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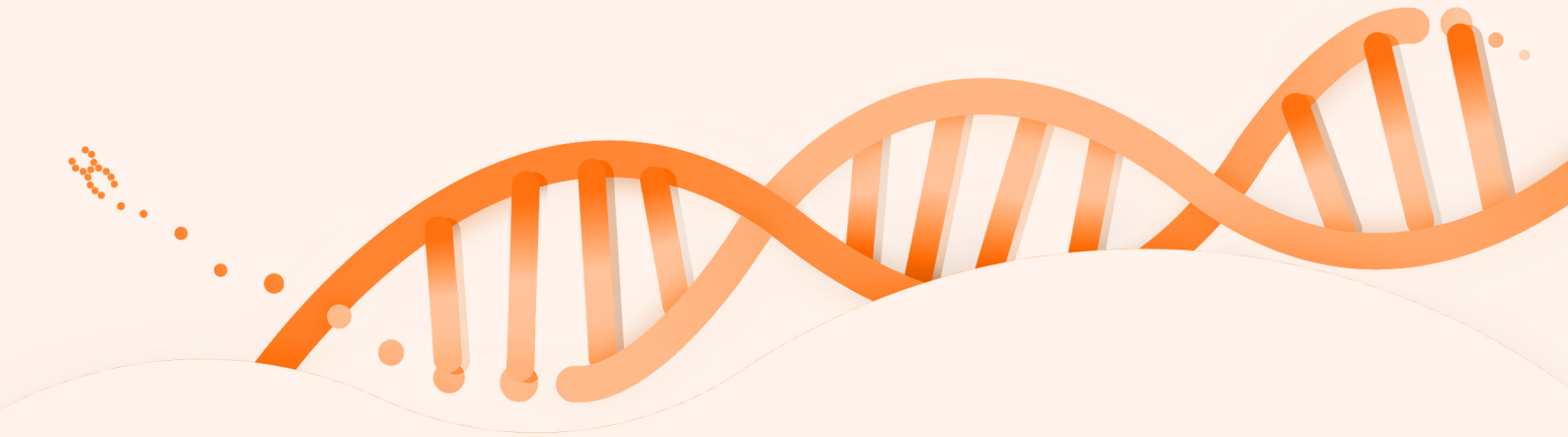

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Nanoplatforms in Gastric Ulcer Therapy: A Narrative Review

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ABSTRACT

Introduction and Aim: Gastric ulcer is a prevalent gastrointestinal disease caused by an imbalance between damaging and protective factors such as gastric acid, oxidative stress, chronic inflammation, and *Helicobacter pylori* infection and protective mucosal mechanisms. Limitations of conventional therapies, including drug resistance, systemic side effects, and suboptimal efficacy in certain patients, underscore the need for novel therapeutic strategies. Nanoparticles, as advanced drug delivery systems, offer promising approaches for targeted gastric mucosal therapy by enhancing drug stability, enabling site-specific delivery, and minimizing adverse effects. This review aims to analyze the therapeutic applications of nanoplatforms in the management of gastric ulcers and associated infections.

Methods: Relevant studies were identified from PubMed, Scopus, and Web of Science for this narrative review from 2010 to 2025. Standardized keywords included "gastric ulcer," "nanoparticles," "targeted drug delivery," "*Helicobacter pylori*," and "mucosal drug delivery systems." Eligible studies comprised preclinical investigations, animal models, and relevant review articles. Data were extracted and analyzed qualitatively and thematically.

Results: The findings indicate that nanocarriers including poly (lactic-co-glycolic acid) (PLGA), chitosan, silver, gold, selenium nanoparticles, and plant-based formulations—enhance drug bioavailability, enable controlled release, improve mucoadhesion, and achieve targeted gastric delivery. These properties collectively facilitate accelerated mucosal healing and tissue regeneration. Key mechanisms include inhibition of inflammatory pathways such as NF-κB, reduction of pro-inflammatory cytokines (e.g., TNF-α, IL-6), attenuation of oxidative stress, enhancement of antioxidant defenses (SOD, CAT, NRF2), and antibacterial activity against *H. pylori*. Conversely, certain nanoparticles, such as titanium dioxide, may provoke local inflammation and mucosal injury, highlighting the importance of nanoparticle type and physicochemical properties.

Conclusion: Targeted nanoparticles and nanocarriers, through controlled drug release, mucoadhesion, anti-inflammatory effects, and antibacterial activity, represent a novel and effective approach for gastric ulcer therapy and *H. pylori* eradication. However, heterogeneity in biological responses and potential toxicity necessitate further extensive preclinical studies and rigorously designed clinical trials to ensure the safety and efficacy of these nanomedicine strategies.

