal/mol) in chain A, although the pump-associated form exhibited reduced binding due to steric hindrance.[29] Stabilization was mediated primarily by hydrogen bonds and van der Waals interactions, with π–alkyl interactions reinforcing hydrophobic complementarity. The observed reduction in binding affinity within the efflux pump context highlights the dynamic influence of protein multimerization on ligand accommodation.[25]

Ellagic acid (CID 5281855) effectively targeted DNA gyrase B (−8.3 kcal/mol), forming stable hydrogen bonds with GLU274 and LYS305, along with π–π stacking and π–anion interactions, indicating potential interference with bacterial replication.[26,30] Partial solvent exposure and moderate hydrophobic stabilization align with its intermediate binding affinity, suggesting a balance between accessibility and pocket encapsulation.[28]

Ramachandran plot analyses confirmed the structural reliability of all protein targets,[24,25] ensuring accurate docking predictions. Hydrophobic mapping and SAS analyses consistently correlated ligand burial with binding strength,[31,32] underscoring the importance of complementary non-polar interactions in complex stabilization. Bond length evaluations further reinforced the stability of top-scoring complexes, with hydrogen bonds in the 2.6–3.0 Å range mitigating the effect of minor unfavorable contacts.[33]

Both ADMET and drug-likeness predictions revealed that luteolin, despite high GI absorption and favorable drug-likeness, displayed potential mitochondrial and metabolic liabilities.[26,28] In contrast, rutin and ellagic acid generally exhibited safer pharmacokinetic and toxicity profiles,[31,33] although rutin showed moderate nephrotoxicity and immunotoxicity.[30] These findings highlight a critical trade-off between binding potency and predicted systemic safety, guiding prioritization for experimental validation.[28]

It is important to note that these predictions are computational and do not substitute for experimental toxicity assessments. The actual cytotoxicity and therapeutic safety of luteolin, rutin, and ellagic acid against human cells and MDR bacteria remain to be established through *in vitro* and *in vivo* studies.

Overall, luteolin, rutin, and ellagic acid were identified as promising computational leads with complementary inhibitory mechanisms against MDR Gram-negative bacteria.[34] The dual-action potential of these phytochemicals, targeting both virulence factors and essential bacterial enzymes, demonstrates the translational value of African medicinal plant scaffolds in rational antimicrobial design.[10] Subsequent *in vitro* and *in vivo* studies are warranted to confirm efficacy, optimize safety, and explore synergistic combinations for novel anti-MDR therapeutics.[35]

5. Conclusion

This study demonstrates that African medicinal plant phytochemicals possess significant dual-action potential against MDR Gram-negative bacteria. Luteolin exhibited strong binding to both LasR and TEM-1 β-lactamase, suggesting simultaneous QS disruption and enzyme inhibition. Rutin and ellagic acid showed high affinities for the AcrB efflux pump and DNA gyrase B, respectively, indicating complementary mechanisms to impair bacterial survival and resistance. *In silico* ADMET and drug-likeness analyses further highlighted luteolin, rutin, and ellagic acid as promising leads with generally favorable pharmacokinetic profiles and manageable toxicity risks.[20] Collectively, these findings provide a computational foundation for prioritizing these phytochemicals in experimental studies aimed at developing novel anti-MDR therapeutics. Although the data suggest that these flavonoids could serve as promising antimicrobial agents, their safety, cytotoxicity, and actual antimicrobial efficacy still require experimental confirmation. Future *in vitro* and *in vivo* studies are essential before any therapeutic claims can be made.

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The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

All data generated or analyzed during this study are included in this manuscript.

Further disclosure

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Safety of the 9-valent human papillomavirus vaccine in pregnancy based on the vaccine adverse event reporting system

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Abstract

Current guidelines recommend against administering the 9-valent human papillomavirus (HPV) vaccine during pregnancy due to limited safety data. We examined adverse events reported in patients receiving the 9-valent HPV vaccine during pregnancy. The Vaccine Adverse Events Reporting System (VAERS) database was queried for "exposure during pregnancy" to the 9-valent HPV vaccine. Cases were excluded if there was no information on pregnancy in the report or the patient was not pregnant at the time of vaccination. Individual reports were reviewed and data were extracted on gestational age and adverse pregnancy events. From the 285 reports identified, 273 were included. 40.3% (110/273) of the reports stated that there were no adverse events following vaccination. There were eight reports of mild maternal reactions (most commonly injection site pain) and one report of a serious reaction (angioedema). The majority of reports (257/273, 94.1%) did not include the pregnancy outcome. There was one case of vaginal bleeding, four miscarriages, one elective abortion, no stillbirths, and two congenital anomalies. There were seven reports of live births. 61.5% (168/273) of the reports included gestational age at the time of vaccination. Few cases of pregnancy-related adverse events were identified following 9-valent HPV vaccine administration. The VAERS database is an electively reported database; thus, the incidence of events could not be determined, and many reports were incomplete. Despite these limitations, the low numbers of adverse events are reassuring. Clinical trials are warranted to conclusively examine the safety and efficacy of HPV vaccination during pregnancy.

Keywords: Human papillomavirus; Vaccination; Vaccine Adverse Events Reporting System; Pregnancy; Gestational age; Congenital vaccine exposure

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1. Introduction

The human papillomavirus (HPV) is the cause of various types of cancer, such as that of the cervix, vagina, vulva, anus, and oropharynx, as well as genital warts. Every year, over 500,000 individuals are diagnosed with cervical cancer, and over 300,000 cases of mortality are recorded worldwide.[1] In the United States (US) alone, there are approximately 10,800 cases of cervical cancer, 2,300 cases of oropharyngeal cancer, 5000 cases of anal cancer, 3000 cases of vulvar cancer, and 700 cases of vaginal cancer annually.[2] Every year, 4,000 of these women die of cervical cancer.[2]

A significant proportion of these deaths is preventable through vaccination. The quadrivalent HPV vaccine was approved by the US Food and Drug Administration (FDA) in 2006. It has since been replaced by the 9-valent HPV vaccine, which was approved by the FDA in December of 2014 and is now recommended for persons aged 9–11 with catch-up vaccination recommended until age 26, and shared decision-making regarding catch-up vaccination until age 45.[3-5] The 9-valent HPV vaccine protects against HPV types 16, 18, 6, 11, 31, 33, 45, 52, and 58, which are the most commonly associated with cervical cancer and genital warts. It has been shown to be highly protective against cervical neoplasia and cancer, with the potential to prevent 90% of cases of cervical cancer.[6,7] In addition to this high efficacy, the HPV vaccine has consistently shown a favorable safety profile, which has improved over time, with fewer adverse events reported with the 9-valent vaccine than previous formulations.[8]

Vaccination rates have risen since the HPV vaccine first debuted but remain stubbornly low, with only 61.4% of US adolescents vaccinated as of 2023.[9] Although many factors contribute to this deficit, infrequent adolescent healthcare visits are a significant reason.[10] In response to these statistics, the American College of Obstetricians and Gynecologists has stated that HPV vaccination rates in the US are unacceptably low and encourages catch-up vaccination in adults up to age 45.[11] Similarly, the Centers for Disease Control and Prevention (CDC) recommends vaccination up until age 26 and states that it should be discussed and offered up until age 45.[12] Although the optimal time for vaccination is before sexual debut, underpinning the recommended target age of 9–11 years, the HPV vaccine is still beneficial even when HPV vaccination does not occur until after exposure to the virus. It has been shown to provide a 30% reduction in high-grade cervical dysplasia (CIN2+) and 25% reduction in the need for excisional procedures.[13] Despite the recommendation for catch-up HPV vaccination, there are still low reported rates of vaccination in patients presenting for antenatal care.[14-16] Indeed, under current guidelines in which antepartum vaccination is contraindicated,[3,11] pregnancy has been identified as a barrier to completion of catch-up vaccination.[17] Postpartum vaccination rates are similarly low, with significant loss to follow-up after the first dose.[16,18] A challenge that has been identified in postpartum vaccination studies is that patients are often lost to follow-up until they become pregnant again[19] at which time the vaccine cannot be given until the postpartum period. While postpartum vaccination rates can be improved to up to 65% completion with targeted interventions,[16] this still leaves many women unvaccinated and susceptible to cervical cancer. Additional strategies are needed to ensure that access to the HPV vaccine is maximized.

While pregnancy has been considered a barrier to vaccination under current guidelines, it has the potential to present an opportunity for vaccination if vaccine safety can be demonstrated in pregnancy. During pregnancy, individuals who may not otherwise seek or have access to healthcare have increased access to medical insurance and frequent contact with healthcare providers. Pregnancy is also a time of high patient motivation that crosses racial and socioeconomic lines, mitigating some social and structural barriers to care.[20] Given active patient engagement with the healthcare system for prenatal care, pregnancy may be an ideal time to complete the full course of HPV vaccination, which would also reduce rates of incomplete vaccination due to loss to follow-up.[18,21,22] This may be particularly valuable in low- and middle-income countries where nearly 90% of cervical cancer-related deaths occurred and where vaccine coverage was only 15% in 2019.[23] In these settings, integration of HPV vaccination into existing health infrastructure has been proposed as a means of combating cervical cancer.[23] Nevertheless, it is worth noting that antenatal visit attendance is also lower in these settings and the HPV vaccine is not currently available in many of these countries.[24]

Safety concerns have been cited for national and international guidelines recommending against administering the 9-valent HPV vaccine in pregnancy. However, unlike vaccines against varicella or rubella, which are live attenuated vaccines and thus contraindicated in pregnancy due to a theoretical potential for crossing the placenta possibly causing congenital infection, the HPV vaccine does not contain live HPV virus. Instead, the HPV vaccine is comprised of recombinant virus-like particles with no risk of causing infection to the mother or the fetus.[25] Another potential safety concern during pregnancy is exposure to adjuvants which are used in vaccines to boost the immune response. The adjuvant in the 9-valent HPV vaccine is an aluminum salt. Aluminum salts are commonly used in vaccines, including the tetanus, diphtheria, and acellular pertussis (Tdap) vaccine, which is routinely administered during pregnancy.[26] Despite the low theoretical risk for harm, the ACOG, the Society for Maternal-Fetal Medicine (SMFM), the World Health Organization (WHO), and the CDC currently recommend against vaccination during pregnancy due to insufficient safety data.[3,11,27,28]

Given the concerns regarding inadequate safety data, we aimed to examine adverse events reported in the Vaccine Adverse Events Reporting System (VAERS) database, a nationally maintained database that accepts voluntary reports of adverse events following vaccine administration, for patients who inadvertently received the 9-valent HPV vaccine during pregnancy. We further aimed to review the existing literature on vaccine safety in pregnancy and interpret our results in the context of these other studies

2. Materials and methods

2.1. Study design

This is a retrospective observational cohort study of adverse events reported to the VAERS database.

2.2. Data collection

VAERS is a national database maintained by the CDC and FDA that accept adverse event reports following receipt of any US-licensed vaccine. The database goes back as far as 1990, although our data set begins in 2015 when the 9-valent HPV vaccine was licensed. VAERS reports are submitted on a voluntary basis by healthcare providers or members of the public.[29] Healthcare providers are required to report certain adverse events, and vaccine manufacturers are required to report all adverse events that they are aware of. VAERS is a passive reporting system and is not designed to assess causality but to help detect unusual patterns of adverse event reporting that may underlie a vaccine safety issue. An essential objective of the VAERS database is to identify possible vaccine safety signals, such as rare adverse events that may be missed in pre-licensure clinical trials. Reported events are often temporally associated with vaccination, and may or may not be causally related.

We queried the VAERS database on July 27, 2022, with the following query parameters: Sex: Female; State/Territory: The United States/Territories/Unknown; Symptoms: EXPOSURE DURING PREGNANCY; Vaccine Products: HUMAN PAPILLOMAVIRUS (TYPES 6, 11,16, 18, 31, 33, 45, 52, 58) RECOMBINANT VACCINE (HPV9); VAERS ID: All; Group By: VAERS ID; Show Totals: False; Show Zero Values: False.[30] Cases were excluded if there was no information on pregnancy in the report, or the patient was stated not to be pregnant at the time of the vaccination. Thus, preconception exposure was excluded. Individual reports were reviewed, and data were extracted on pregnancy status, gestational age at time of vaccination, adverse events, and pregnancy outcome. Report severity is determined on the basis of criteria established by the Code of Federal Regulations. Serious adverse events include those that result in death, life-threatening illness, hospitalization, prolongation of hospitalization, disability or permanent damage, congenital anomaly, or other medically important condition. Demographic characteristics of cases were not routinely reported in the database and could not be collected.

2.3. Outcomes

Adverse events were characterized as pregnancy-related or unrelated. Pregnancy-related adverse events included miscarriage, vaginal bleeding, elective abortion, stillbirth, and congenital anomaly. Miscarriage was defined as occurring before 20-week gestational age. Stillbirth was defined as occurring at 20-week gestational age or later. Since most reports in VAERS documented gestational age in whole weeks (*e.g.*, 15 weeks rather than 15.29 weeks or 15 weeks and 2 days), gestational age was treated as a categorical variable. Gestational age was determined from the report or calculated from the last menstrual period (LMP) or estimated due date stated in the report, rounding to the number of weeks of gestation completed. Gestational age abstracted from VAERS was categorized by trimester for analysis. The first trimester was defined as less than 14-week gestational age, the second trimester was defined as 14–27-week gestational age, and the third trimester was defined as 28-week gestational age or more.

2.4. Analysis

Frequencies and proportions were calculated using Microsoft Excel 2019. Graphs were generated in Microsoft Excel 2019. Causality between vaccination and adverse events could not be assessed due to the nature of the VAERS reporting system.

Because VAERS is a publicly available database, this study was exempted from institutional review board (IRB) review under federal regulation 45 CFR 46.104, and informed consent is not required.

3. Results

There were 285 reports identified in the VAERS system under our search criteria, with the earliest in 2015 less than 1 year after licensing of the 9-valent HPV vaccine. After excluding those reports in which the patient was not pregnant at the time of vaccination, or pregnancy status could not be determined from the report, 273 were included in this analysis (Figure 1). Among these, 110 (40.3%) reports expressly stated that there were no adverse events following vaccination and the reason for submission of the report was vaccine exposure during pregnancy. Of the remaining 163 reports, there were 21 reports of mild adverse events. The most common adverse event ($n = 8$) was injection site pain. Other adverse events, as reported in VAERS, included urinary tract infection,

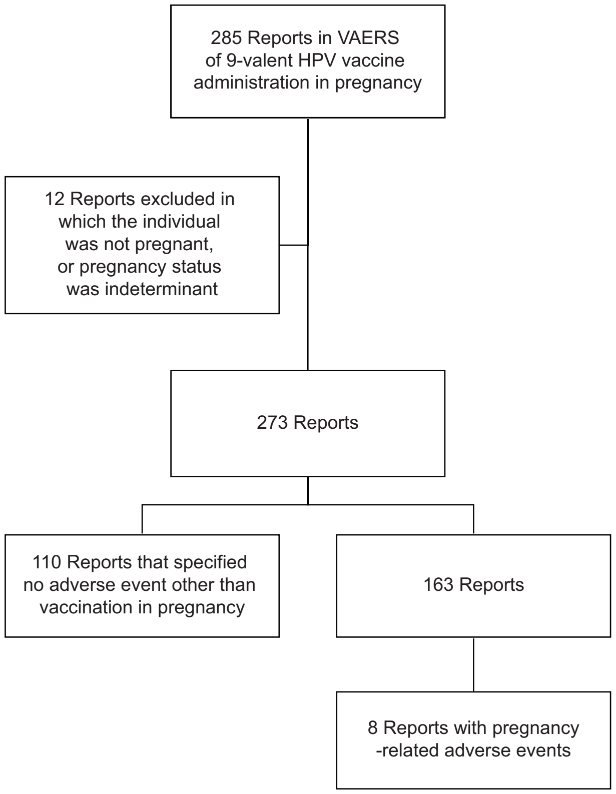

Figure 1. Flow diagram of Vaccine Adverse Events Reporting System database. The initial query identified 285 reports of exposure to the 9-valent human papillomavirus vaccine during pregnancy. On review, 12 reports were excluded due to the vaccine exposure happening outside of pregnancy, or at an indeterminate time in relation to the pregnancy. Of the remaining 273 reports, 110 specified that there was no adverse event other than administration during pregnancy. Of the remaining 163 reports, 7 reported adverse pregnancy events.

abdominal pain, nausea, fatigue, nasal congestion, weakness, discomfort, syncope, headache, injection site swelling, irregular menses, testing positive for HPV, and weight gain. There was one report of a serious maternal reaction (angioedema). There were no reports of maternal death.

The majority of reports (257, 94.1%) did not include the pregnancy outcome. Of pregnancy-related events, there was one report of pre-eclampsia, one case of vaginal bleeding, four reported miscarriages, one elective abortion, no stillbirths, and two congenital anomalies (Table 1). One of the congenital anomalies was reported as Trisomy 18, and the other did not have details reported. Of note, this unspecified congenital anomaly was also associated with a miscarriage. There were seven reports of live births.

Gestational age at the time of vaccination was included in 168 (61.5%) reports. Vaccination occurred in all trimesters of pregnancy and ranged from 1 to 38 weeks of gestational age (Figure 2). Among those exposures with reported gestational ages, 54.8% occurred in the first trimester, 13.7% occurred in the second trimester, and 31.5% occurred in the third trimester. The average gestational age at time of vaccination was 15.6 weeks, the median gestational age at vaccination was 11 weeks and the most common week of vaccination was 28-week gestational age.

Table 1. Adverse pregnancy-related events and gestational age at vaccination

Adverse event	Gestational age at time of vaccination
Vaginal bleeding	7 weeks
Miscarriage	6 weeks
Miscarriage*	Unreported
Miscarriage	Unreported
Miscarriage	Unreported
Elective abortion	Unreported
Congenital anomaly (Trisomy 18)	3 weeks
Congenital anomaly (Unreported type)*	Unreported
Pre-eclampsia	4 weeks

Note: *These two adverse events occurred in the same pregnancy.

While HPV vaccine exposures occurred across all gestational ages, the data demonstrate a bimodal vaccine administration distribution. Many patients received the HPV vaccine in the first trimester before they were aware of the pregnancy. These exposures were often reported as the urine pregnancy test came back positive after the vaccine was administered. A second spike occurred around the time of the 28-week visit when the Tdap vaccine is administered. Many of these reports stated that the provider inadvertently administered the 9-valent HPV vaccine rather than the Tdap vaccine.