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INTRODUCTION

Gastric ulcers represent a frequent pathological condition affecting the digestive system, arising from disruption of the gastric mucosal layer and an

imbalance between aggressive and protective factors (1). Clinically, it can manifest as epigastric pain, gastrointestinal bleeding, nausea, vomiting, and, in severe cases, perforation of the gastric wall, leading to life-threatening complications (2). The global burden of gastric ulcer is driven by a combination of infectious, pharmacological, and lifestyle-related factors, particularly *H. pylori*, NSAIDs, stress, alcohol, smoking, and diet. Moreover, the growing antibiotic resistance in *H. pylori* has complicated the management of this condition (1, 2).

Gastric ulcerogenesis arises from the interaction between damaging stimuli and impaired mucosal protection. Acid-peptic activity, microbial infection, oxidative stress, and inflammatory pathways accelerate tissue injury, whereas endogenous defense systems preserve gastric barrier function (3, 4). In many patients, elevated oxidative stress and ROS production lead to cellular damage, lipid peroxidation, and epithelial cell destruction (3, 4). Concurrently, upregulation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 exacerbates mucosal injury, highlighting the need for therapies that not only suppress gastric acid but also mitigate inflammation, oxidative stress, and infection (5).

Conventional treatment strategies for gastric ulcer primarily include proton pump inhibitors (PPIs), H2 receptor antagonists, antibiotics, mucosal protectants, and antacids (6). Although these therapies are effective for many patients, they are limited by low bioavailability, acid-mediated drug degradation, frequent dosing requirements, systemic side effects, disease recurrence, and antibiotic resistance (7). Additionally, several therapeutic agents cannot remain at the ulcer site for prolonged periods, and targeted delivery to the damaged mucosa remains challenging (8). Therefore, the development of novel drug delivery systems capable of enhancing drug stability, therapeutic efficacy, and minimizing adverse effects is imperative (7, 8).

Nanotechnology-based approaches have attracted increasing attention for their potential in modern healthcare and therapy (9). Nanoparticles, owing to their small size, high surface area, surface functionalization potential, and ability to carry diverse therapeutic agents, have opened new avenues for gastrointestinal disease management (10). These systems can protect drugs from acidic degradation, improve bioavailability, and enable controlled and targeted drug release (10). Furthermore, certain nanoparticles possess intrinsic antibacterial, anti-inflammatory, and antioxidant properties, which can directly contribute to gastric ulcer healing (11).

Various types of nanoparticles including polymeric, lipid-based, metallic nanoparticles, liposomes, nanoemulsions, dendrimers, and nanocomposites have been investigated for gastric ulcer therapy (12). These platforms facilitate targeted delivery of PPIs, antibiotics, plant-derived compounds, antioxidants, and mucosal protectants to the ulcer site (13). Studies have demonstrated that nanoparticles enhance mucosal adhesion, improve tissue penetration, and prolong gastric residence time, thereby increasing therapeutic efficacy (12, 13). Metallic nanoparticles, such as silver, gold, and zinc oxide, exhibit potent antibacterial activity against *H. pylori* and may contribute to overcoming antibiotic resistance (13).

Beyond drug delivery, nanoparticles play a significant role in promoting gastric mucosal regeneration (14). Preclinical studies indicate that nanocarriers can accelerate tissue repair by reducing inflammation, mitigating oxidative stress, stimulating angiogenesis, and enhancing epithelial cell proliferation (15). Moreover, nanoparticles can serve as vehicles for growth factors, small RNAs, bioactive compounds, and natural products, enabling precision-targeted ulcer therapy (15). Some nanoplatforms are also responsive to gastric pH, allowing intelligent, site-specific drug release (16).

Despite these advantages, the application of nanoparticles in gastric ulcer therapy faces several challenges (17). Potential cytotoxicity, tissue accumulation, unintended immune responses, biosafety concerns, and difficulties in standardizing nanoparticle formulations represent major limitations (18). Additionally, gastric acidity, peristaltic movement, and mucosal barriers may affect the performance of nanocarrier systems. Hence, comprehensive evaluation of biocompatibility, toxicity, and stability is essential before clinical application (18).