4. Discussion

We identified few cases of pregnancy-related adverse events among the 273 reports in the VAERS database following administration of the 9-valent HPV vaccine during pregnancy. The most common adverse event was injection site pain (*n* = 8) which is a known side effect of the vaccine, and the most common pregnancy-related adverse event was miscarriage (*n* = 4). Many of the reported adverse events were likely only temporally related and others may have been symptoms of pregnancy, such as nausea and fatigue. Only two cases of congenital anomalies were identified. Miscarriage is a common early pregnancy outcome, seen in approximately 10% of pregnancies, and these numbers were not higher than would be expected. Likewise, congenital anomalies complicate roughly 3% of pregnancies, and rates observed were not higher than

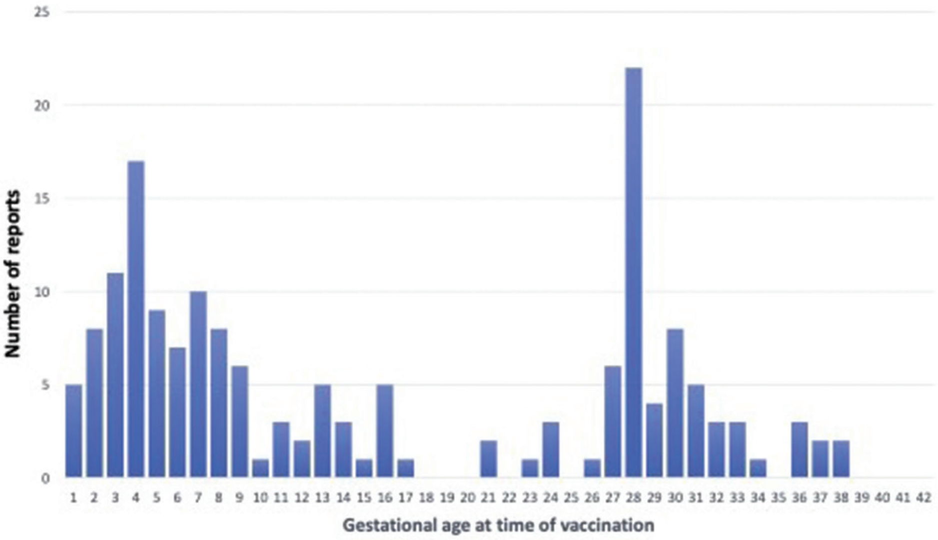

Figure 2. Distribution of timing of inadvertent 9-valent human papillomavirus vaccination during pregnancy. There were 168 reports with data available on gestational age at the time of vaccine exposure. Gestational age is reported as weeks completed.

expected. The only serious adverse event reported was angioedema, which was not a pregnancy-specific adverse event. Overall, these findings provide reassuring data on the safety of the 9-valent HPV vaccine in pregnancy.

Our data are consistent with prior reports from both the quadrivalent and 9-valent vaccines, supporting the safety of vaccination in pregnancy. In a voluntary post-marketing registry from Merck of the quadrivalent HPV vaccine, rates of spontaneous abortion and major birth defects were no greater than the general population.[31] Similarly, in a study of VAERS reports for the quadrivalent HPV vaccine inadvertently administered during pregnancy, there were no unexpected patterns of adverse maternal or fetal outcomes. The most common pregnancy-specific adverse event was miscarriage, and the most common adverse event overall was maternal fever.[32] Neonatal anomalies included one each of absence of lower extremities, total anomalous pulmonary venous return, and Trisomy 21. A positive safety profile was also seen in a retrospective study using data from a national registry in Denmark, in which women exposed to the quadrivalent HPV vaccine in pregnancy showed no increased risk of spontaneous abortion, preterm birth, small-for-gestational-age neonate, or stillbirth.[33] In a combined analysis of five phase III clinical trials of the quadrivalent HPV vaccine, there was also no increased risk of fetal loss, spontaneous abortion, or congenital anomalies.[34] A meta-analysis of 11 clinical trials and cohort studies including the 2-, 4-, and 9-valent vaccine showed no association with stillbirth, preterm birth, birth defects, small-for-gestational-age newborn, or ectopic pregnancy.[35] Similarly, pre-licensure studies of pregnant individuals who were inadvertently vaccinated with the 9-valent HPV vaccine showed good safety.[25,36] A post-marketing surveillance study from China noted 50 cases of exposure during pregnancy, with no cases of stillbirth.[37] A recent cohort study of 1,493 patients from seven Vaccine Safety Datalink sites found that the 9-valent HPV vaccine was not associated with spontaneous abortion, preterm birth, small-for-gestational-age birth, or birth defects.[38] There was no pattern to the identified birth defects, which included ventricular septal defects, microcephaly, cleft palate, septo-optic dysplasia, encephalocele, renal agenesis, and pyloric stenosis. However, this study only examined vaccination up to 19-week gestation. Consistent with these data, in a meta-analysis of phase III clinical trials of the 9-valent HPV vaccine, rates of spontaneous abortion, stillbirth, and congenital anomalies were similar to the general population.[36] Fetal anomalies that were identified were diverse across organ systems and showed no pattern. The most common adverse events reported were injection site-related, including pain, swelling, and erythema, similar to the common adverse events seen in our VAERS data.

Other studies, in addition to ours, have examined the VAERS data on the 9-valent HPV vaccine in pregnancy. A prior study by Landazabal *et al.*[39] examining VAERS reports from 2014 to 2017 identified 82 reports of 9-valent HPV vaccine administration during pregnancy, compared to the 273 cases reported here. Our data overlapped with Landazabal *et al.*'s study,[39] but we included more recent reports and many more reports of vaccination occurring in the third trimester. A more recent retrospective pharmacovigilance analysis of VAERS reports from 2015 to

2024 for the 9-valent HPV vaccine using disproportionality analyses to detect potential safety signals by Liu *et al.*[40] found no positive signals for reports in pregnant individuals. Of note, despite a time frame that overlapped that of our study and the study by Landazabal *et al.*,[39] Liu *et al.*[40] only identified reports from 18 pregnant individuals. This discrepancy is likely due to differences in VAERS search parameters. While the study did not delineate the full set of search parameters used, they did note that they included reports involving "GARDASIL 9" in the vaccine name field. It is also possible that their exclusion criteria were more stringent than those employed by us or Landazabal *et al.*[39] Interestingly, the authors identified four cases of fetal cardiac disorders, which were not identified in our analysis and did not mention the congenital anomalies that we noted (Trisomy 18 and an unspecified anomaly). It is possible that the cardiac cases were all seen after July 27, 2022, when our data collection occurred. Alternatively, different search criteria may have been responsible. Landazabal *et al.*[39] did not note any congenital anomalies in their results. Despite the differences in sample size and fetal effects for our studies, our conclusions regarding the safety of the vaccine in pregnancy are consistent.

There are two notable exceptions to the overall positive safety data. The first is a meta-analysis which included two randomized controlled trials (RCTs) with the 9-valent HPV vaccine and found that vaccination during the period from 90 days before the LMP to 45 days after LMP seemed to be related to an increased risk of spontaneous abortion (RR = 2.04, 95% CI: 1.28–3.24).[35] This was based on the results of four RCTs, only one of which, Moreira *et al.*[36] showed an increased risk of spontaneous abortion, while the others trended toward a decreased risk of spontaneous abortion. Interestingly, from their own data, Moreira *et al.*[36] concluded that they observed increased rates of spontaneous abortion with the 9-valent as compared to the quadrivalent vaccine, but that both rates were similar to those in the general population. The authors of the systematic review noted that given the paucity of studies, their findings should be interpreted with caution. Only four miscarriages were noted in our study, although we did not examine the preconception time period, possibly omitting some miscarriages that would have been included in the meta-analysis. Thus, further research is indicated on periconception exposure to the 9-valent HPV vaccine, and patients with exposure during this period should be counseled on the insufficient data on the risk of miscarriage. For recommendations regarding vaccination during pregnancy, it may be reasonable to suggest waiting until after the first trimester. The other study that identified potential safety signals reviewed VAERS data for the quadrivalent HPV vaccine and raised concerns for increased risk of venous thromboemboli,[41] especially in pregnant women. This is of particular concern because pregnancy is at baseline a prothrombotic state, and increased risk of thromboembolism could have significant health ramifications. However, this concern was not borne out in population-based studies.[41,42] Consistent with these population-based studies, we saw no cases of venous thromboembolism among the 273 reports reviewed.

We further considered our data in the context of other vaccines that are administered in pregnancy. Safety data from VAERS on the 9-valent HPV vaccine are similar to those reported for the COVID-19 vaccines, with most adverse events being unrelated to pregnancy, and the most common pregnancy-related adverse event being miscarriage.[43] Conversely, pregnant women vaccinated with cell-based influenza vaccines were more likely to report pregnancy-related adverse events in VAERS, and the most commonly reported adverse event was premature delivery.[44]

Our results have implications for future research. While our data and the studies reviewed above are reassuring, further research is warranted on HPV vaccination in pregnancy. First, it will be critical to assess patient and provider acceptance of HPV vaccination in pregnancy. The acceptance rate of vaccines varies during pregnancy; for example, the acceptance rate of influenza, Tdap, and COVID vaccines during pregnancy is 61.2%, 56.6%, and 28.5%, respectively.[45,46] One study of patient attitudes surrounding antepartum vaccination showed that one-third of patients would accept the HPV vaccine during pregnancy if recommended by their obstetrician.[14] However, this study was from over a decade ago, and attitudes toward vaccination have changed since the COVID-19 pandemic. In addition, patients may be more familiar with the HPV vaccine since it has been on the market longer, and more vaccines are now recommended during pregnancy, which could both alter patient perceptions of antepartum HPV vaccination. Second, it would be valuable to gather population-level data on individuals inadvertently vaccinated with the 9-valent HPV vaccine during pregnancy, as has been done for the quadrivalent vaccine.[33] Long-term follow-up of the children born to these pregnancies would also be of value and is becoming feasible now that the 9-valent vaccine has been in circulation for a decade. Large databases such as national records maintained in Europe or the Kaiser system in California could help to provide further reassurance of the safety of the 9-valent vaccine. Third, it will be important to demonstrate the immunogenicity of the vaccine when administered during pregnancy. This could be done through retrospective serology studies of women who were inadvertently exposed, or ideally with prospective clinical trials. It is possible that immunologic changes of pregnancy could alter the efficacy or durability of vaccination during this time. Moreover, immunogenicity may vary with gestational age. Sex steroid hormone levels are thought to be responsible for sex differences in vaccine immune responses.[47] However, data from other vaccines administered in pregnancy are reassuring. The hepatitis B,[48] Tdap,[49] H1N1 influenza,[50] quadrivalent inactivated influenza,[51] COVID-19,[52] and respiratory syncytial virus (RSV)[53] vaccines have all demonstrated high immunogenicity during pregnancy. Moreover, postpartum vaccination data show high immunogenicity even with a modified two-dose regimen.[54] Finally, cost-effectiveness studies would also be valuable. The direct medical costs of cervical dysplasia have been estimated at over eight billion dollars annually.[55] Increased vaccine uptake during pregnancy has the potential to reduce these costs. Other vaccines in pregnancy have benefited from cost-effectiveness analyses. Screening and vaccinating for hepatitis B has been shown to be cost-effective during pregnancy[56] and helped to reshape SMFM guidance on vaccination during pregnancy.[57]

Beyond future research implications, our results have significant clinical implications as well. First, this study still has clinical value in counseling patients who were inadvertently exposed to the 9-valent HPV vaccine during pregnancy. While ACOG recommends against administration of the HPV vaccine during pregnancy, it also advises against pregnancy testing before administration of the HPV vaccine.[11] Thus, there will continue to be women who are inadvertently vaccinated before pregnancy. Patients may feel anxious having received a contraindicated vaccine during pregnancy. Providers can provide reassurance that our data, along with previous studies, do not show an elevated risk of severe adverse maternal or fetal effects and that the most common adverse events are mild, related to the injection site, and not pregnancy-specific. Another important clinical implication of our data is the finding of a spike in inadvertent vaccine administration around 28-week gestation when pregnant women receive the Tdap vaccine. This was by far the most frequent timing of vaccine administration. In the case of wrong vaccine administration with the 9-valent HPV vaccine, the risks to the pregnant patient are minimal, but it may result in anxiety or mistrust of the healthcare system, which can hopefully be ameliorated with counseling as previously discussed. However, misadministration of live-attenuated vaccines poses a greater potential risk. It is reasonable to assume that wrong vaccine administration during around 28-week gestation is not limited to the HPV vaccine but may include other vaccines that providers stock. Identifying this common timing of vaccine administration errors creates an opportunity for targeted intervention. Quality improvement initiatives in clinics and pharmacies where vaccines are administered have the potential to reduce these mistakes and improve patient care. This is increasingly important as more vaccines are recommended during pregnancy, such as the RSV vaccine and other vaccines that are currently in development. Perhaps, the greatest clinical implication of our data is its contribution to the growing body of literature on the safety of the 9-valent HPV vaccine in pregnancy. Safety data may ultimately lead to a revision of the national and international guidelines that contraindicate vaccination with the 9-valent HPV vaccine in pregnancy. If guidelines were less restrictive, pregnancy presents an opportunity to complete vaccination. Lack of frequent contact with the healthcare system has been proposed as a reason for poor vaccine uptake among adolescents. Given active engagement with the healthcare system during pregnancy, it may be an ideal time to complete the full course of HPV vaccination, which would also reduce rates of incomplete vaccination due to loss to follow-up.[18] Indeed, SMFM now recommends vaccination for hepatitis B during pregnancy for women who are non-immune or who have not previously been vaccinated, due to the fact that prenatal care presents an opportunity to complete a multi-dose vaccine series[57] and compliance rates for vaccine completion are high in pregnancy.[58] Underscoring the importance of VAERS data in guideline development, safety data from VAERS studies were used in the decision-making process for the updated hepatitis B vaccine recommendations.[57,59] While one of the great potential benefits of vaccination during pregnancy is the chance to administer a multiple-shot vaccine, it is important to note that studies examining the efficacy of single-dose HPV vaccination are currently being performed. Thus far, these studies have focused on adolescents,[60-63] but it is possible that they could be extended to pregnancy. Studies have already demonstrated the immunogenicity of a two-shot series postpartum. If a single-shot regimen was found to be effective in adult women, the single dose could be administered postpartum, obviating the benefit of frequent healthcare contact during pregnancy for completion of the three-shot series. While earlier vaccination is ideal, and many patients may prefer to wait until postpartum for vaccination, vaccination during pregnancy still represents an opportunity to increase vaccine coverage and improve the health of women. Indeed, the WHO lists safety and efficacy in pregnant women as a desirable HPV vaccine characteristic under its considerations for vaccine implementation.[64] Furthermore, it is possible that there may still be benefits to vaccination antepartum. HPV vaccination has been shown to reduce the risk of preterm birth and small-for-gestational-age infants.[65,66] However, these data should be interpreted cautiously as they were from women who were vaccinated in adolescence rather than antepartum. Further research is needed on any pregnancy-specific benefits of antepartum vaccination.

Our study has multiple strengths. First, it is a contemporary evaluation of the currently utilized 9-valent HPV vaccine. Data are uploaded to the system in near real time, so there was no lag in data collection from the time of entry to our data pull. To our knowledge, this is the most comprehensive analysis of the VAERS database for 9-valent HPV administration during pregnancy, encompassing 273 reports. Second, we present data not only on reported adverse events but also on the timing of vaccination in pregnancy. This allowed us to identify peak times of inadvertent vaccine administration and note the high rates of wrong vaccine administration around 28-week gestation. This is a novel and highly clinically relevant finding. Third, we included exposures that occurred at any gestational age. Prior studies focused on preconception and early gestation. However, adverse events are not limited to early pregnancy and may vary over the course of pregnancy due to physiologic adaptations of the pregnant woman and fetus. It is well known that drugs can have different impacts on a pregnancy in different trimesters. While miscarriage and congenital anomalies occur early in gestation, some adverse events, such as fetal growth restriction, may be more impacted by exposures later in pregnancy. In addition, any deliberate vaccination campaign during pregnancy would likely be implemented in the second and third trimesters, making it critical to collect these data.[21]

There are several limitations to note from our study. Many of these limitations are inherent to the design of the VAERS database. Many of the features that allow the VAERS database to quickly identify adverse events and capture rare events are associated with some drawbacks. As a voluntary reporting system, VAERS is at risk of both over- and underreporting; reporting bias; incomplete, coincidental, or inaccurate data; unverified reports; and inconsistent quality of reports.[29] Since there are no data on the total number of pregnant persons vaccinated in VAERS, the incidence of adverse events cannot be calculated nor can causation be determined. Events close to the time of the vaccination are more likely to be reported than remote events. Consequently, few reports included information on the delivery outcome. In addition, data on how many doses of the vaccine the patient received during pregnancy were lacking. Finally, due to the nature of the data, it was not possible to access medical records for verification of the exposure and the adverse event, or follow-up from the event. Despite these limitations, our study adds to the literature and should be viewed in the context of the aforementioned studies which also favor the safety of the vaccine in pregnancy. To this effect, ACOG and the CDC do not advise pregnancy testing before vaccination and report that existing safety data in pregnant persons who are inadvertently vaccinated is reassuring.[3,11]

5. Conclusion

HPV is a significant cause of morbidity and mortality, and despite the availability of a highly effective vaccine, vaccination remains underutilized in the target adolescent population. Vaccination during pregnancy has been proposed as a time to reach patients when they are highly engaged with the healthcare system. Our study examined the currently utilized 9-valent vaccine and did not identify concerning rates of adverse events, pregnancy-related, or otherwise. These findings, in the context of the existing literature, can provide reassurance to women inadvertently vaccinated during pregnancy. Phase I clinical trials are warranted to demonstrate immunogenicity of the vaccine in pregnancy, and national databases should be examined for the safety of the 9-valent HPV vaccine in pregnancy, similar to those completed for the quadrivalent vaccine. Qualitative research on patient and provider attitudes regarding HPV vaccination is also warranted to comprehensively evaluate the possible risks and benefits of 9-valent HPV vaccination during pregnancy. Given the increasingly large body of literature showing reassuring safety data among women inadvertently exposed to the 9-valent HPV vaccine during pregnancy, the high efficacy of the vaccine, the significant morbidity, mortality, and cost from cervical cancer, as well as the fact that for some women pregnancy may be a rare touchpoint with the healthcare system, we argue that national and international bodies should consider adopting guidelines that permit catch-up vaccination during pregnancy, using shared decision-making to weigh the risks and benefits of vaccination antepartum versus waiting until the postpartum period.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Sarah Boudova, Rupsa C. Boelig
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Ethical approval and consent to participate

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Availability of data

Data are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Diagnostic performance of RNA extraction-free dilution and heating method for the detection of severe acute respiratory syndrome coronavirus 2

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Abstract

Real-time quantitative polymerase chain reaction remains the gold standard for COVID-19 diagnosis, but RNA extraction is time-consuming, expensive, and associated with increased biosafety requirements. This study evaluated an extraction-free dilution and heating (EFDH) method as a simplified alternative to conventional extraction-based (EB) real-time quantitative polymerase chain reaction for severe acute respiratory syndrome coronavirus 2 detection. A total of 300 archived nasopharyngeal specimens, including 190 positives and 110 negatives, from the National Virology Reference Laboratory at the Ethiopian Public Health Institute, were analyzed. Samples were diluted 1:2 with RNase-free water, heated at 72°C for 15 min, and analyzed using an ABI 7500 Fast instrument. The EFDH method showed a sensitivity of 92%, a specificity of 100%, and an overall accuracy of 85.8%, producing 15 false-negative and 12 invalid results. Agreement with the EB method was high, with 95% concordance and a kappa coefficient of 0.89. Performance was strongest in samples with high viral loads (cycle threshold [Ct] < 20) and declined in those with low viral loads (Ct > 35). A significant correlation was observed between the two methods ($R^2 = 0.99$, $p=0.001$). These findings indicate that the EFDH approach reliably detects moderate-to-high viral loads and may serve as a practical testing option in resource-limited settings, especially during outbreaks when rapid and simplified workflows are needed.

Keywords: SARS-CoV-2; Extraction free; Heating; Dilution; Diagnostic performance

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1. Introduction

Coronaviruses belong to the *Coronaviridae* family. They are RNA viruses with an enveloped structure and a positive single-stranded genome. Their genome contains over 29,891 nucleotides, which encode for 9,890 amino acids. It also contains numerous open-reading frames (ORFs) that encode both structural and non-structural proteins. These proteins play crucial roles in the viral life cycle and pathogenic processes.[1] The structural proteins consist of S (spike protein), E (envelope protein), M (membrane protein), and N (nucleocapsid protein). Furthermore, there are 16 non-structural proteins that assist in viral metabolism and interaction with the host immune system.[2] The genetic information of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for Coronavirus Disease 2019 (COVID-19), plays a multifaceted role in diagnostic tool development, encompassing rapid assay development, enhanced detection sensitivity, variant identification, and informing public health responses.[3,4] Its significance underscores the critical intersection of genetics, diagnostics, and public health in effectively managing infectious disease threats.[4]

In viral outbreaks such as COVID-19, it is crucial to effectively manage and control the pandemics.[5] It is reported that up to 40% of individuals with SARS-CoV-2 infection may be asymptomatic or pre-symptomatic, yet have the potential to spread the virus.[6] Therefore, a dependable laboratory diagnosis is the key to preventing and controlling infections.[7]

COVID-19 can be diagnosed primarily by either immunological or molecular tests. Immunological tests include serological tests that detect antibodies (immunoglobulin [Ig]M and IgG) in blood or viral antigens in respiratory secretions obtained through nasal or throat swabs. These tests can be performed using point-of-care platforms, rapid testing systems that provide results within minutes and are particularly useful in non-laboratory settings. Serological tests are particularly important for diagnosing patients with mild to moderate disease when molecular diagnostics are not available. Serological tests play a crucial role in identifying individuals who have developed immunity against COVID-19.[8] On the other hand, molecular tests detect SARS-CoV-2 RNA in various specimens, primarily nasopharyngeal samples. Unlike serological tests, most molecular tests require specialized laboratory infrastructure, including advanced equipment and highly trained staff. This limitation restricts their widespread use compared to simpler immunological tests.[9] In addition to these primary diagnostic approaches, other laboratory parameters have been utilized to monitor patients with COVID-19.[10-12]

The specimen of choice for SARS-CoV-2 detection can be swabs from the nasopharynx, oropharynx, and anus, as well as saliva and sputum samples. In addition, the virus can be present in other bodily fluids, such as blood, urine, and feces.[13] The nasopharyngeal swab collection technique is among the most commonly used methods, with sensitivities reaching up to 98%.[14] Nasopharyngeal swabbing is regarded as the standard method for safely and conveniently collecting SARS-CoV-2 samples, as it minimizes direct exposure of the healthcare provider to respiratory secretions.[15]

The "gold standard" assay for diagnosing both symptomatic and asymptomatic cases is real-time quantitative polymerase chain reaction (RT-qPCR).[16] The first protocol, recommended by the World Health Organization (WHO), was published in 2020 by the Charité Institute at Berlin University in Germany.[4] This protocol utilizes TaqMan technology and specific primers and probes to detect the RNA-dependent RNA polymerase (*RdRp*), *E*, and *N* genes. In addition, Table 1 lists several in-house methods reported by WHO that are currently being validated in partner laboratories.[4]

Typically, three steps should be completed before conducting quantitative polymerase chain reaction (qPCR). These steps include: (i) purifying total RNA from the sample, (ii) eluting and concentrating the material, and (iii) synthesizing complementary DNA (cDNA) from the template RNA.[17] However, it is important to note that RNA extraction can be a labor-intensive, expensive, and time-consuming process that requires manual handling and carries a risk of exposure to infectious material, which may also introduce experimental errors.[17,18]

The process begins with the isolation of viral RNA from collected specimens, typically performed using commercially available RNA extraction kits. Proper handling during this step is essential to ensure accuracy and to prevent contamination. Once isolated, the RNA undergoes reverse transcription to synthesize cDNA. This

Table 1. List of real-time polymerase chain reaction protocols indicated by the World Health Organization

Institute	Gene targets
China CDC, China	*ORF1ab* and *N*
Institute Pasteur, France	Two targets in *RdRP*
United States CDC, USA	Three targets in the *N* gene
National Institute of Infectious Diseases, Japan	Multiple targets in coronavirus panel
Charité, Germany	*RdRP*, *E*, *N*
Hong Kong University, Hong Kong SAR	*ORF1b-NSP14*, *N*
National Institute of Health, Thailand	*N*

Abbreviations: CDC: Centers for Disease Control and Prevention; SAR: Special administrative region.
Source: https://www.who.int/who-documents-detail/molecular-assays-to-diagnose-COVID-19-summary-table-of-available-protocols.

step uses a master mix containing reverse transcriptase enzyme and is carried out in a thermal cycler to initiate the reaction. The resulting cDNA is then prepared for qPCR by mixing it with primers, distilled water, and either a DNA probe or SYBR Green dye. This mixture is then pipetted into a 96-well plate, which is subsequently placed in an RT-qPCR instrument programmed with the appropriate thermal cycling settings. To ensure reliable results, a negative control is included to confirm the absence of contaminants, and a positive control is used to validate the assay accuracy.[19]

The qPCR amplification process involves three key steps: denaturation, annealing, and extension. During denaturation, the temperature is raised to denature double-stranded DNA (dsDNA) into single strands. Annealing follows, during which the temperature is lowered to allow primers to bind to their specific target sequences on the DNA. In the extension step, DNA polymerase attaches nucleotides to synthesize the cDNA strand, completing the replication process. These three steps are repeated approximately 40 times, doubling the DNA in each cycle. A fluorescent signal is generated when either DNA probes are cleaved or SYBR Green binds to newly formed dsDNA. The intensity of this fluorescence increases with the number of DNA copies, enabling real-time monitoring of the amplification process. The cycle threshold (Ct) value is determined by the number of cycles needed for fluorescence to surpass a predetermined threshold and provides quantitative insights into the amount of target nucleic acid present. Lower Ct values indicate higher viral loads, while higher Ct values indicate lower viral loads.[19,20]

Real-time qPCR was the first standard molecular technique for SARS-CoV-2 detection since the emergence of COVID-19. This molecular diagnostic technique identifies nucleic acids by targeting and amplifying specific genes, producing millions of copies from a small initial sample. This amplification is monitored in real time, enabling precise and timely analysis.[19,21,22] Primers play a critical role in this process by binding specifically to the target gene. To ensure accuracy, primers must be well-designed with high specificity to avoid non-specific amplification or the formation of unwanted structures, such as primer dimers, which can lead to false-positive results.

Despite its effectiveness, RT-qPCR often exhibits variability in performance, largely influenced by the primers and probes used.[23,24] False results in viral polymerase chain reaction (PCR) testing often stem from a mix of biological, technical, and procedural issues. False negatives are especially common when sample quality is poor, viral load is low, or RNA extraction is inefficient. Problems during collection, handling, storage, or transport can further degrade the sample. Contaminants in clinical specimens may inhibit PCR, while cross-contamination during processing can lead to false positives. Mutations in primer or probe binding sites, as well as faulty equipment or reagents, also contribute to inaccurate results.[25,26] On the other hand, false positives may occur due to contamination, cross-reactivity with non-target organisms, or detection of residual viral material post-infection.[27] RNA extraction is a major bottleneck in the workflow due to its labor-intensive and specialized nature, particularly during high-demand periods such as the COVID-19 pandemic. The global shortage of RNA extraction kits has further strained testing capacities. However, numerous countries have adopted in-house RT-qPCR protocols as their standard diagnosis tool. This highlights the need for new diagnostic platforms that are fast, sensitive, and accessible to address this problem.[28]

To address the challenges in conventional RT-qPCR, it is crucial to develop novel testing methods that do not require advanced resources or extended processing time. Ideally, a test that delivers results within 2–3 h would support timely preventive measures, as patients could immediately receive the necessary support and care.[29] Globally, various direct approaches that avoid RNA extraction have been suggested, including heat-processed methods.[30,31] Our method is novel in that it does not use chelating agents, proteinase K, or other additional chemicals. We use heat and dilution to reduce the cost of the RNA extraction step. The cost reduction is particularly important in low-income countries such as Ethiopia when testing large numbers of samples. This consideration provided the main rationale for the present study. The objective of this study is to compare the diagnostic performance of our extraction-free dilution and heating (EFDH) method with that of extraction-based methods for SARS-CoV-2 detection.

2. Materials and methods

2.1. Study population, sample size calculation, and selection of stored specimens

Individuals tested for SARS-CoV-2 during the fifth wave of the pandemic (June–August 2020) were considered the study population. The study involved random selection and retrieval of stored nasopharyngeal specimens. The sample size was calculated using the double-population proportion formula, yielding 300 samples. Accordingly, a total of 300 samples (190 positive and 110 negative) were retrospectively obtained from the National Virology Reference Laboratory at the Ethiopian Public Health Institute in Addis Ababa, Ethiopia, which serves as the first national COVID-19 testing laboratory. They were initially collected for clinical diagnosis of COVID-19 in viral transport media. The samples were retrieved using the unique numbers assigned to each specimen in the laboratory register. These specimens were retested using the EFDH technique, and the results were compared with those from the extraction-based detection method. The clinical samples were handled and processed in a Biosafety Level 2 laboratory at the virology laboratory.

2.2. Method validation

We intentionally chose six specimens with an average Ct value of 19 and an internal control (with Victoria [VIC] dye) having an average Ct value of 19. These specimens were diluted 1:2 and 1:4 with RNase-free water. The diluted specimens were then heated at 62°C and 72°C for 10, 15, 20, and 30 min, and at 96°C for 5, 10, 15, and 20 min using an Eppendorf thermomixer. Considering these heated and diluted specimens as an RNA eluate, we added 10 μL of the eluate to 20 μL of master mix and performed RT-qPCR on a thermal cycler (ABI 7500, Thermo-Fisher Inc., United States of America) using DAAN detection kits (Cat. no. DA0932, Daan Gene Co., Ltd, China). For the SARS-CoV-2 DAAN detection kits, the primary targets are the *N* and *ORF1ab* genes. The kit targets the *N* and *ORF1ab* genes, with the *N* gene detected on the FAM channel, the *ORF1ab* gene on the VIC channel, and the internal control on the Cy5 channel. These genes are crucial for detecting and identifying SARS-CoV-2 using PCR-based assays. The results show that after 15 min and 30 min incubation at 72°C, the average *N* and *ORF1ab* Ct values were 24 and 26, respectively; and the VIC average values were 26 and 27, respectively. The validation experiment showed that 1:2 diluted specimens exposed to 72°C for 15 min yielded results comparable to the standard RNA extraction-based method for SARS-CoV-2 detection. Therefore, we evaluated this EFDH method on the 300 samples using a 1:2 dilution and exposure at 72°C for 15 min.

2.3. Extraction-based protocol (reference method)

Extraction was performed manually using the QIAamp Viral RNA Kit (Cat. no. 52904, QIAGEN, Germany) following the standard protocol.[32] The process began with sample lysis under highly denaturing conditions, effectively inactivating RNases and protecting the integrity of viral RNA. These conditions ensured that the RNA remained intact throughout the extraction process. Next, the buffering conditions were carefully adjusted to facilitate the binding of RNA to the specialized membrane of the QIAamp plates. This step was crucial for capturing the RNA while preventing the co-binding of contaminants. To ensure the removal of impurities, the bound RNA underwent a series of washes using two wash buffers followed by a final ethanol wash. These steps effectively eliminated contaminants, including proteins, nucleases, and other potential inhibitors, yielding high-purity RNA. The purified RNA was then eluted using a specially formulated RNase-free buffer, making it immediately available for downstream applications or safe for long-term storage without degradation. The unique QIAamp membrane played a pivotal role in the process, ensuring exceptionally high recovery rates of pure and intact RNA. The methodology avoided the use of phenol/chloroform extraction or alcohol precipitation, making it a safe and efficient alternative for obtaining high-quality RNA suitable for sensitive molecular diagnostic procedures.

2.4. PCR reagent preparation (master mix preparation)

The PCR solutions A and B were thawed at room temperature and thoroughly mixed. The solution was then centrifuged at 8,000 rpm for a few seconds. The number of specimens to be tested, along with the number of *ORF1ab* and *N* negative controls and positive controls, was labeled on the PCR tubes. Next, 20 μL of the solution was aliquoted into each PCR tube, then 5 μL of the negative control material, RNA from the specimens to be tested, or positive control material was added to the respective PCR tubes. After securely covering the tubes, they were transferred to the amplification detection area following a brief centrifugation at 8,000 rpm. Finally, the reaction tubes were placed in the instrument's sample sink.

2.5. EFDH protocols (index method)

Each sample (100 μL) was transferred to a heat-resistant 1.5 mL microcentrifuge tube and heated at 72°C for 15 min using a thermomixer (Eppendorf, Germany). The eluates were then stored at −20°C for up to 4 h while waiting for other clinical samples to be processed in batches. Afterward, 10 μL of the eluate was mixed with 20 μL of the master mix, following a procedure similar to that for the extraction-based eluted samples. RT-qPCR was then performed on a thermocycler (ABI7500, Thermo-Fisher Inc., United States of America) following the protocol as shown in Figure 1.

2.6. Detection of SARS-CoV-2 (RT-qPCR)

The detection of SARS-CoV-2 using RT-qPCR was performed using a thermocycler (ABI7500, Thermo-Fisher Inc., United States of America), along with the DAAN kits (Cat. no. DA0932, Daan Gene Co., Ltd, China). The protocol consisted of a single cycle of reverse transcription at 50°C for 15 min, followed by a single cycle of polymerase activation at 95°C for 2 min. This was followed by 40 amplification cycles, each consisting of

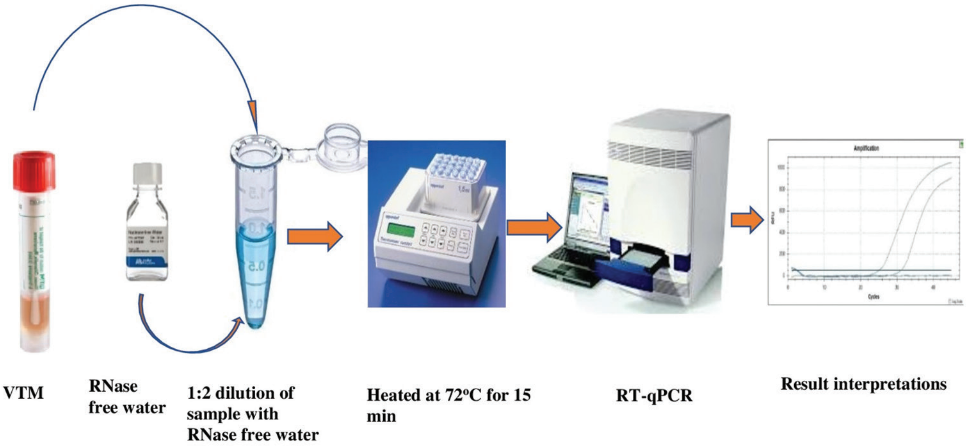

Figure 1. Process of sample dilution and heating for the detection and interpretation of severe acute respiratory syndrome coronavirus 2
Abbreviations: RT-qPCR: Real-time quantitative polymerase chain reaction; VTM: Viral transport media.

15 s at 95°C and 60 s at 60°C. The purpose of this assay was to detect the presence of SARS-CoV-2 using primers and probes targeting the *N* and *ORF1ab* gene. For comparison, the standard EB technique served as the reference method, alongside the EFDH method. The RT-qPCR results were then interpreted. A Ct value greater than 40, or the absence of an amplification curve in both FAM and VIC channels, indicated the absence of SARS-CoV-2 RNA in the sample. Conversely, a clear amplification curve in the VIC channel with a Ct value of 40 or less indicated a positive result. Samples with borderline amplification or ambiguous VIC signals were retested, and the result of the repeat run was considered final. The repeated result was considered the final result. At each step of both methods, strict quality control measures were taken. In addition to the positive and negative controls provided in the kit for the extraction method, we also used an extraction control. All measuring devices in the laboratory, including the thermal cycler, were within their calibration dates.

2.7. Ethics statement

This study was reviewed and approved by the Institutional Review Board of Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University in Ethiopia, under protocol number ALIPB IRB/52/2013/21. A permission letter was obtained from the Ethiopian Public Health Institute with reference number WG12/19 on April 21, 2021. The studies were conducted in accordance with the local legislation and institutional requirements.