Given the rapid advancements in nanotechnology and gastrointestinal therapeutics, a comprehensive synthesis of current evidence is increasingly necessary (19). Although numerous studies have explored the application of nanoparticles in gastric ulcer therapy, detailed insights regarding their mechanisms, benefits, limitations, and future prospects remain scattered (19). Therefore, the objective of this narrative review is to examine the therapeutic applications of nanoparticles in the management of gastric ulcer disease

METHODS

This review synthesized recent evidence on the application of nanoparticles in gastric ulcer treatment and gastrointestinal delivery systems. Publications available between 2010 and 2025 were screened using multiple scientific databases (PubMed, Scopus, Web of Science, SID, and ISC).

The search strategy was developed using a combination of specialized keywords and Boolean operators. The key terms included “Gastric Ulcer,” “Peptic Ulcer,” “Nanoparticles,” “Nanomedicine,”

"Drug Delivery," "Helicobacter pylori," "Targeted Therapy," "Nano-carriers," "Gastroretentive Systems," and "Mucosal Healing." These terms were combined using the Boolean operators AND and OR. An example of the search string was as follows: (Gastric Ulcer OR Peptic Ulcer) AND (Nanoparticles OR Nanomedicine OR Nanocarriers) AND (Drug Delivery OR *Helicobacter pylori* OR Targeted Therapy).

Eligible studies included original research articles, preclinical investigations, animal models, in vitro studies, review articles, and systematic reviews related to nanoparticle applications in gastric ulcer therapy. Inclusion criteria were: (1) publication in English, (2) availability of full-text articles, (3) direct relevance to the therapeutic application of nanoparticles in gastric ulcers, and (4) evaluation of mechanisms of action, drug delivery systems, or anti-inflammatory, antibacterial, and mucosal healing effects of nanoparticles. Exclusion criteria comprised unrelated studies, conference abstracts, letters to the editor, articles lacking reliable scientific data, and studies focusing exclusively on nanoparticle toxicity without therapeutic relevance.

Following the initial search, all retrieved records underwent a multi-stage screening process. Titles and abstracts were first reviewed, and duplicate studies were removed. Subsequently, full-text articles meeting the eligibility criteria were carefully assessed. Data extraction was performed independently using a standardized form. The extracted information included author name, year of publication, study type, animal or cell model, type of nanoparticle, drug delivery system, mechanism of action, therapeutic objectives, anti-inflammatory and antibacterial effects, main findings, and study limitations.

To enhance the methodological quality and scientific validity of the review, selected studies were further evaluated based on study design, methodological rigor, sample size, data reliability, and relevance to the research objectives. The extracted data were organized and analyzed descriptively and thematically. Findings were categorized according to nanoparticle type, drug delivery mechanisms, antibacterial activity against *Helicobacter pylori*, antioxidant properties, gastric mucosal healing effects, and advanced therapeutic platforms. Finally, the advantages, limitations, safety challenges, and future perspectives of nanoparticle-based therapies in gastric ulcer management were critically discussed.

RESULTS

The reviewed studies demonstrated that metallic nanoparticles, metal oxides, and polymeric nanoparticles particularly those prepared via green synthesis exhibited significant effects on gastric ulcer healing. These nanoplatforms primarily acted through the reduction of oxidative stress, inhibition of inflammatory pathways, and enhancement of mucosal defense mechanisms. Additionally, remarkable improvements in gastric tissue regeneration and decreased ulcer indices were reported in animal models. Drug-loaded nanocarriers further enhanced therapeutic efficacy by increasing drug stability and bioavailability, thereby significantly improving the performance of conventional treatments. Overall, the findings highlight the high potential of nanotechnology in developing novel therapies for gastric ulcer management.

The detailed results are presented in Table 1 and 2.

DISCUSSION

Gastric ulcerogenesis involves the interaction between injurious stimuli and weakened mucosal defenses, leading to gastric tissue damage (47). Although proton pump inhibitors, H2 receptor antagonists, and *Helicobacter pylori* eradication regimens have significantly improved therapeutic outcomes, challenges such as antibiotic resistance, disease recurrence, drug-related adverse effects, and reduced efficacy in certain patients persist. The findings of this review indicate that nanoparticle-based drug delivery platforms can mitigate many of these limitations by enhancing mucosal targeting, improving drug bioavailability, and providing controlled release.

A major advantage of nanoparticle carriers in gastric ulcer therapy is their ability to prolong drug residence

Table 1. Nanoparticles with Table 1. Systematic review studies on the application of nanoparticles in the prevention and treatment of gastric ulcers.