2.8. Statistical analysis

The data were summarized as mean ± standard error of the mean (SEM) and coefficient of determination (R^2). The D'Agostino and Pearson test, using a 95% confidence interval (CI), was used to assess normality. CIs for sensitivity, specificity, positive and negative predictive values, and accuracy were calculated using R software (v.3.6.0, R Foundation for Statistical Computing, Austria). The Wilcoxon signed-rank test was used to compare the two groups. The correlation between the Ct values of the two techniques was determined with the two-tailed Pearson's correlation coefficient using STATA (v17, StataCorp LP, United States of America). A $p<0.05$ indicated statistical significance. The Ct values were grouped accordingly (Ct < 20, 20–35, and >35), and performance characteristics in each group were compared.

3. Results

The EFDH method detected 85.8% (163/190) of the positive samples and 100% (110/110) of the negative samples. However, 15 false-negative results were recorded, most of which had average Ct values >36 by the extraction-based detection method. In addition, the EFDH method yielded 12 invalid results where the internal control did not amplify. To compare the two methods, we excluded the invalid results, using 178 positive and 110 negative results detected by the extraction-based detection method, as shown in Table 2.

The extraction-based method yielded a mean Ct value of 24.1 (95% CI: 23.3–24.8) with an SEM of 0.4. This method detected a range of Ct values from 16.0 to 34.1. On the other hand, the EFDH method yielded an average Ct value of 27.7 (95% CI: 26.8–28.7) with an SEM of 0.5. This method also detected a range of Ct values from 18.0 to 38.9. The true prevalence among study participants was 62% (178/288) (95% CI: 56–67%). The apparent prevalence, defined as the proportion of true positives out of the total tested, was 57% (163/288) (95% CI: 51–62%).

3.1. Diagnostic performance of the EFDH method

The EFDH method showed an overall sensitivity and specificity of 92% (95% CI: 86–95%) and 100% (95% CI: 97–100%), respectively. The positive predictive value was 100% (95% CI: 97–100%), while the negative predictive value was 88% (95% CI: 81–93%). The negative likelihood ratio, which measures the ability of a test to rule out a disease, was found to be 0.08 (95% CI: 0.05–0.14). The study also revealed an overall agreement (accuracy) of 95% (95% CI: 92–97%) for the EFDH method, with a kappa value of 0.89 ($p<0.001$) (Table 3).

The correlation between the two methods is represented by Equation (1):

$$Y = -0.12 + 1.16\ X \tag{1}$$

With $R^2 = 0.99$, $p=0.001$, showing a strong and significant linear relationship between the extraction-based and EFDH detection methods. For every unit increase in the Ct value by the extraction-based detection methods, the Ct value by the EFDH detection method increases by approximately 1.16 units (Figure 2). The Wilcoxon signed-rank test ($z = 11.08$, $p=0.0001$) indicated a statistically significant difference in Ct values between the extraction-based and EFDH detection methods. The extraction-based method generally produces lower Ct values compared to the EFDH method across all observations.

Table 2. Comparison of the extraction-based method and the extraction-free dilution and heating (EFDH) methods

EFDH	Extraction-based method		
	Positive	Negative	Total
Positive	163	0	163
Negative	15	110	125
Total	178	110	288

Note: Pearson's χ^2<0.001.

Table 3. Performance characteristics of the extraction-based and extraction-free dilution and heating methods for severe acute respiratory syndrome coronavirus in Ethiopia

Statistic	Value (%)	95% CI
Apparent prevalence	57	51–62%
True prevalence	62	56–67%
Sensitivity	92	86–95%
Specificity	100	85–100%
Negative likelihood ratio	0.08	0.05–0.14
Positive predictive value	100	98–100%
Negative predictive value	88	81–93%
Accuracy/agreement	95	92–97%

Abbreviation: CI: Confidence interval.

3.2. Comparison of performance characteristics in terms of cycle threshold value

Regarding the samples with a Ct value <20 (38 samples), both techniques correctly identified all samples as true positives. Of the 124 results with a Ct value ranging from 20 to 35, 121 (98%) were true positives. For the remaining 119 test results with Ct values higher than 35, the EFDH method yielded nine false negatives. These findings demonstrated that both techniques were highly sensitive to the Ct < 20 group, accurately identifying all samples as positive. In the 20–35 Ct value group, both techniques detected most of the positive samples, but the EFDH method showed 2% false-negative results (3 samples). Finally, the EFDH technique showed low sensitivity in the group with Ct > 35. These results suggested that the EFDH method is more reliable for detecting SARS-CoV-2 in samples with higher viral loads (Table 4).

4. Discussion

We compared the EFDH method to the standard extraction-based SARS-CoV-2 detection method. The EFDH method showed high sensitivity, specificity, and positive and negative predictive values, especially at high SARS-CoV-2 viral loads. In addition, the EFDH method showed a remarkable agreement and correlation with the extraction-based method. The mean Ct values for the EFDH method were slightly higher compared to the extraction-based method; however, they were generally comparable to the gold standard method. The findings

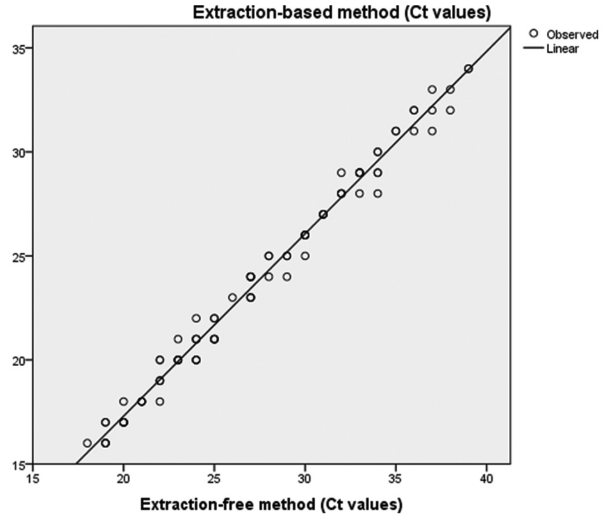

Figure 2. Correlation between extraction-free heating and dilution method and extraction-based severe acute respiratory syndrome coronavirus 2 detection methods in Ethiopia

Table 4. Performance of extraction-free and extraction-based methods across different ranges of cycle threshold value

Extraction-free method	Extraction-based method		
	Positive	Negative	Total
Ct<20			
Positive	38	0	38
Negative	0	0	0
Total	38	0	38
Ct=20–35			
Positive	121	0	121
Negative	3	0	3
Total	124	0	124
Ct>35			
Positive	0	0	0
Negative	9	110	119
Total	9	110	119

Abbreviation: Ct: Cycle threshold.

suggest that the EFDH method is particularly reliable for detecting SARS-CoV-2 in samples with higher viral loads. Despite limitations in samples with low viral loads or high Ct values, the EFDH method demonstrated overall high accuracy and closely aligned with the extraction-based method, indicating a strong performance and reliability.

In this study, the performance of the EFDH method was comparable to that of the extraction-based method. The EFDH method demonstrated good sensitivity and specificity. This finding aligns with a study conducted in India, which reported an overall sensitivity of 79% (95% CI: 71–86%) and specificity of 99% (95% CI: 98–100%).[33] Another study on extraction-free, multiplexed amplification of SARS-CoV-2 demonstrated a sensitivity of 86% and specificity of 100%.[34] Similarly, a study conducted at the Karolinska University Hospital in Stockholm, Sweden, in 2020, using a heating technique at 95°C for 5 min, reported a sensitivity of 96.0% and specificity of 99.8%.[17] In contrast, a study conducted in India and Italy reported lower overall sensitivity, specifically 78.9% (95% CI: 71–86%) and 57.3% (95% CI: 47.3–66.8%), respectively. However, both studies reported comparable specificity, at 99.9% (95% CI: 98–99.6%) and 100% (95% CI: 94.4–100%), respectively.[35]

This study reports higher positive and negative predictive values compared to previous studies. These findings indicate excellent specificity in identifying true positives, thus enhancing the reliability of the method for confirming the presence of the diseases. However, they also highlight the need to reduce false-negative rates, which would further enhance the method's utility for ruling out infection. False viral PCR results are caused by a combination of procedural and biological factors, often due to poor sample quality, low viral load, or inefficient RNA extraction, and may also arise from issues during specimen storage. In addition, contaminants in the sample can inhibit PCR amplification and compromise detection.[29,30] Similar findings were reported in a 2021 study conducted in the United Arab Emirates (UAE), showing positive and negative predictive values of 92% and 91%, respectively.[36] The finding aligns with another 2021 study in India, with positive and negative predictive values of 92% and 97%, respectively.[33]

In this study, the overall agreement between the extraction-based and EFDH methods was found to be 95%, with a kappa value of 0.89 (p=0.000). This indicates a high level of reliability in detecting SARS-CoV-2 infections. Similar findings were reported in a study conducted in India, where the agreement was 96.8% (k = 0.83, SEM = 0.03).[36]In addition, a study conducted in Sweden showed an accuracy of 98.8% (95% CI: 97.5–99.5%),[17] while a study in the UAE reported an overall agreement (kappa coefficient) of 0.797 (p<0.001).[36] However, different findings were reported from a study conducted at the clinical laboratory of the Institut Pasteur of M'sila, Algeria, where the overall agreement between extracted and heat-inactivated (65°C for 30 min) samples was only 45%, with a 95% CI of 37–52%.[37] In the current study, a near-perfect correlation (R^2 = 0.99, p=0.001) was found between the extraction-based and EFDH methods, supporting the consistency and reliability of the EFDH method in detecting SARS-CoV-2. These findings are further supported by a 2020 study conducted in Singapore, which showed an R^2 of 0.9986 for the detection of SARS-CoV-2 without extraction. Similar results were reported from the Karolinska University Hospital in Stockholm, Sweden, with an R^2 of 0.987.[17]

The consistent findings across studies utilizing the extraction-free method for SARS-CoV-2 detection can be attributed to the method's simplicity, the inherent properties of SARS-CoV-2, and its validation against established standards. These factors contribute to the method's robustness and reliability in detecting SARS-CoV-2, making it a valuable tool in overcoming the COVID-19 pandemic.[38] Despite the challenges, the technique's overall high performance supports its continued use in surveillance and outbreak control efforts. However, the potential for false negatives emphasizes the importance of comprehensive testing strategies, including targeted testing of high-risk populations and asymptomatic individuals.[39] This method's advantages in terms of speed and simplicity, combined with its high sensitivity and specificity, make it a compelling alternative for SARS-CoV-2 detection, especially in resource-limited settings or during rapid response scenarios.[40]

These findings highlight the strengths and limitations of the extraction-free method in detecting SARS-CoV-2 across different viral loads. Notably, the method demonstrated high sensitivity and specificity in samples with lower Ct values (<35). However, its performance decreases in detecting samples with low viral loads (higher Ct values), as evidenced by an increased rate of false negatives. A study conducted in Austria to evaluate extraction-free RT-qPCR methods for SARS-CoV-2 diagnostics showed an 8.2% false-negative rate for the EFDH method in samples with a Ct value >30.[38] Another study from Algeria on the detection of SARS-CoV-2 using heat inactivation at 65°C for 30 min correctly identified 100% of clinical samples with a high viral load (Ct value <30).[37] Accurate detection of SARS-CoV-2, even at high viral loads (lower Ct value), is crucial for early case identification, contact tracing, and isolation measures.

5. Limitations

The study utilized previously stored samples instead of freshly collected ones and compared the retest results with the initial findings. It did not assess the performance of the EFDH method across different storage conditions and time intervals. Furthermore, it did not determine the method's limit of detection.

6. Conclusion

The findings suggest that the EFDH method may serve as a reliable option for detecting SARS-CoV-2 in samples with low Ct values, corresponding to higher viral loads. The EFDH method offers a potential advantage in settings where resources are limited or rapid turnaround times are required. Despite the promising results, the study highlights areas for improvement. The number of invalid and false-negative results obtained with the EFDH method necessitates further investigation into the causes of these failures and the development of new strategies to mitigate them.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. Ethical review and approval were obtained from the Institutional Review Board of the Aklilu Lemma Institute of Pathobiology at Addis Ababa University, under protocol number ALIPB IRB/52/2013/21. A permission letter was obtained from the Ethiopian Public Health Institute to use leftover samples from repositories.

Consent for publication

Not applicable.

Availability of data

Data used in this work is available from the corresponding author upon reasonable request.

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MINI-REVIEW

Intratumoral Bacillus Calmette–Guérin as a dual immunometabolic therapy for triple-negative breast cancer: A comprehensive narrative review of mechanistic insights and translational potential

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive and metabolically distinct subtype of breast cancer characterized by immune evasion, a high reliance on glycolysis, and poor treatment outcomes. Given the limitations of conventional therapies, there is an urgent need for novel, targeted approaches that integrate immune stimulation with metabolic disruption. This review explores the potential of intratumoral Bacillus Calmette–Guérin (BCG) therapy as a dual-action strategy in TNBC, focusing on its ability to convert immunologically "cold" tumors into "hot" tumors when simultaneously targeting TNBC's metabolic vulnerabilities. A comprehensive narrative review was conducted using PubMed, Scopus, and Web of Science, identifying 60 peer-reviewed studies published between 2000 and 2024. The selection criteria focused on BCG's role in oncology, its immunological and metabolic effects, and its application in solid tumors. Studies were assessed for methodological rigor using the Scale for the Assessment of Narrative Review Articles checklist. BCG enhances antitumor immunity by engaging Toll-like receptors, triggering proinflammatory cytokine release (*e.g.*, tumor necrosis factor alpha, interferon gamma, and interleukin-12), and promoting the infiltration of tumor-infiltrating lymphocytes, including cytotoxic T-cells and natural killer cells. This immune activation reprograms the tumor microenvironment, increasing susceptibility to immunotherapy. Simultaneously, BCG disrupts TNBC's glycolytic dependence by downregulating hexokinase 2 and pyruvate kinase M2, forcing a metabolic shift toward oxidative phosphorylation. This metabolic stress induces mitochondrial dysfunction, reactive oxygen species accumulation, and tumor cell apoptosis. In addition, BCG-induced "trained immunity" epigenetically reprograms innate immune cells, enhancing long-term tumor surveillance and reducing recurrence risk. Intratumoral BCG presents a promising immunometabolic intervention for TNBC by simultaneously enhancing immune activation and disrupting tumor metabolism. Future studies should focus on optimizing its clinical application, developing sustained-release formulations, and exploring synergistic combinations with immune checkpoint inhibitors and metabolic inhibitors. By addressing TNBC's dual vulnerabilities, this strategy may redefine treatment paradigms and improve patient outcomes.

Keywords: Triple-negative breast cancer; Bacillus Calmette–Guérin; Intratumoral injection; Warburg effect; Glycolysis inhibition; Immunotherapy; Trained immunity

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1. Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer, distinguished by its lack of estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor 2 (HER2) expression. Accounting for approximately 15 – 20% of all breast cancers, TNBC is notably more common in younger women and individuals with BReast CAncer gene 1 (BRCA1) mutations.[1] The absence of these receptors limits therapeutic options, as hormone and HER2-targeted treatments are ineffective, rendering chemotherapy the primary systemic approach. Unfortunately, this reliance on chemotherapy is associated with high recurrence rates, significant toxicity, and a poor overall prognosis due to TNBC's aggressive nature and high metastatic potential. Thus, the search for innovative, targeted treatments for TNBC is critical.[2] The Bacillus Calmette–Guérin (BCG) vaccine, originally developed for tuberculosis (TB), has shown notable success as an immunotherapy in oncology, especially in non-muscle invasive bladder cancer (NMIBC), by activating both innate and adaptive immune responses.[3] BCG's antitumor activity is primarily mediated through macrophage and dendritic cell activation, leading to the release of inflammatory cytokines and enhanced T-cell cytotoxicity within the tumor microenvironment.[4] These effects, along with the potential to stimulate natural killer (NK) cells, position BCG as a compelling candidate for TNBC immunotherapy.[5] Furthermore, TNBC cells exhibit a pronounced reliance on aerobic glycolysis a phenomenon known as the Warburg effect which promotes glucose consumption to sustain rapid growth and invasiveness even in hypoxic conditions.[6] This metabolic reprogramming, integral to TNBC cell survival and proliferation, is a promising therapeutic target.[7] Emerging research suggests that BCG might counteract the Warburg effect by modulating glycolytic pathways, potentially inhibiting key enzymes involved in glucose metabolism and shifting cells toward oxidative phosphorylation.[8] This metabolic disruption, when combined with immune activation, could impair TNBC cell viability and augment immune-mediated cytotoxicity, particularly when BCG is administered intratumorally. In this perspective, we explore the potential of BCG as a localized therapeutic approach for TNBC, hypothesizing that its dual action immune activation and metabolic restriction could establish a novel, specialized immunometabolic strategy, thereby offering a promising avenue for targeted TNBC treatment.

2. Methodology

A comprehensive narrative review was conducted through a systematic search of PubMed, Scopus, and Web of Science, focusing on studies related to the immunological and metabolic mechanisms of BCG therapy in cancer, with a specific emphasis on TNBC. The search strategy employed a combination of Medical Subject Headings terms and free-text keywords such as "Bacillus Calmette-Guérin," "TNBC immunotherapy," "BCG and Warburg effect," "intratumoral BCG," "trained immunity in cancer," and "BCG metabolic modulation," using Boolean operators (AND/OR) to refine the results and ensure comprehensive coverage of relevant literature. Only peer-reviewed studies published in English from 2000 to 2024 were included, and reference lists of key articles were manually screened to identify additional relevant publications. Inclusion criteria encompassed studies investigating BCG's immunological effects in oncology, its metabolic impact on glycolysis and oxidative phosphorylation, and experimental or clinical research exploring intratumoral BCG administration in solid tumors. Exclusion criteria included non-English studies, case reports with limited statistical power, studies lacking direct relevance to TNBC or intratumoral BCG therapy, and articles without full-text availability. The study selection process followed a two-stage screening approach, retrieving an initial pool of 745 studies. After removing 162 duplicates, title and abstract screening excluded 413 studies due to irrelevance, leaving 170 full-text articles for in-depth evaluation. Of these, 110 were excluded due to methodological limitations or insufficient focus on TNBC, resulting in a final inclusion of 60 studies. Data extraction was conducted independently by all authors, assessing study design, sample size, key findings, and relevance to the proposed hypothesis. Any discrepancies were resolved through discussion or consultation with a third reviewer. Quality assessment was performed using the Scale for the Assessment of Narrative Review Articles checklist, evaluating justification, clear aims, literature search rigor, inclusion of primary and relevant literature, evidence-based reasoning, and structured presentation. Only studies scoring ≥9/12 were considered methodologically robust. This review synthesizes existing evidence on the dual role of BCG in TNBC, integrating immunological and metabolic perspectives. Specifically, it examines BCG's ability to reshape the tumor microenvironment by converting immune-cold tumors into immune-hot phenotypes, its influence on tumor-infiltrating lymphocytes (TILs), and its disruption of TNBC's glycolytic dependence through the downregulation of key enzymes such as hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2). By consolidating these findings, this review constructs an integrated model of BCG's immunometabolic actions in TNBC, establishing a foundation for further empirical validation and suggesting novel therapeutic strategies to optimize BCG's clinical application in breast oncology.

3. Characterization of TNBC: Mechanisms of aggressiveness, metabolic reprogramming, and immune microenvironment modulation

TNBC is an aggressive subtype of breast cancer characterized by the absence of ERs, PRs, and HER2 overexpression. This receptor-negative profile differentiates TNBC from other breast cancer subtypes, as it limits therapeutic targets to non-hormonal and non-HER2-directed options, contributing to a poorer prognosis and high rates of recurrence and metastasis.[9]

TNBC's onset is believed to be driven by genetic mutations and epigenetic modifications, often involving *BRCA1* and *BRCA2* mutations, which play critical roles in DNA repair.[10] These genetic alterations lead to genomic instability and an accumulation of DNA damage, fostering malignant transformation. Additional mutations affecting the p53 tumor suppressor gene and various pathways, such as Phosphoinositide 3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin, drive cellular proliferation, resistance to apoptosis, and tumor progression.[11]

As TNBC cells proliferate, they develop further alterations in cellular metabolism and signaling that enhance their survival and invasiveness. One hallmark of TNBC is the reprogramming of cellular energy production, notably through the Warburg effect, where the cells rely predominantly on glycolysis for ATP generation, even in oxygen-rich environments.[12] This metabolic shift not only supports rapid cellular proliferation but also generates an acidic microenvironment that promotes invasion and metastasis. This glycolytic dependency increases glucose uptake, supporting TNBC's high energy demands and further promoting its aggressive behavior.[13]

The immune microenvironment in TNBC also undergoes significant modifications. Unlike other subtypes of breast cancer, TNBC tumors exhibit substantial infiltration by immune cells, including tumor-associated macrophages (TAMs), regulatory T-cells (Tregs), and myeloid-derived suppressor cells (MDSCs), which often create an immunosuppressive milieu.[14] TNBC cells evade immune surveillance through the upregulation of immune checkpoint proteins, such as programmed cell death ligand 1 (PD-L1), which inhibit T-cell activity and enable immune escape.[15] Simultaneously, TNBC induces a proinflammatory microenvironment via cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor alpha (TNF-α), fostering chronic inflammation that supports tumor growth and metastasis.[16] This inflammatory setting promotes the recruitment of immunosuppressive cells, including Tregs and MDSCs, which further suppress the antitumor immune response and contribute to a vicious cycle of immune evasion and tumor promotion.[17]

As TNBC progresses, systemic effects emerge due to the tumor's influence on distant organs and the release of circulating tumor cells and extracellular vesicles (EVs), which facilitate metastasis. TNBC metastasizes preferentially to the lungs, liver, and brain, where it adapts to the unique microenvironments of these organs.[18] Through the secretion of exosomes and EVs, TNBC cells influence distant tissues to create a premetastatic niche that promotes metastatic colonization. These vesicles carry prometastatic signals, including proteins, RNA, and microRNA, which modify the local immune landscape, enhance vascular permeability, and promote inflammation at distant sites. Furthermore, metabolic alterations in TNBC also increase oxidative stress, which can lead to systemic inflammation and immune dysregulation.[19]

TILs play a crucial role in the immune microenvironment of TNBC, serving as key mediators of antitumor immunity. TNBC is characterized by a high degree of immune cell infiltration compared to other breast cancer subtypes, with varying levels of CD8+ cytotoxic T lymphocytes, CD4+ helper T-cells, Tregs, and TAMs.[20] The presence of TILs before treatment has been strongly correlated with prognosis, as higher baseline levels of CD8+ T-cells are associated with better overall survival and a stronger response to chemotherapy.[21] However, TNBC tumors often develop immunosuppressive mechanisms that limit the effectiveness of TILs, including the upregulation of immune checkpoint molecules such as PD-L1 and the recruitment of immunosuppressive cells such as Tregs and MDSCs.[15] These factors contribute to T-cell exhaustion and functional impairment, allowing the tumor to evade immune surveillance. The ability of TILs to mount an effective immune response before treatment is therefore a critical determinant of TNBC progression and therapeutic response, making them a key focus for immunotherapeutic interventions such as BCG therapy.

The development and progression of TNBC are influenced by a complex interplay of genetic, metabolic, and environmental factors, including exposure to environmental toxins and trace element imbalances. Epidemiological studies suggest that environmental pollutants such as endocrine-disrupting chemicals, heavy metals, and persistent organic pollutants may contribute to TNBC risk by inducing oxidative stress, DNA damage, and epigenetic modifications that promote tumor initiation and progression. In particular, exposure to cadmium, arsenic, and lead has been linked to increased breast cancer risk due to their ability to mimic estrogenic activity, disrupt cellular redox balance, and interfere with tumor suppressor pathways.

Trace elements, essential for various metabolic and enzymatic processes, also play a crucial role in TNBC pathophysiology. Imbalances in elements such as zinc (Zn), selenium (Se), and copper (Cu) have been associated with altered antioxidant defense mechanisms and immune dysregulation in TNBC patients.

Zn, a key cofactor for antioxidant enzymes such as superoxide dismutase, is often deficient in TNBC, leading to increased oxidative stress and genomic instability. Conversely, elevated Cu levels have been observed in TNBC tumors, contributing to enhanced angiogenesis and tumor progression via the activation of proangiogenic factors such as vascular endothelial growth factor. Se, known for its role in redox homeostasis, has been shown to exert protective effects against TNBC by modulating glutathione peroxidase activity and reducing oxidative DNA damage. The intricate relationship between environmental exposures, trace element homeostasis, and TNBC metabolism underscores the need for further research into potential preventive and therapeutic strategies targeting these metabolic vulnerabilities.[22]

4. From TB vaccine to oncological immunotherapy: Mechanisms of action and efficacy of BCG in cancer treatment

The BCG vaccine was originally developed in the early 20th century by Albert Calmette and Camille Guérin as a preventative vaccine for TB. BCG is derived from a live attenuated strain of *Mycobacterium bovis*, modified to maintain its immunogenicity when minimizing virulence.[23] Since its introduction, BCG has been widely utilized as a TB vaccine, particularly in countries with high TB incidence.[24] However, BCG's potent immunostimulatory properties soon led researchers to investigate its potential as an anticancer agent. In the 1970s, studies demonstrated that BCG could effectively treat NMIBC, marking it as one of the first successful immunotherapies in oncology.[25] Today, intravesical BCG is a standard treatment for NMIBC, significantly reducing recurrence and progression rates by stimulating an antitumor immune response directly within the bladder.

The success of BCG in NMIBC stems from its robust activation of both innate and adaptive immune responses, mediated through a series of precise immunological mechanisms.[26] Upon administration into the bladder, BCG is internalized by urothelial cells and professional antigen-presenting cells, such as macrophages and dendritic cells.[27] This uptake initiates a cascade of immune responses, beginning with the activation of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), on antigen-presenting cells. TLR activation leads to the release of proinflammatory cytokines, including IL-1, TNF-α, and interferon gamma (IFN-γ), creating an inflammatory microenvironment conducive to antitumor activity.[28]

These cytokines recruit a diverse array of immune cells to the tumor site, including neutrophils, monocytes, NK cells, and T-cells, each contributing uniquely to the antitumor response. Neutrophils and NK cells are among the first responders, releasing cytotoxic molecules such as reactive oxygen species (ROS) and perforin, which directly kill tumor cells. In addition, NK cells, activated by IFN-γ, engage in direct cytotoxicity and secrete additional cytokines to recruit more immune cells.[29]

The adaptive immune response is also highly engaged through BCG's action. Dendritic cells, after processing BCG antigens, migrate to the lymph nodes, where they present BCG-derived peptides on major histocompatibility complex (MHC) molecules to T-cells, thereby priming a robust T-cell response. CD4+ T-helper cells become activated and further amplify the immune response through cytokine production, particularly IFN-γ and IL-2, which are essential for cytotoxic T-cell (CD8+) activation. These CD8+ T-cells then recognize and destroy BCG-infected and tumor cells, exerting a specific and sustained antitumor effect.[30] Importantly, BCG also induces a "trained immunity" effect in innate immune cells. This phenomenon, characterized by epigenetic reprogramming in monocytes and macrophages, enhances their responsiveness to subsequent infections and tumors, potentially providing lasting protection against recurrence.[31]

BCG has demonstrated efficacy primarily in NMIBC, but studies suggest its potential in other cancer types, including melanoma, colorectal cancer, and certain hematologic malignancies. However, its success is context-dependent and varies according to the tumor's immune microenvironment and accessibility to immune cells.[32]

5. Intratumoral BCG injection: A dual strategy of immune activation and metabolic disruption in TNBC treatment

The novel approach of utilizing BCG vaccine as an intratumoral injection directly within TNBC tumors represents a promising strategy to harness both immune activation and metabolic disruption against this aggressive cancer. TNBC, known for its lack of ERs, PRs, and HER2, poses a therapeutic challenge due to its limited treatment options and high rate of recurrence and metastasis. The cancer cells in TNBC exhibit an enhanced dependency on glycolysis, even in the presence of oxygen a metabolic adaptation known as the Warburg effect. This shift to glycolytic metabolism facilitates rapid proliferation and supports an immune-suppressive microenvironment, which are essential for TNBC survival and progression.[33] BCG, through a combination of immune and metabolic actions, could counteract these characteristics and offer a multifaceted assault on TNBC.[34]

5.1. Immune activation through BCG injection in TNBC tumors

Intratumoral administration of BCG in TNBC tumors directly exposes tumor cells and the surrounding immune microenvironment to the live attenuated bacteria. This proximity is crucial for stimulating a robust immune response. Upon injection, BCG is rapidly recognized by the immune system due to the presence of pathogen-associated molecular patterns (PAMPs) on its cell wall. These PAMPs, such as lipopolysaccharides and peptidoglycans, interact with PRRs, specifically TLRs, on tumor-resident macrophages and dendritic cells. This receptor-ligand binding triggers a strong release of proinflammatory cytokines, including TNF-α, IL-6, and IFN-γ, creating an inflammatory response that attracts additional immune cells to the site of injection.[35]

The local inflammation induced by BCG leads to the recruitment of neutrophils, monocytes, NK cells, and both CD4+ and CD8+ T-cells. Neutrophils, as one of the initial responders, release cytotoxic molecules, including ROS and proteolytic enzymes, which can directly damage TNBC cells.[36] NK cells, once activated by the cytokine environment and the presence of stressed tumor cells, release perforin and granzymes, inducing apoptosis in tumor cells.[37]

Following the initial innate response, dendritic cells that have phagocytozed BCG components and tumor antigens migrate to regional lymph nodes. Here, they present processed antigens on MHC molecules to T-cells, particularly activating CD4+ helper T-cells and CD8+ cytotoxic T-cells. CD4+ T-helper cells further amplify the immune response by secreting IL-2 and IFN-γ, promoting the expansion and activation of CD8+ cytotoxic T-cells. These CD8+ T-cells then home back to the tumor site, recognizing and killing TNBC cells with high specificity.[38]

5.2. Modulation of tumor metabolism: Targeting the Warburg effect

One of the key characteristics of TNBC is its dependency on glucose metabolism, or the Warburg effect, which is critical for maintaining its rapid growth and survival. The Warburg effect enables TNBC cells to generate energy predominantly through glycolysis, even under oxygen-rich conditions, leading to an acidic microenvironment that fosters immune evasion and further promotes tumor aggression.[39] This glycolytic reliance, however, creates a therapeutic vulnerability that BCG may exploit.

Recent research suggests that BCG may disrupt cancer cell metabolism by altering glycolytic pathways. When BCG is injected into the tumor, the resulting immune activation generates an inflammatory milieu that can impact TNBC's metabolic pathways.[40] Specifically, the release of cytokines such as IFN-γ and TNF-α can inhibit key enzymes in the glycolytic pathway, including HK2 and PKM2, thereby reducing glucose uptake and utilization by TNBC cells.[41] By interfering with glycolysis, BCG forces TNBC cells to rely more on oxidative phosphorylation, a less favorable pathway for these cells, given their adaptations for glycolytic metabolism. This metabolic disruption limits the tumor's energy production and growth capacity.[42]

Moreover, the induction of ROS by immune cells recruited to the tumor site can lead to oxidative stress, which further impairs mitochondrial function and induces metabolic strain on TNBC cells. The combined inhibition of glycolysis and oxidative stress places TNBC cells in a bioenergetic crisis, leading to decreased proliferation, increased apoptosis, and enhanced susceptibility to immune-mediated destruction.[43]

BCG exerts its metabolic and immunomodulatory effects on multiple cell types within the TNBC tumor microenvironment, targeting not only tumor cells but also key immune regulators such as dendritic cells, macrophages, and T lymphocytes.[44] One of the primary metabolic vulnerabilities of TNBC is its reliance on glycolysis via the Warburg effect, where tumor cells predominantly generate ATP through aerobic glycolysis, rather than oxidative phosphorylation. This metabolic adaptation is driven by overexpression of glycolytic enzymes such as HK2 and PKM2, which enhance glucose uptake and lactate production, fostering an immunosuppressive microenvironment.[45] BCG disrupts this metabolic program by inducing a proinflammatory cytokine response, particularly through IFN-γ and TNF-α signaling, which has been shown to downregulate HK2 and PKM2 expression in tumor cells. The inhibition of these enzymes forces TNBC cells to shift toward oxidative phosphorylation, increasing ROS production and promoting metabolic stress, ultimately leading to tumor cell apoptosis and reduced proliferation.[46]

Beyond direct metabolic disruption in tumor cells, BCG also modulates the function of dendritic cells and macrophages, which play essential roles in tumor antigen presentation and immune activation. Upon exposure to BCG, dendritic cells undergo metabolic reprogramming, shifting from glycolysis to oxidative phosphorylation, a process essential for their maturation and enhanced antigen presentation capabilities.[47] This shift allows dendritic cells to more effectively process and present tumor antigens, leading to the priming and activation of cytotoxic T lymphocytes. In addition, BCG influences macrophage polarization, promoting an M1-like phenotype characterized by increased IL-12 and TNF-α production when reducing the immunosuppressive activity of M2 macrophages that are typically associated with tumor progression.[46] The metabolic and immunological effects of BCG converge to reshape the TNBC microenvironment, counteracting tumor immune evasion strategies and making tumor cells more susceptible to immune-mediated destruction. By simultaneously targeting glycolytic metabolism in cancer cells and enhancing antigen presentation in Dendritic cells, BCG represents a dual-action therapeutic strategy that integrates metabolic and immune-based interventions in TNBC treatment.

The metabolic vulnerabilities of TNBC extend beyond the Warburg effect to include a broader phenomenon of glucolipotoxicity, a metabolic dysfunction characterized by excessive intracellular accumulation of glycolipids that disrupts normal cellular homeostasis. Recent research suggests that glucolipotoxicity plays a pivotal role in cancer progression by inducing oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction, all of which contribute to immune evasion and tumor survival.[48] BCG, through its dual immunometabolic action, has the potential to counteract these metabolic disruptions by not only inhibiting key glycolytic enzymes such as HK2 and PKM2 but also modulating lipid metabolism.[49] Proinflammatory cytokines released upon BCG injection, including TNF-α and IFN-γ, have been shown to interfere with lipid accumulation pathways, potentially restoring metabolic balance in TNBC cells.[30] Furthermore, BCG-induced metabolic stress forces TNBC cells to shift away from anaerobic glycolysis, thereby reducing lactate accumulation and mitigating the acidic tumor microenvironment that facilitates immune suppression. This metabolic reprogramming aligns with the proposed glucolipotoxicity hypothesis, which highlights how dysregulated glycolipid metabolism fuels tumor progression. By integrating these insights, BCG therapy emerges as a promising strategy not only for immune activation but also for disrupting tumor-promoting metabolic adaptations, providing a novel framework for TNBC treatment.[48]

5.3. "Trained immunity" and long-term immunological memory

In addition to these immediate effects, BCG also has the potential to induce "trained immunity" in innate immune cells, particularly monocytes and macrophages. Trained immunity is an epigenetic reprogramming of innate immune cells that results in enhanced responsiveness to subsequent immune challenges. BCG-induced trained immunity could lead to a long-lasting, heightened immune state against TNBC, maintaining an antitumor immune response even after initial treatment. This could be particularly valuable in TNBC, where recurrence rates are high and long-term immune vigilance is crucial.[8]

5.4. Establishing a localized, sustained antitumor environment

The continuous release of cytokines and chemokines, as well as the infiltration of activated immune cells into the tumor microenvironment, results in a sustained antitumor response that can potentially limit TNBC's capacity for recurrence and metastasis.[50] The localized inflammatory response helps reshape the immune-suppressive tumor microenvironment into one that favors immune activation. This reprogramming not only enhances the effectiveness of immune effector cells but also sensitizes TNBC cells to further immunologic attacks.[51] The hypoxic and acidic conditions created by the Warburg effect are disrupted, thereby reducing the immune escape mechanisms typically employed by TNBC.[52]

6. Limitations and challenges of intratumoral BCG therapy in TNBC

The application of BCG as an intratumoral therapy for TNBC presents several challenges that must be addressed to optimize its clinical potential. One major limitation is the variability in immune responses among patients, which can significantly impact treatment efficacy. BCG exerts its immunostimulatory effects through PRRs, particularly TLRs, which activate macrophages and dendritic cells to release key proinflammatory cytokines such as TNF-α, IL-6, and IFN-γ.[27] However, genetic polymorphisms in TLRs and other immune-regulatory genes, differences in trained immunity responses, and variations in the tumor microenvironment including immune checkpoint expression and the presence of immunosuppressive cells such as Tregs and MDSCs may lead to inconsistent immune activation across patients.[53] In addition, systemic factors such as the gut microbiome, which modulates host immunity, may further contribute to differential responses to BCG therapy. Another critical challenge is the potential for excessive inflammatory responses and immune-related toxicities. While BCG-induced inflammation is essential for tumor eradication, uncontrolled activation of nuclear factor-kappa B (NF-κB) and STAT3 signaling pathways may lead to excessive production of IL-1β and TNF-α, resulting in severe tissue damage, tumor necrosis, and even systemic cytokine release syndrome.[54] This is particularly concerning in TNBC tumors with dense stromal fibrosis, where increased immune cell infiltration could exacerbate tissue edema and impair therapeutic outcomes. The risk of prolonged inflammation, granuloma formation, and chronic immune activation also raises concerns about the long-term effects of intratumoral BCG administration. Furthermore, the safety profile of BCG outside bladder cancer remains uncertain, as the breast tumor environment differs significantly from the bladder, which provides a confined setting for intravesical BCG instillation.[55] Intratumoral injection in breast tissue poses risks of bacterial leakage into surrounding tissues or systemic dissemination, potentially leading to disseminated BCG infection (BCGosis), particularly in immunocompromised individuals.