Author & Year	Nanoparticle / System	Drug / Active Agent	Study Type	Key Mechanisms	Main Outcomes	Ref.
Bahramikia & Izadi (2023)	Green-synthesized nanoparticles (metallic, oxide, polymeric)	Plant extracts / phytocompounds	Systematic review	Antioxidant and anti-inflammatory activity; inhibition of *Helicobacter pylori*	Reduced gastric mucosal damage; enhanced tissue regeneration and ulcer healing	20
Yadav et al. (2025)	Green metal and metal oxide nanoparticles	Bioactive plant compounds	Review study	Free radical scavenging; anti-inflammatory and antibacterial activity	High therapeutic efficacy with improved biocompatibility and reduced toxicity	21

Table 2. Preclinical (in vitro and in vivo) studies on nanoparticle-based therapies for gastric ulcer treatment.

Author & Year	Nanoparticle / System	Drug / Active Agent	Study Model	Key Mechanisms	Main Outcomes	Ref.
Alai & Lin (2015)	PLGA and Eudragit nanoparticles	Lansoprazole	Rat	Enhanced bioavailability and targeted delivery	92–95% ulcer healing within 7 days	22
Li et al. (2023)	Bletilla polysaccharide nanoparticles	Omeprazole	Rat	Reduction of ROS, TNF-α, and IL-6	90–95% mucosal lesion recovery	23
Zewail et al. (2024)	Lecithin–chitosan nanoparticles	Famotidine	Rat	Increased mucoadhesion and antioxidant activity	~96% gastric mucosal protection	24
Wang et al. (2025)	TiO_2 nanoparticles	No drug	Rat	Pro-inflammatory activation; mast cell stimulation	Gastric mucosal injury and gastrointestinal dysfunction	25
Alai & Lin (2014)	PLGA and Eudragit nanoparticles	Lansoprazole	In vitro / in vivo	Enhanced cellular uptake and drug transport	89–92% ulcer healing improvement	26
Albalawi & Khateeb (2025)	Glycyrrhizic acid nanoparticles	Glycyrrhizic acid	Rat	NF-κB inhibition; SIRT1/FOXO pathway modulation	Significant improvement in ulcer healing	27
El-Sheikh & Hamad (2025)	Starch nanoparticles	None	Mouse	Mucosal barrier reinforcement	Reduced inflammatory and oxidative lesions	28
Huang et al. (2021)	Chitosan–bilirubin nanoparticles	Bilirubin	In vitro / in vivo	Potent antioxidant activity; ROS scavenging	Marked reduction in acute gastric ulcers	29
Abd El Hady et al. (2019)	PLGA–diosmin nanoparticles	Diosmin	Rat	NF-κB pathway inhibition	Improved histopathological parameters	30
Zhang et al. (2025)	Curcumin–oleanolic acid nanoparticles	Curcumin	Mouse	Anti-inflammatory and antioxidant effects	Reduced fibrosis, oxidative stress, and apoptosis	31
Aman et al. (2021)	CS/STPP nanoparticles	Allantoin	Rat	Mucoadhesion and tissue regeneration	Significant reduction in ulcer area	32
Salem et al. (2018)	Silver oxide nanoparticles	Silver	Rat	TNF-α and MDA reduction	Superior efficacy compared to omeprazole	33
Kumar et al. (2022)	Cellulose-based CuO nanoparticles	Copper oxide	Rat	TNF-α and IL-1β suppression	Enhanced tissue regeneration	34
Sreelakshmy et al. (2016)	Biosynthesized silver nanoparticles	Silver	In vitro (*H. pylori*)	Direct antibacterial activity	Growth inhibition of *H. pylori* (MIC ~250 µg/mL)	35
Abd El-Rady et al. (2021)	Pomegranate extract-loaded polymeric nanoparticles	Polyphenols	Rat	Increased IL-10; reduced IL-6	Enhanced efficacy vs free extract	36
Moradi et al. (2025)	Green-synthesized AgNPs (Lavandula)	Silver	Rat	Increased SOD; reduced oxidative stress	Comparable efficacy to famotidine	37
Sun et al. (2017)	Solid lipid nanoparticles (SLNs)	Sanguinarine	Mouse	NF-κB inhibition	Significant anti-ulcer activity	38
Prasad et al. (2013)	CeO_2 nanoparticles	Cerium oxide	Rat	ROS scavenging; antioxidant activity	~80% gastric mucosal protection	39
Elsisi et al. (2023)	CORM2 nanoparticles	CO donor	Rat	NRF2 and HO-1 activation	Strong anti-inflammatory and cytoprotective effects	40

Table 2. Preclinical (in vitro and in vivo) studies on nanoparticle-based therapies for gastric ulcer treatment.