Another concern is the persistence of BCG within TAMs, which, instead of sustaining an antitumor immune response, may lead to immune suppression through increased IL-10 and transforming growth factor-beta (TGF-β) secretion, thereby facilitating tumor immune escape.[56] Pre-existing exposure to mycobacteria or prior BCG vaccination may further complicate host immune responses, potentially causing either heightened reactivity or immune tolerance. Addressing these challenges requires several strategic interventions, including patient stratification based on immune profiling to predict responsiveness, combination therapies that integrate BCG with immune checkpoint inhibitors or glycolysis inhibitors to counteract tumor immune evasion, and the development of controlled-release formulations such as biodegradable poly(lactic-co-glycolic acid) (PLGA) hydrogels to ensure localized and sustained immune activation when minimizing systemic exposure. Moreover, regular monitoring for systemic BCG dissemination using polymerase chain reaction-based detection methods could help mitigate the risks of widespread infection. By addressing these limitations, future research can refine intratumoral BCG therapy, enhancing its safety and efficacy as a novel immunometabolic intervention in TNBC.

7. Preclinical evaluation of intratumoral BCG therapy in an orthotopic TNBC model

To evaluate the therapeutic efficacy of intratumoral BCG injection TNBC, a well-structured preclinical animal study should be designed using an orthotopic TNBC mouse model. Female immunocompetent C57BL/6 or BALB/c mice, aged 6 – 8 weeks, are used to ensure an intact immune system capable of responding to BCG-mediated immune activation. TNBC cells, such as 4T1 (BALB/c) or E0771 (C57BL/6), are cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. A total of 1 × 10^6 TNBC cells in 100 μL of phosphate-buffered saline (PBS) are orthotopically injected into the fourth mammary fat pad to establish tumors. Tumor growth is monitored every 2 days using calipers, and once tumors reach an average volume of 100 mm^3, the mice are randomized into experimental groups: (1) Control (PBS), (2) BCG monotherapy, (3) BCG + immune checkpoint inhibitor (antiprogrammed cell death protein 1 [PD-1]), and (4) BCG + glycolysis inhibitor (2-deoxyglucose). For intratumoral BCG administration, 10^6 colony-forming unit of live attenuated BCG suspended in 50 μL PBS is injected directly into the tumor using an insulin syringe with a 30G needle, ensuring uniform dispersion.

The treatment is administered twice weekly for 3 weeks. Afterward, tumor growth is assessed by measuring volume using the formula (length × width^2)/2, whereas tumor burden reduction is statistically analyzed using two-way analysis of variance.

To assess immune activation, flow cytometry is performed on TILs extracted via enzymatic digestion of tumors using collagenase IV and DNase I. The cells are stained with fluorophore-conjugated antibodies targeting CD8, CD4, NK1.1, CD11c, PD-1, and IFN-γ, and analyzed using a flow cytometer. Cytokine levels, including TNF-α, IL-6, IL-12, and IFN-γ, are quantified in tumor lysates and serum using enzyme-linked immunosorbent assay. To investigate metabolic alterations, tumor sections are analyzed for glycolytic enzyme expression, including HK2 and PKM2, using immunohistochemistry and Western blotting. Lactate production is assessed using a lactate assay kit, and mitochondrial oxidative phosphorylation activity is evaluated by measuring oxygen consumption rates using a Seahorse XF Analyzer. Histopathological examination of tumors is conducted using hematoxylin and eosin staining to assess necrosis, inflammatory infiltration, and granuloma formation. To determine systemic safety, complete blood count and liver and kidney function tests are performed on serum samples. In data analysis, statistical significance is set at $p<0.05$. This protocol will provide a comprehensive evaluation of BCG's immunometabolic effects in TNBC, supporting its potential translation into clinical applications.

8. Discussion

The innovative approach of using intratumoral BCG injections for the treatment of TNBC represents a paradigm shift in cancer therapy by leveraging both immunological and metabolic vulnerabilities of this aggressive subtype. TNBC is notoriously difficult to treat due to its lack of hormone receptors and its dependence on glycolysis via the Warburg effect to sustain rapid proliferation. Unlike conventional therapies, BCG offers a dual mechanism of action: it induces a localized, potent immune response and disrupts the tumor's metabolic dependencies, providing a multifaceted therapeutic strategy.

Upon intratumoral injection, BCG engages PRRs, primarily TLRs such as TLR2 and TLR4, expressed on antigen-presenting cells including macrophages and dendritic cells. This interaction triggers the activation of NF-κB and interferon regulatory factors, leading to the secretion of proinflammatory cytokines such as TNF-α, IL-6, and IFN-γ.

These cytokines, in turn, promote the recruitment and activation of cytotoxic T lymphocytes, NK cells, and additional antigen-presenting cells, creating an inflammatory microenvironment conducive to tumor destruction. Furthermore, BCG facilitates dendritic cell maturation, leading to efficient antigen presentation via MHC molecules, thereby priming an adaptive immune response that extends beyond the primary tumor site.

A key immunological consequence of BCG administration is its ability to convert immunologically "cold" tumors characterized by low immune infiltration and an immunosuppressive milieu into "hot" tumors with high levels of TILs. This conversion is driven by increased expression of chemokines such as CCL2, CCL5, and CXCL10, which promote the trafficking of effector T-cells into the tumor microenvironment. In addition, BCG-induced trained immunity leads to epigenetic reprogramming of monocytes and macrophages through histone modifications and metabolic shifts in the tricarboxylic acid cycle, enhancing long-term immune responsiveness and reducing immune evasion. Clinical and preclinical studies have demonstrated that BCG increases TIL density in tumors that previously exhibited immune exclusion, potentially sensitizing TNBC to immune checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 therapies.

On the metabolic front, BCG disrupts TNBC's reliance on glycolysis by downregulating key glycolytic enzymes, including HK2 and PKM2, thereby impairing glucose uptake and ATP generation. The suppression of these enzymes forces TNBC cells to rely on oxidative phosphorylation, leading to increased mitochondrial stress and ROS production. Elevated ROS levels induce DNA damage and mitochondrial dysfunction, sensitizing tumor cells to apoptosis and reducing their proliferative capacity. Furthermore, BCG-mediated metabolic disruption mitigates the tumor's acidic and hypoxic microenvironment by reducing lactate production, which is known to suppress cytotoxic immune cell function and promote the recruitment of immunosuppressive MDSCs. By alleviating these metabolic constraints, BCG creates a more favorable immune landscape that enhances effector T-cell activity and reduces immune escape mechanisms.

Despite its potential, several challenges must be addressed to optimize BCG's efficacy in TNBC. One major limitation is the variability in immune responses among patients, which can significantly impact treatment outcomes. Differences in TLR expression, genetic polymorphisms in immune-regulatory genes, and variations in the gut microbiome may contribute to differential responses to BCG therapy.

In addition, TNBC tumors with dense stromal fibrosis may limit immune cell infiltration, reducing BCG's efficacy. Another critical concern is the risk of excessive inflammation, as BCG-induced activation of NF-κB and STAT3 signaling pathways can lead to an overproduction of IL-1β and TNF-α, potentially resulting in severe tissue damage, tumor necrosis, and even systemic cytokine release syndrome. The formation of granulomas and persistent local inflammation may further complicate treatment outcomes, necessitating careful patient stratification and monitoring.

Safety considerations regarding BCG use in non-bladder tumors also warrant attention. Unlike the bladder, which provides a contained environment for BCG instillation, intratumoral administration in breast tissue poses a risk of bacterial leakage and systemic dissemination, particularly in immunocompromised patients. Persistent BCG infection within TAMs could paradoxically induce immune suppression through increased IL-10 and TGF-β secretion, facilitating tumor immune escape rather than enhancing immune clearance. Addressing these concerns requires the development of controlled-release formulations, such as biodegradable PLGA hydrogels, which could ensure sustained and localized immune activation while minimizing systemic exposure.

To translate this approach into clinical practice, rigorous quality control measures must be implemented in the development of BCG-based formulations, including sterilization, stability assays, and bioactivity testing in compliance with Good Manufacturing Practice standards. Preclinical studies should focus on validating the hydrogel's biocompatibility, release kinetics, and therapeutic efficacy before advancing to clinical trials. In addition, combination strategies integrating BCG with immune checkpoint blockade or metabolic inhibitors could further enhance therapeutic outcomes by overcoming tumor immune evasion mechanisms.

By addressing these limitations, BCG therapy could establish a new immunometabolic paradigm for TNBC treatment, leveraging its ability to simultaneously modulate immune responses and disrupt tumor metabolism. Future research should focus on optimizing patient selection criteria, refining delivery methods, and integrating BCG-based immunotherapy into multimodal treatment regimens to enhance its clinical applicability and long-term therapeutic benefits.

9. Conclusion

Intratumoral BCG therapy represents a novel and promising dual-action strategy for TNBC, integrating robust immune activation with metabolic disruption. By converting immune-cold tumors into immune-hot phenotypes, BCG enhances antitumor immunity, increasing TIL recruitment and priming adaptive responses. Concurrently, BCG disrupts TNBC's reliance on glycolysis by downregulating HK2 and PKM2, inducing metabolic stress that sensitizes tumor cells to immune-mediated destruction. This immunometabolic synergy not only suppresses tumor progression but also establishes a sustained antitumor response through BCG-induced trained immunity. However, challenges such as interpatient immune variability, potential inflammatory toxicities, and delivery optimization must be addressed to maximize clinical efficacy. Future research should prioritize controlled-release formulations, biomarker-driven patient stratification, and combination therapies integrating BCG with immune checkpoint blockade or metabolic inhibitors.

If successfully translated into clinical practice, this approach has the potential to revolutionize TNBC treatment, offering a durable and targeted alternative to conventional therapies in a subtype with historically limited options.

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ACCSCIENCE
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Microbes & Immunity

CASE REPORT

Acute disseminated encephalomyelitis following intralesional MMR vaccination: A rare case report and mini review

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Abstract

Acute disseminated encephalomyelitis (ADEM) is a demyelinating, immune-mediated inflammatory condition of the central nervous system (CNS). It can affect individuals of all ages. The case definition for monophasic ADEM includes polyfocal clinical CNS events of inflammatory origin, with magnetic resonance imaging showing large (>1 cm) diffuse white matter lesions, absence of relapse for 3 months, and exclusion of other acute demyelinating disorders. ADEM can also be associated with various infections and vaccinations. Our case study suggests that ADEM occurs following intralesional measles, mumps, and rubella (MMR) vaccination to treat cutaneous warts. This case highlights the importance of making a well-informed decision regarding the risk of demyelinating disease in patients receiving vaccines. In addition, it emphasizes the need to obtain a detailed vaccination history when assessing patients with suspected ADEM. This is the first documented case of ADEM following intralesional MMR vaccination.

Keywords: Vaccination; Neurological; Measles; Mumps; Rubella

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1. Background

Acute disseminated encephalomyelitis (ADEM) is a demyelinating, immune-mediated inflammatory disease affecting the central nervous system (CNS) that can affect individuals of all ages. ADEM usually presents with encephalopathy and multiple neurological symptoms,[1,2] along with diffuse white matter lesions observed on imaging. Although monophasic course is a common symptom of ADEM, other relapsing varieties, such as multiphasic disseminated encephalomyelitis,[3] have also been described in the literature.

2. Case presentation

A young male in his early twenties from North India presented with weakness in the left upper and lower limbs, along with progressive behavioral disturbances, such as decreased verbal output, apathy, and anhedonia, for the past 7 days. The patient reported receiving three doses of intralesional measles, mumps, and rubella (MMR) vaccination to treat facial warts (Figure 1). Each dose was 0.3 mL of reconstituted MMR vaccine (diluted in sterile water) and was administered over the course of 1 month, after which the warts completely resolved. However, after 3 weeks of the final MMR vaccination, he began experiencing the abovementioned symptoms. There was no history of fever, skin lesions, or seizures associated with the symptoms.

The general physical examination was unremarkable, with normal pulse rate, blood pressure, afebrile, no skin changes, and no lymphadenopathy. Higher mental function assessment revealed a Montreal cognitive assessment (MoCA) scale of 28/30. In addition, mild impairment in the frontal lobar was observed during a detailed lobar function examination. Of the cranial nerve, the examination was normal, along with normal visual acuity and fundus. Motor examination revealed a reduced power in the left upper and lower limbs, with a Medical Research Council grading of 4/5. In addition, brisk deep tendon reflexes and extensor plantar response were observed. Sensory and cerebellar examinations were normal, with no extra-pyramidal signs. Routine laboratory investigations – including complete hemogram profile (hemoglobin level, total leukocyte count, and platelet count), renal profile (urea and creatinine levels), thyroid profile (thyroid-stimulating hormone, triiodothyronine, and thyroxine levels), and serum electrolytes (sodium and potassium levels) – were in normal ranges. Tests for hepatitis B surface antigen, hepatitis C virus immunoglobulin M, human immunodeficiency virus (using enzyme-linked immunosorbent assay), and COVID-19 (using reverse transcription polymerase chain reaction) were all negative. In addition, Vitamin B12 and folate levels were normal, while antineutrophil antibody, anti-aquaporin-4, and myelin oligodendrocyte glycoprotein antibody tests were negative. Cerebrospinal fluid (CSF) analysis revealed a leucocyte count of 6 cells/µL (44% neutrophils), a protein level of 15.5 mg/dL, and a glucose level of 60 mg/dL, with concomitant blood serum glucose of 90 mg/dL. In addition, CSF analysis was negative for Gram stain, acid-fast bacilli, India ink, and malignant cells. Both serum and CSF analyses for oligoclonal bands were negative. Nerve conduction studies of all four limbs, visual evoked potentials, and brainstem auditory evoked responses showed normal values (Table 1). Radiological investigations, including chest X-ray and computerized tomography of the chest and abdomen, were normal. Gadolinium-enhanced magnetic resonance imaging (MRI) of the brain revealed multiple discrete and confluent areas of altered signal intensities with a peripheral rim of enhancement in the bilateral cerebral or cerebellar hemispheres and brainstem, suggesting possible demyelination (Figure 2).

Figure 1. Index case forehead showing facial warts

Table 1. Relevant investigations of the case

Serial no.	Investigation	Values/report
1	HB/TLC/ESR	13.5 g/7,780/15 mm in the 1st h
2	Antineutrophil antibody	Negative
3	HBsAg, HCV (using ELISA), HIV (using ELISA), and COVID-19 (using RT-PCR)	Negative
4	Visual evoked potentials	Bilateral P100 latencies showed normal values
5	Brainstem auditory evoked response	Bilateral I – III, III – V, and I – V inter-peak latencies showed normal values
6	Nerve conduction studies of all four limbs	Normal
7	CSF examination	CSF analysis revealed a leucocyte count of 6 cells/µL (3 – 5 cells) with 44% neutrophils, a protein level of 15.5 mg/dL (normal range: 15 – 45 mg/dL), and a glucose level of 60 mg/dL, with concomitant blood serum glucose of 90 mg/dL, a negative result for CSF analysis of oligoclonal bands using isoelectric focusing, a negative result for CSF analysis of AFB, gene expert, and HSV-PCR. India ink and CSF analysis of culture sensitivity was sterile.
8	Anti-aquaporin-4 (NMO) antibody	Negative
9	Myelin oligodendrocyte glycoprotein antibody	Negative

Abbreviations: AFB: Acid-fast bacilli; CSF: Cerebrospinal fluid; ELISA: Enzyme-linked immunosorbent assay; ESR: Erythrocyte sedimentation rate; HB: Hemoglobin; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HSV-PCR: Herpes simplex virus polymerase chain reaction; NMO: Neuromyelitis optica; RT-PCR: Reverse transcription polymerase chain reaction; TLC: Total leukocyte count.

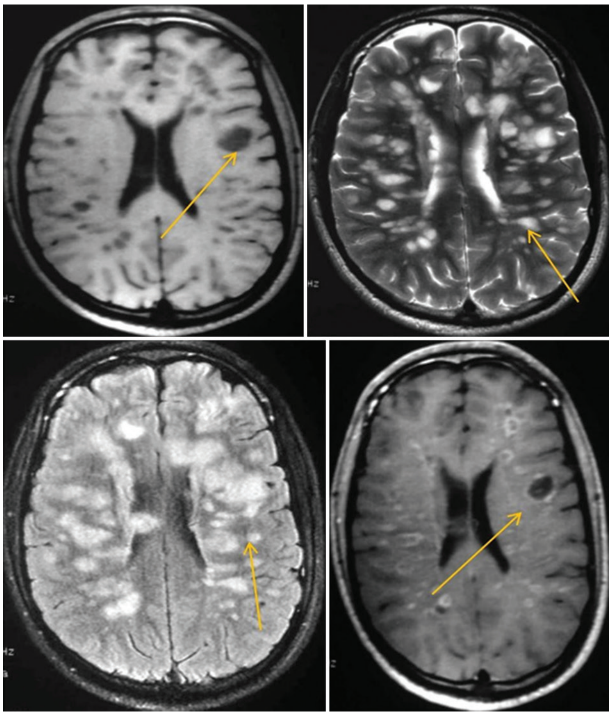

Figure 2. The brain MRI revealed multiple discrete and confluent altered SI lesions with a peripheral rim of enhancement in both bilateral cerebral hemispheres (indicated by yellow arrows)
Abbreviations: MRI: Magnetic resonance imaging; SI: Signal intensity.

Due to the typical clinical history, temporal association with MMR vaccination, and neuro-imaging findings, a demyelinating illness, such as neuromyelitis optica spectrum disorder, myelin oligodendrocyte glycoprotein antibody disease, or ADEM was considered. Multiple sclerosis (MS) was also considered; however, there was no dissemination in time, and the CSF analysis was negative for oligoclonal bands, making it less likely to be considered. Based on the clinical, laboratory, and radiology findings, the patient's profile aligns with a diagnosis of monophasic ADEM. Hence, a diagnosis of ADEM was made, and the patient was treated accordingly with intravenous steroids (one gram of methylprednisolone daily for 5 days), followed by oral steroids in tapering dosages over 6 weeks.

The patient's condition improved during the hospital stay, and the patient was discharged in a favorable condition, with improvements in sensorium, speech, and motor functions. To further improve his condition, the patient continued treatment with tapering doses of oral steroids. After a 3-month follow-up visit, the patient's condition improved with a normal MoCA scale, and no motor deficits were observed during the examination. Follow-up imaging revealed scans with complete resolution of lesions in the brain MRI.

3. Discussion

ADEM is a monophasic, demyelinating disease usually involving cerebral hemispheres, optic nerves, cerebellum, brainstem, and spinal cord. ADEM is triggered by infectious or other environmental agents and is caused by immune system dysregulation, leading to widespread demyelination. MS is an important differential diagnosis of ADEM, which usually presents with optic neuritis, brainstem syndrome, or sub-acute myelopathy. However, optic neuritis in ADEM mainly occurs simultaneously and bilaterally, whereas in MS, it is usually unilateral. In addition, other characteristics of ADEM – such as fever, meningism, and behavioral symptoms – do not typically occur in MS.[4]

ADEM can occur in all age groups. However, it primarily affects children with no gender predominance and usually occurs after a week following a viral infection or vaccination.[5] An abnormal, exaggerated immune response to an infection or extraneous trigger is most commonly implicated in the pathology of this condition. Post-infection ADEM cases are usually associated with MMR, varicella zoster, Epstein-Barr, cytomegalovirus, herpes simplex, hepatitis A, influenza, and enterovirus infections. In addition, <5% of ADEM cases occur following immunization for rabies, hepatitis B, influenza, Japanese B encephalitis, diphtheria, pertussis, tetanus, MMR, pneumococcal, polio, smallpox, and varicella, suggesting that the pathogenesis of ADEM is immunologically mediated.[6,7] Rabies, MMR, influenza, and COVID-19 vaccination have been associated with vaccination-induced immune dysregulation leading to ADEM. Initially, post-vaccination ADEM was thought to be due to the vaccine's viral component but later it was hypothesized that it is due to contamination in the CNS tissue where the vaccine was propagated.[2,3] The significant reduction in ADEM cases following the development of recombinant protein-based vaccines and the results of experimental allergic encephalomyelitis studies further support this theory. However, the occurrence of ADEM following vaccination with vaccines manufactured without the involvement of neural tissue does not fully support this theory. Immune dysregulation caused by extrinsic agents is the central pathway for the development of ADEM.[5] Pathologically, there is a presence of perivenous inflammation and demyelination surrounding small vessels in both the grey and white matter. However, the lesions could be vasculitic and hemorrhagic, leading to a devastating clinical course in patients with acute hemorrhagic leukoencephalitis, a rapidly progressing form of ADEM.[2] High doses of corticosteroids, intravenous immunoglobulins, and plasmapheresis constitute the pillars of treatment in ADEM.[2] Here, we describe a case of a young male who suffered from ADEM following MMR vaccination. Non-neural MMR vaccination has been shown to be involved in ADEM cases; however, ADEM following intralesional instillation of MMR vaccination for treating warts is rare. The association between severe acute respiratory syndrome coronavirus 2 vaccination and ADEM has emerged in recent years. However, the exact incidence is yet to be deciphered from population-based studies.[8]

Our patient had a history of MMR vaccination 1 month before symptom onset. The patient presented with an acute onset of a progressive neurological syndrome affecting the pyramidal tracts, cerebellum, and bilateral cerebellar hemispheres. Evidence supporting the diagnosis of ADEM includes a prior history of MMR vaccination, the presence of multiple neurologic symptoms, MRI findings showing typical demyelinating lesions with no other etiology to explain these events, and a positive response to intravenous methylprednisolone. However, the literature suggests that MMR vaccination-induced ADEM associated with myelin oligodendrocyte glycoprotein antibody-positive requires prolonged immunomodulation due to the high probability of relapse.[9]

The literature search found that CSF can be normal in about one-third of such patients.[10] During a 3-month follow-up visit, the patient never developed any new neurological symptoms and showed improvement in his condition. The patient's clinical presentation was likely due to post-vaccination ADEM, as no other etiology could explain these events.

4. Conclusion

In conclusion, ADEM is a multifocal illness characterized by various neurological symptoms and signs. A recent vaccination history should be considered when evaluating patients with ADEM or other demyelinating diseases. A high index of suspicion is essential in patients with monophasic or polyphasic demyelinating disorders to identify the cause or inciting event precipitating central or peripheral nervous system demyelination. Factors disrupting or altering the immune system, such as vaccination and infection, can trigger central or peripheral nervous system demyelination. Steroid treatment remains the mainstay of ADEM treatment.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

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Ethics approval and consent to participate

Consent was obtained from the patient.

Consent for publication

Consent for publication was obtained from the patient.

Availability of data

Data are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

Microbes & Immunity

COMMUNICATION

C_7Mab-2: A novel monoclonal antibody against mouse CCR7 established by immunization of the extracellular loop domain

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Abstract

The chemokine receptors possess seven transmembrane helices connected by an extracellular N-terminal region, three extracellular loops (ECL1–3), three intracellular loops, and an intracellular C-terminal region. Specific monoclonal antibodies (mAbs) against chemokine receptors for flow cytometry have been developed using Cell-Based Immunization and Screening, and the N-terminal peptide immunization methods. However, there are few reports on the establishment of anti-chemokine receptor mAbs through immunization with ECL peptides. Here, an anti-mouse C–C chemokine receptor type 7 (mCCR7) mAb, C_7Mab-2 (rat immunoglobulin G_{2b}, kappa), was established through immunization with the ECL3 peptide. C_7Mab-2 demonstrated reactivity to mCCR7-overexpressed Chinese hamster ovary-K1 (CHO/mCCR7) cells in flow cytometry, which was inhibited by the ECL3 peptide. C_7Mab-2 did not show cross-reactivity with other mouse CC, CXC, CX3C, and XC chemokine receptors. The dissociation constant value of C_7Mab-2 was determined to be 2.8×10^{-9} M for CHO/mCCR7 cells. Furthermore, C_7Mab-2 detected mCCR7 in immunohistochemistry. This strategy could accelerate the development of novel chemokine receptor mAbs with high affinity and specificity.

Keywords: Mouse C–C chemokine receptor type 7; Monoclonal antibody; Extracellular loop; Peptide immunization; Flow cytometry; Immunohistochemistry

1. Introduction

Chemotactic trafficking is regulated by G protein-coupled receptors (GPCRs) on immune cells.[1] With four conserved cysteine residues forming two disulfide bonds, chemokines are cytokines that guide immune cells to the appropriate locations. They can be classified into four subfamilies: CC, CXC, XC, and CX3C, based on the number and position of cysteine residues at the N-terminus.[2] The biological effects of chemokines are mediated through a family of GPCRs. The chemokine receptors possess seven transmembrane helices connected by an extracellular N-terminal region, three extracellular loops (ECL1–3), three intracellular loops, and an intracellular C-terminal region.[3] Several disulfide bonds connect the N-terminus to ECL3 and ECL1 to ECL2.[4] The binding of chemokines to their specific receptors induces conformational changes and activates the chemokine signaling pathways to regulate the migration, integration, adhesion, and proliferation of immune cells.[5,6]

C–C chemokine receptor 7 (CCR7) is expressed on naïve T and B cells, natural killer cells, regulatory T cells, central memory T cells, dendritic cells, and tumor cells.[7] C–C motif chemokine ligands (CCL) 19 and 21 are high-affinity CCR7 ligands that promote the migration of CCR7-positive cells to secondary lymphoid organs, including the thymus, spleen, and lymph nodes.[8-12] Genome-wide association studies have revealed a relationship between CCL21/CCR7 and disease severity in patients with systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, or asthma.[7] Disrupting the CCL21/CCR7 interaction with monoclonal antibodies (mAbs) or inhibitors suppresses the migration of CCR7-positive cells to inflammatory sites, thereby inhibiting disease progression.

Metastasis to the lymph node is an important predictive factor for patients with cancer.[13] The elevated expression of CCR7 is associated with lymph node metastasis in many solid tumors, including esophageal,[14] gastric,[15] colorectal,[16] pancreatic,[17] thyroid,[18] oral,[19] and non-melanoma skin cancers.[20] CCR7, but not others, specifically drives cancer cell homing to lymph nodes and other secondary lymphoid organs, where the ligands CCL19 and CCL21 are constitutively expressed by stroma cells.[21] Therefore, developing specific mAbs against mouse CCR7 (mCCR7) is essential to targeting the CCR7-expressing cells in preclinical mouse disease models.

Specific mAbs against various chemokine receptors have been developed, including mouse CXCR1 (mCXCR1; clone Cx_1Mab-1),[22] mouse CXCR3 (mCXCR3; clone Cx_3Mab-4),[23] mouse CXCR4 (mCXCR4; clone Cx_4Mab-1),[24] mouse CCR1 (mCCR1; clone C_1Mab-6),[25] mouse CCR3 (mCCR3; clones C_3Mab-2, C_3Mab-3, and C_3Mab-4),[26] mouse CCR5 (mCCR5; clone C_5Mab-2),[27] mCCR7 (clone C_7Mab-7),[28] and mouse CCR8 (mCCR8; clones C_8Mab-1, C_8Mab-2, and C_8Mab-3)[29] using the Cell-Based Immunization and Screening (CBIS) method. This method involves immunization with antigen-overexpressed cells and flow cytometry-based high-throughput screening. Furthermore, specific mAbs against mouse CCR2 (mCCR2; clone C_2Mab-6),[30] mCCR3 (clones C_3Mab-6 and C_3Mab-7),[31] mouse CCR4 (mCCR4; clone C_4Mab-1),[32] mouse CCR6 (mCCR6; clone C_6Mab-13),[33] mouse CCR9 (mCCR9; clone C_9Mab-24),[34] mouse CXCR5 (mCXCR5; clone Cx_5Mab-3),[35] and mouse CXCR6 (mCXCR6; clone Cx_6Mab-1)[36] have also been established using the N-terminal peptide immunization. In contrast, there are few reports on the establishment of anti-chemokine receptor mAbs by immunization with ECL peptides.

2. Materials and methods

2.1. Cell lines

Mouse myeloma cell line P3X63Ag8.U1 (P3U1) and Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (USA). The mCCR7-overexpressed CHO-K1 (CHO/mCCR7) cell line was previously established.[28] Stable transfectants of the following chemokine receptors were previously established:[35] CHO/mCCR1, CHO/mCCR2, CHO/mCCR3, CHO/PA-mCCR4, CHO/mCCR5, CHO/PA-mCCR6, CHO/mCCR8, CHO/mCCR9, CHO/PA-mCCR10, CHO/mCXCR1, CHO/mCXCR2, CHO/mCXCR3, CHO/mCXCR4, CHO/mCXCR5, CHO/mCXCR6, CHO/mCX3CR1, and CHO/mXCR1. These cells were maintained as described previously.[35]

2.2. Peptides

Eurofins Genomics KK (Japan) synthesized partial sequences of the ECLs of mCCR7 as follows: mCCR7-1 (SEAKSWIFGVYLC), mCCR7-2 (ELLYSGLQKNSGEDTLRC), and mCCR7-3 (CETSKQLNIAYDVTYS). Subsequently, the keyhole limpet hemocyanin (KLH) was conjugated to the N-terminus of mCCR7-3 or the C-terminus of mCCR7-1 and mCCR7-2.

2.3. Hybridoma production

The Animal Care and Use Committee of Tohoku University approved the animal study (Permit number: 2022MdA-001). Three 6-week-old female Sprague–Dawley (SD) rats (CLEA Japan, Japan) were intraperitoneally immunized with 100 μg of the KLH-conjugated mCCR7 peptides mixed with 2% Alhydrogel adjuvant (InvivoGen, USA). Hybridomas were generated as described previously.[36]

2.4. Enzyme-linked immunosorbent assay

The synthesized mCCR7 peptides (1 μg/mL) were immobilized on immunoplates. After blocking with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Nacalai Tesque, Inc., Japan) and 1% bovine serum albumin (BSA), the plates were treated with hybridoma supernatants. Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Japan). Optical density was detected at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).[36]

2.5. Flow cytometry

Cells were incubated with C_7Mab-2 in a blocking buffer (0.1% BSA in PBS) at 4°C for 30 min. For the peptide inhibition assay, C_7Mab-2 (2 μg/mL) was pre-incubated with dimethyl sulfoxide (DMSO) or 1 μg/mL mCCR7-3 peptide for 15 min, and then incubated with the cells for 30 min at 4°C. Data were collected using the SA3800 Cell Analyzer (Sony Biotechnology, Japan) and analyzed as described previously.[36]

2.6. Determination of dissociation constant

Cells were treated with C_7Mab-2 (0.006 to 100 μg/mL). The cells were then incubated with Alexa Fluor 488-conjugated anti-rat immunoglobulin G (IgG) at 4°C for 30 min. The dissociation constant (K_D) of C_7Mab-2 for CHO/mCCR7 was determined using GraphPad PRISM 6 (USA), as described previously.[36]

2.7. Immunohistochemical analysis

The preparation of cell sections and antigen retrieval were performed as described previously.[28] After blocking, the sections were treated with C_7Mab-2 (20 μg/mL) for 1 h. For the peptide inhibition assay, C_7Mab-2 (20 μg/mL) was pre-incubated with 2 μg/mL of the mCCR7-3 peptide or DMSO for 15 min and then incubated with the cell blocks for 1. Color development was achieved as described previously.[28]

3. Results

3.1. Development of an anti-mouse CCR7 monoclonal antibody, C_7Mab-2, by immunization with three extracellular loop peptides

Three SD rats were immunized with the KLH-conjugated mCCR7 peptides, respectively (Figure 1A). Hybridomas were produced by fusion with P3U1 cells (Figure 1B). Then, positive wells for each unconjugated mCCR7 peptide were selected using enzyme-linked immunosorbent assay (ELISA) (Figure 1C). The ELISA screening identified 11 of 1,534 wells for mCCR7-1 (0.7%), 78 of 1,534 wells for mCCR7-2 (5.1%), and 93 of 1,438 wells for mCCR7-3 (6.5%) that strongly reacted with the respective mCCR7 peptide. Second screenings were subsequently performed using flow cytometry (Figure 1C). Among the 93 ELISA-positive wells for mCCR7-3, 11 wells showed reactivity with CHO/mCCR7 cells but not with CHO-K1 cells. No flow cytometry-positive wells were obtained from hybridomas derived from mCCR7-1–KLH- and mCCR7-2–KLH-immunized rat. The anti-mCCR7 mAb-producing hybridomas derived from KLH-mCCR7-3-immunized rat were further cloned by limiting dilution, and C_7Mab-2 (rat IgG_{2b}, kappa) was finally established (Figure 1D).

3.2. Flow cytometry using C_7Mab-2

Flow cytometry was performed using C_7Mab-2 against CHO/mCCR7 cells and CHO-K1 cells. C_7Mab-2 recognized CHO/mCCR7 cells at concentrations ranging from 0.01 to 10 μg/mL, whereas CHO-K1 cells were not recognized even at 10 μg/mL (Figure 2A). A peptide-blocking assay demonstrated that C_7Mab-2 reacted with CHO/mCCR7 cells, and this reactivity was completely neutralized by the mCCR7-3 peptide (Figure 2B).

3.3. Reactivity of C_7Mab-2 to various chemokine receptor-expressing CHO-K1 cells

Anti-mouse CC, CXC, CX3C, and XC chemokine receptor mAbs have previously been established and evaluated using CHO-K1 cells expressing these receptors.[35] Among 18 CHO-K1 cells expressing mouse CC, CXC, CX3C, and XC chemokine receptors, C_7Mab-2 recognized only CHO/mCCR7 cells, but not others (Figure 3).

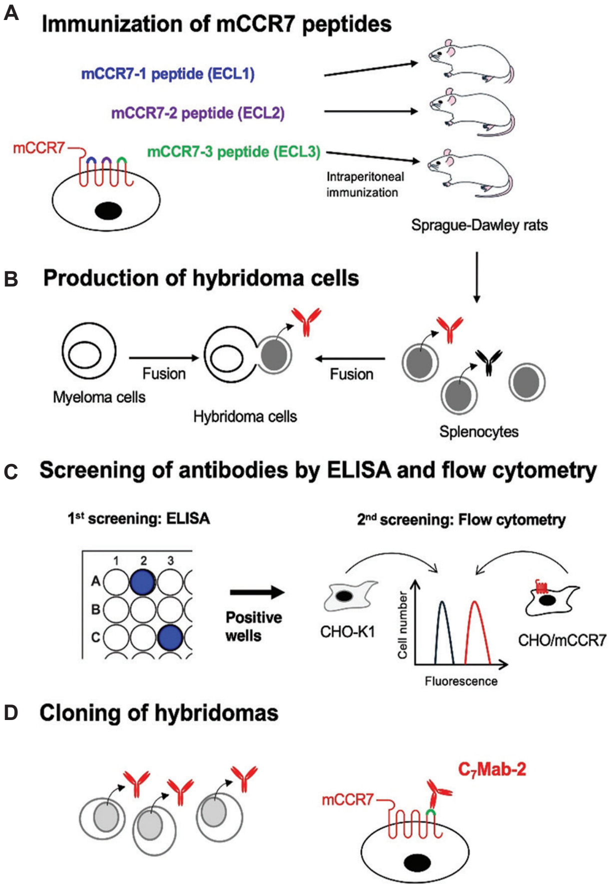

Figure 1. Schematic representation of anti-mCCR7 mAb production. (A) The KLH-conjugated mCCR7 ECL peptides (mCCR7-1, mCCR7-2, and mCCR7-3) were immunized into Sprague–Dawley rats. (B) The spleen cells were fused with P3U1 cells. (C) To select anti-mCCR7 mAb-producing hybridomas, the supernatants were screened by ELISA and flow cytometry using CHO-K1 cells and CHO/mCCR7 cells. (D) The anti-mCCR7 mAb-producing hybridomas from KLH-mCCR7-3-immunized rat were further cloned by limiting dilution, and C_7Mab-2 (rat IgG_{2b}, kappa) was finally established.