Shareef et al. (2022)	Gold nanoparticles	Gold	Rat	Increased SOD and CAT activity	Reduced mucosal damage and oxidative stress	41
Perumcherry Raman et al. (2022)	Chitosan–anthocyanin nanoparticles	Anthocyanins	Rat	Immune modulation (IL-4/IFN-γ balance)	Reduced gastric inflammation	42
Fath-All et al. (2025)	Yeast-derived selenium nanoparticles	Selenium	Rat	Gut microbiota modulation; anti-inflammatory effect	Accelerated epithelial regeneration	43
Alamoudi et al. (2024)	Hesperidin nanoparticles	Hesperidin	Rat	SIRT1/PGC-1α activation; NF-κB inhibition	Strong mucosal protective effect	44
Nagarajana et al. (2015)	Chitosan–lansoprazole nanoparticles	Lansoprazole	In vitro	Controlled drug release	Sustained release up to 24 hours	45
Manivannan et al. (2023)	CS–OME–ATM nanoparticles	Omeprazole + H_2S donor	Rat	Increased NO and H_2S; improved perfusion	Enhanced mucosal healing and regeneration	46

time in the stomach and enhance contact with the injured mucosa (48). Mucoadhesive systems, such as chitosan-based nanoparticles, can adhere to the gastric epithelium, extending local drug retention. This property not only improves therapeutic efficacy but also reduces dosing frequency and stabilizes local drug concentrations, thereby optimizing clinical outcomes. In addition, protecting drugs from the acidic gastric environment and enabling sustained release further contribute to the therapeutic benefits of nanoparticles.

This review also highlights that a substantial part of the therapeutic effects of nanoparticles in gastric ulcers is mediated through modulation of inflammatory processes. Chronic mucosal inflammation is a key factor in sustained tissue damage and delayed ulcer healing (49). Several studies reported that therapeutic nanoparticles inhibit critical inflammatory pathways, including NF-κB, and reduce pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (50). Suppression of these pathways limits inflammatory cell infiltration, reduces oxidative damage, and provides a favorable environment for gastric epithelial regeneration (50).

ROS overproduction contributes substantially to gastric mucosal injury through oxidative damage to cellular components and compromised healing processes. Studies suggest that selenium nanoparticles and bioactive plant-based nanocomposites exert gastroprotective effects by restoring antioxidant capacity and upregulating enzymes such as SOD and CAT (51). Activation of NRF2-dependent signaling by nanoparticles further promotes resistance to oxidative stress and facilitates mucosal repair (52).

Another important observation from this review is the role of nanoparticle platforms in managing *H. pylori* infection (53). This bacterium is the most recognized risk factor for gastric ulcers and gastric cancer, and rising antibiotic resistance has compromised standard treatment efficacy (53). Nanocarriers improve the delivery of antibiotics to the infection site and provide controlled, localized drug release, ensuring therapeutically effective concentrations at the gastric mucosa. Additionally, metallic nanoparticles, such as silver and gold, exhibit intrinsic antibacterial activity against *H. pylori*, positioning nanoparticle-based therapies as promising strategies against drug-resistant infections (53).

Despite these therapeutic advantages, the review underscores the critical need to evaluate the biocompatibility and safety of nanoparticles. While many carriers demonstrate favorable biocompatibility, certain inorganic nanoparticles, such as titanium dioxide, can induce local inflammation, disrupt cellular function, and exacerbate tissue damage under specific conditions. These effects are highly dependent on physicochemical properties, including particle size, surface charge, morphology, dose, and exposure duration. Therefore, careful selection of materials and optimization of nanoparticle design are essential to maximize efficacy and minimize toxicity.

Although preclinical results are promising, the translation of nanomedicine from laboratory models to clinical applications faces several challenges. Most data are derived from animal studies, with limited information on long-term safety, pharmacokinetics, and biodistribution in humans. Moreover, the lack of standardized protocols for nanoparticle design and evaluation complicates cross-study comparisons. Consequently, well-designed, multicenter clinical trials are needed to establish the safety and efficacy of these nanoplatforms.

CONCLUSION

Current evidence indicates that nanoparticle-based drug delivery systems hold significant potential to enhance gastric ulcer therapy. These platforms can accelerate ulcer healing by improving drug bioavailability, targeting the gastric mucosa, modulating inflammation, reducing oxidative stress, and controlling *H. pylori* infection. However, successful clinical translation requires the development of safer nanocarriers, standardization of manufacturing processes, and extensive clinical evaluation. Future advances in nanomedicine and smart delivery systems are expected to enable more effective, safer, and personalized therapies for patients with gastric ulcers.