Abbreviations: CHO-K1: Chinese hamster ovary-K1; ECL: Extracellular loop; ELISA: Enzyme-linked immunosorbent assay; KLH: Keyhole limpet hemocyanin; mAb: Monoclonal antibody; mCCR7: Mouse C–C chemokine receptor type 7.

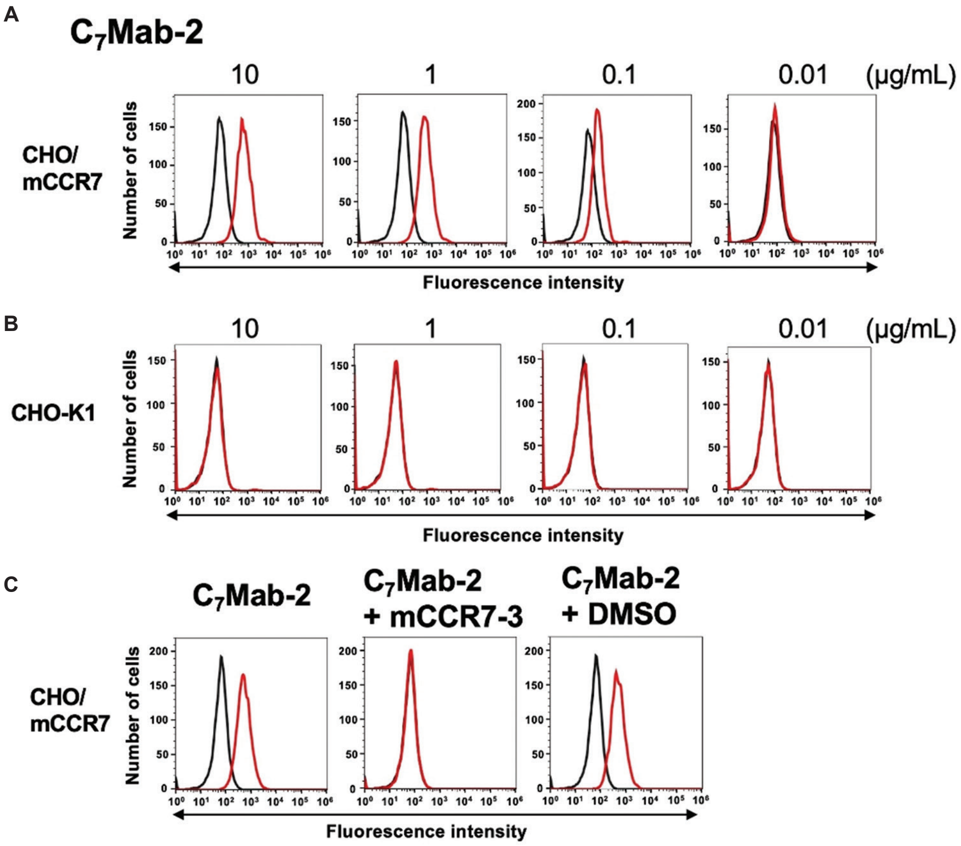

Figure 2. Flow cytometry analysis of C$_7$Mab-2 against CHO/mCCR7 cells and CHO-K1 cells. (A and B) CHO/mCCR7 cells (A) and CHO-K1 cells (B) were treated with 0.01, 0.1, 1, and 10 µg/mL of C$_7$Mab-2 (red line). The mAb-treated cells were further incubated with Alexa Fluor 488-conjugated anti-rat IgG. The black line represents the negative control (blocking buffer). The dose-dependent reactivities of C$_7$Mab-2 to CHO/mCCR7 cells were investigated in at least three independent experiments. (C) Peptide-blocking assay using C$_7$Mab-2 with mCCR7-3 peptide. C$_7$Mab-2 (2 µg/mL) with mCCR7-3 (1 µg/mL, red line) or control (1% DMSO in blocking buffer, red line) was reacted with CHO/mCCR7 for 30 min at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. The black line represents the negative control (blocking buffer).

Abbreviations: CHO-K1: Chinese hamster ovary-K1; DMSO: Dimethyl sulfoxide; IgG: Immunoglobulin G; mCCR7: Mouse C–C chemokine receptor type 7.

3.4. Dissociation constant of C$_7$Mab-2

The binding affinity of C$_7$Mab-2 was evaluated using flow cytometry. The K_D of C$_7$Mab-2 for CHO/mCCR7 cells was $2.8 \pm 0.3 \times 10^{-9}$ M (Figure 4).

3.5. Immunohistochemistry using C$_7$Mab-2

To examine the suitability of C$_7$Mab-2 for immunohistochemistry, the sections of CHO-K1 cells and CHO/mCCR7 cells were stained with C$_7$Mab-2. The membranous and cytoplasmic staining were observed in CHO/mCCR7 cells (Figure 5A), but not in CHO-K1 cells (Figure 5B). Furthermore, this reactivity was completely neutralized by the mCCR7-3 peptide (Figure 5C and D).

4. Discussion

This study established an anti-mCCR7 mAb, C$_7$Mab-2, by immunizing with the ECL3 peptide. C$_7$Mab-2 can be applied to flow cytometry (Figure 2) and immunohistochemistry (Figure 5) to detect mCCR7-positive cells. The reactivity (Figure 2) and affinity (Figure 4) of C$_7$Mab-2 are similar to another anti-mCCR7 mAb, C$_7$Mab-7, which was established through the CBIS method.[28] It is critical to investigate whether C$_7$Mab-2 can detect endogenous mCCR7 using cells from secondary lymphoid organs, including the lymph node, thymus, and spleen. Furthermore, this study also confirmed that C$_7$Mab-2 recognizes mCCR7, but not other CC, CXC, CX3C, and XC chemokine receptors (Figure 3). The ECL1–3 peptides were immunized, and the ELISA-positive wells in hybridomas were obtained from each peptide-immunized rat. However, it was unable to obtain flow cytometry-positive wells in hybridomas derived from ECL1 and ECL2 peptide-immunized rats. Among hybridomas from the ECL3 peptide-immunized rat, only 10% of ELISA-positive supernatants recognized CHO/mCCR7 in flow cytometry, indicating that conformational changes and modifications, including glycosylation[37] or disulfide bond formation,[4] would restrict the recognition

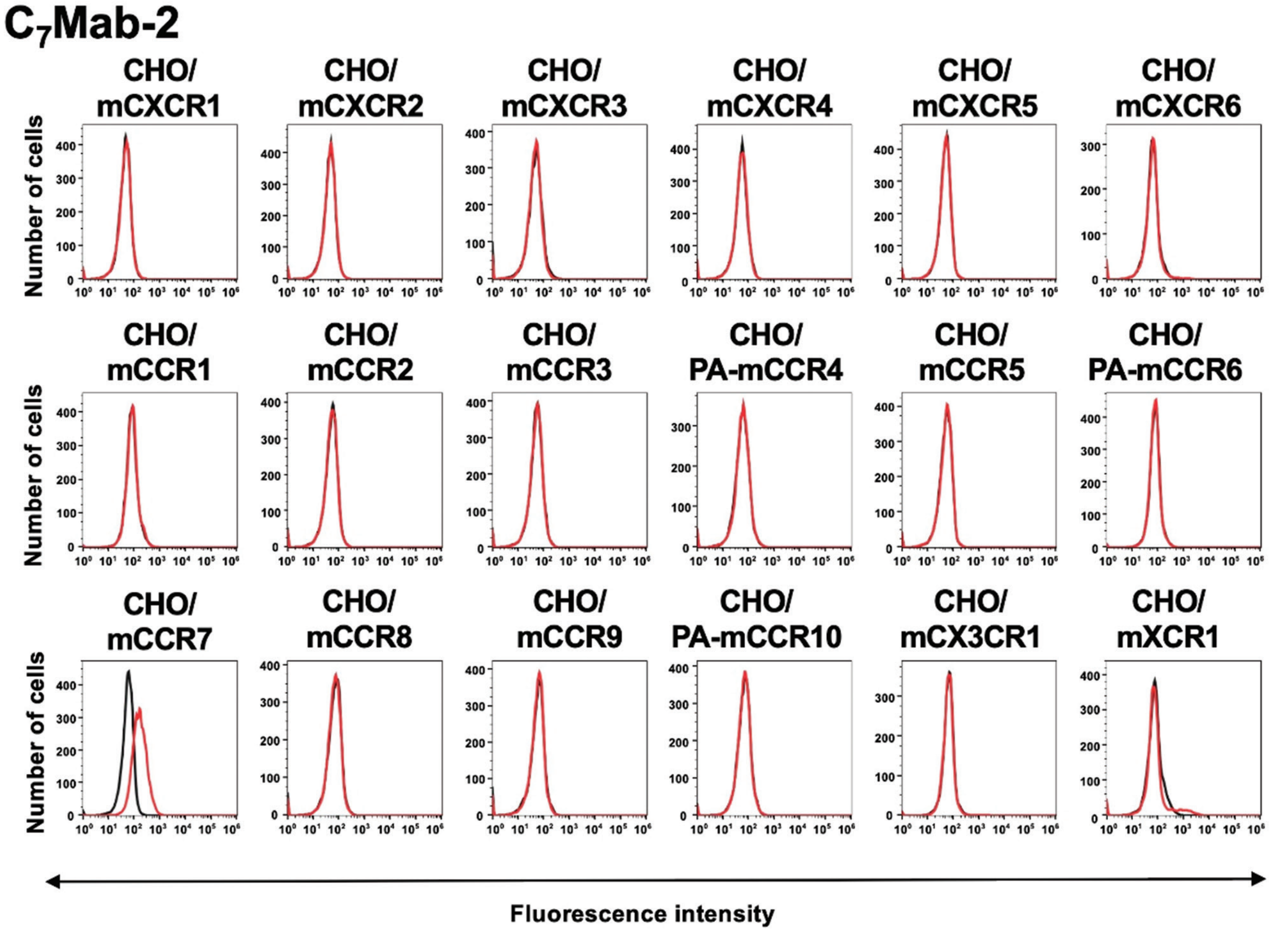

Figure 3. Flow cytometry analysis of C_7Mab-2 in CC, CXC, CX3C, and XC chemokine receptor-expressing CHO-K1 cells. Eighteen mouse CC, CXC, CX3C, and XC chemokine receptor-expressing CHO-K1 cells were treated with 1 μg/mL of C_7Mab-2 (red line) or control blocking buffer (black line), followed by treatment with Alexa fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 cell analyzer. Each receptor expression was previously confirmed by flow cytometry.

Abbreviations: CHO-K1: Chinese hamster ovary-K1; IgG: Immunoglobulin G; mCCR: Mouse C–C chemokine receptor; mCXCR: Mouse C–X–C motif chemokine receptor; mXCR: Mouse X–C motif chemokine receptor.

of mAbs. Future investigation should involve the determination of the critical epitope of C_7Mab-2, which may help the understanding of the recognition mechanism of mCCR7. Notably, the Cx_6Mab-1 epitope was previously identified using 1× and 2× alanine scanning methods.[38]

Structural information on chemokine receptors is required to develop drugs that fulfill the requirements. Much effort has been made to determine the structures in complex with either synthetic ligands[39-41] or native chemokines[42,43] by X-ray crystallography. The cryo-electron microscopy (cryo-EM) has reportedly determined the structures of several chemokine receptor-ligand complexes.[44-47] Although the structure of the CCR7–ligand complex has not been determined, the crystal structure of CCR7 with Cmp2105, an intracellular allosteric CCR7 receptor antagonist, was previously determined.[48] Recently, the structures of chemokine receptor–mAb complexes have been determined by means of the cryo-EM, providing a detailed structural and mechanistic framework of chemokine receptor activation and inhibition.[49] Since C_7Mab-2 is known to recognize ECL3 of mCCR7, it could help the structural analysis of mCCR7 in future studies.

Several *in vitro* and *in vivo* preclinical tumor models have demonstrated that increased CCR7 expression promotes tumor growth and metastasis, whereas reduced CCR7 expression suppresses these processes.[50] For example, in an orthotopic model, mCCR7-overexpressing mouse mammary tumor cells (PyVmT) demonstrated enhanced metastasis to the lymph nodes. In contrast, the control cells did not migrate to the lymph nodes but metastasized to the lungs. Additionally, mCCR7 overexpression significantly increased tumor growth in

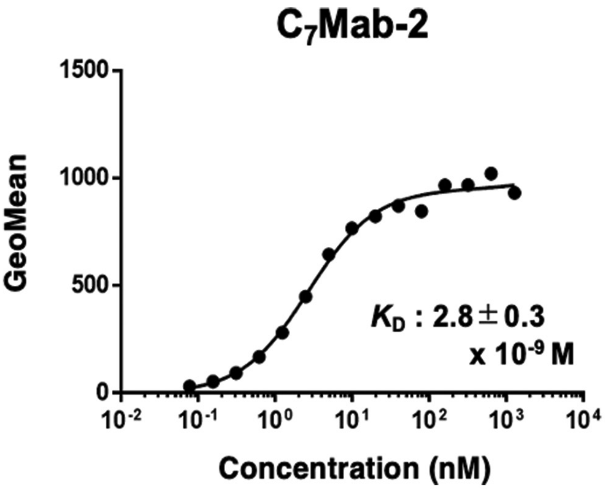

Figure 4. Binding affinity of C_7Mab-2. CHO/mCCR7 cells were incubated with serially diluted C_7Mab-2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 cell analyzer, and the K_D was calculated using GraphPad PRISM 6. The representative results were shown. Three independent experiments were conducted to determine the average K_D (mean ± standard deviation). Abbreviations: CHO: Chinese hamster ovary; IgG: Immunoglobulin G; K_D: Dissociation constant; mCCR7: Mouse C–C chemokine receptor type 7.

PyVmT cells both *in vitro* and *in vivo* compared to the control.[51] Furthermore, in a mouse melanoma model, mCCR7-overexpressed B16 melanoma cells exhibited a significantly higher rate of lymph node metastasis than control cells, although the primary tumor size remained unchanged.[52] To target the mCCR7-positive tumors *in vivo*, C_7Mab-2 (rat IgG_{2b}) should be converted to mouse IgG_{2a} mAb. Through the determination of heavy-chain variable domain and light-chain variable domain sequences of C_7Mab-2, a large amount of recombinant mAbs can be generated for use in preclinical studies.

In a syngeneic mouse model of oral cancers, the growth of tumors was significantly decreased in mCCR7-knockout (KO) mice.[53] Single-cell RNA sequencing analysis showed that the M2 macrophage proportion in the KO group was lower compared to the control.[53] mCCR7 stimulates the polarization of M2 macrophages, which promotes the migration, invasion, and proliferation of tumor cells.[53] Therefore, the depletion of mCCR7-expressing cells by anti-mCCR7 mAbs, such as class-switched and defucosylated mouse IgG_{2a}-type C_7Mab-2, could help investigate the effect of mCCR7-expressing cell depletion on tumor growth.

5. Conclusion

An anti-mCCR7 mAb, C_7Mab-2, was established by immunization with the ECL3 peptide. C_7Mab-2 can be used in flow cytometry and immunohistochemistry experiments and is expected to provide proof of concept in preclinical studies.

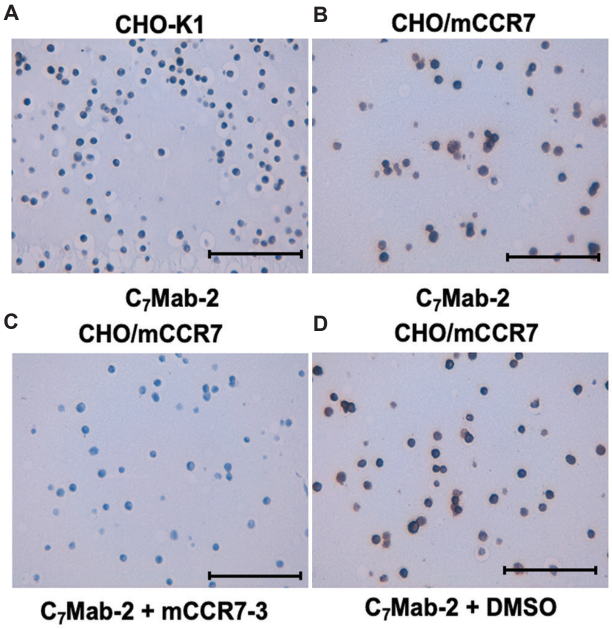

Figure 5. Immunohistochemistry of paraffin-embedded cell sections of CHO/mCCR7 cells and CHO-K1 cells using C_7Mab-2. (A and B) Sections of CHO-K1 cells (A) and CHO/mCCR7 cells (B) were treated with 20 μg/mL of C_7Mab-2, followed by treatment with Histofine Simple Stain Mouse MAX PO (Rat). (C and D) Peptide-blocking assay using C_7Mab-2 with mCCR7-3 peptide. C_7Mab-2 (20 μg/mL) with mCCR7-3 (2 μg/mL, C) or control (1% DMSO in blocking buffer, D) were reacted with the sections of CHO/mCCR7 cells, followed by treatment with Histofine Simple Stain Mouse MAX PO (rat). Color was developed using DAB, and counterstaining was performed using hematoxylin. Scale bar: 100 μm. Magnification: x200.
Abbreviations: CHO: Chinese hamster ovary; DAB: 3,3'-diaminobenzidine tetrahydrochloride; DMSO: Dimethyl sulfoxide; mCCR7: Mouse C–C chemokine receptor type 7.

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Conflict of interest

The authors declare that they have no competing interest.

Author contributions

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Writing–original draft: Hiroyuki Suzuki
Writing–review & editing: Yukinari Kato

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Consent for publication

Not applicable.

Availability of data

The data of this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (doi: 10.20944/preprints202503.0581.v1).

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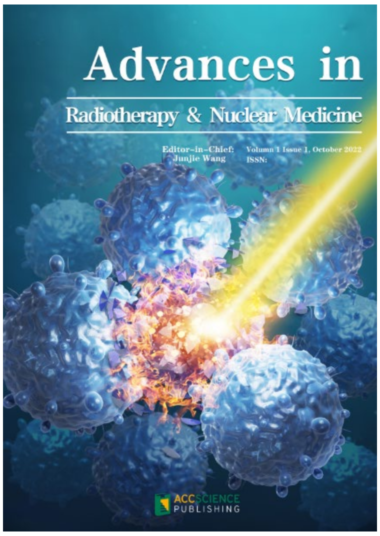
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