Declaration

Conflict of Interest

The authors declare no competing interests.

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Consent for Publication

The authors have given their approval for the publication of this manuscript.

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Ethical Considerations

The authors declare that the study complies with ethical publishing guidelines and adheres to principles of research integrity.

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Phytotherapy of Brucellosis: A Pharmacological Review of Anti-Brucellosis Medicinal Plants in Iranian Flora

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ABSTRACT

Background: Brucellosis is a persistent zoonotic infection endemic in countries such as Iran. Conventional antibiotic therapies have limitations, including adverse effects and incomplete efficacy, which has led to growing interest in medicinal plants as complementary or alternative treatments.

Methods: This study reviews Iranian medicinal plants with potential anti-brucellosis activity, examining their mechanisms based on both traditional knowledge and contemporary scientific research. A comprehensive literature search was conducted using keywords including "brucellosis," "Malta fever," "traditional medicine," "medicinal plants," and "treatment" in databases such as Google Scholar, SID, Magiran, and Scopus. Relevant articles were screened, and data were systematically extracted and analyzed to provide an integrative review.

Results: The analysis identified several medicinal plants with notable therapeutic potential against brucellosis, including *Zingiber officinale* Roscoe, *Mentha piperita* L., *Cinnamomum verum* J. Presl, *Thymus vulgaris* L., *Salvia officinalis* L., *Origanum vulgare* L., *Matricaria chamomilla* L., *Aloe vera* L., *Cuminum cyminum* L., *Alcea rosea* L., *Achillea millefolium* L., *Calendula officinalis* L., *Allium cepa* L., *Anethum graveolens* L., *Apium graveolens* L., *Allium sativum* L., *Coriandrum sativum* L., *Cucurbita* spp. L., *Rosa canina* L., *Elaeagnus angustifolia* L., *Scrophularia deserti* Eig, *Berberis vulgaris* L., *Ziziphora tenuior* L., and *Alhagi maurorum* Medik. Taxonomic analysis showed that the Lamiaceae family was most represented (24%), followed by Asteraceae and Apiaceae (16% each). Mechanistic analysis indicated that antibacterial activity accounted for 32% of therapeutic effects, followed by anti-inflammatory (24%) and antioxidant (20%) activities.

Conclusion: Native Iranian medicinal plants may serve as effective complementary agents in the management of brucellosis. They show promise, but further high-quality clinical studies are required to establish their efficacy and safety.

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INTRODUCTION

Brucellosis (Malta fever) is a bacterial zoonosis caused by *Brucella* species (1). The disease also referred to as Mediterranean fever, undulant fever, or contagious abortion in animals is notable for inducing early-stage pregnancy loss and for its ability to spread across various mammalian species (2). Infected livestock, particularly during early pregnancy, produce contaminated secretions that can readily pollute pastures, farms, and the surrounding environment (3).

A primary route of transmission to humans is the consumption of unpasteurized dairy products, as well as direct contact with infected animals such as cattle, goats, sheep, and camels (4). Individuals who work closely with animals including veterinarians, farm workers, slaughterhouse staff, and microbiologists are at higher risk of infection (5). *Brucella* bacteria can enter the human body through contact with blood or other secretions from infected animals, potentially leading to chronic complications if untreated (6).

Common symptoms include fever, night sweats, arthralgia, fatigue, and weight loss, and without timely diagnosis and treatment, the disease may progress to severe complications (7, 8). Similar symptoms are observed in children, often accompanied by weakness and lymphadenopathy (9). Accurate diagnosis requires specialized laboratory tests alongside clinical evaluation, as brucellosis symptoms can resemble those of other illnesses, such as influenza (10).

The standard treatment for brucellosis relies on antibiotics; however, this approach may be limited by side effects or incomplete efficacy (11). In such cases, Iranian traditional medicine offers complementary strategies for symptom management and patient support through the use of medicinal plants (12). Bioactive medicinal plants may complement conventional therapy through their antibacterial, anti-inflammatory, and immunomodulatory effects (13).

This study aims to investigate the therapeutic potential of medicinal plants with anti-brucellosis activity in the Iranian flora, through a comprehensive analysis of their ecological, morphological, phytochemical, phenological, and pharmacological characteristics.

METHODOLOGY

This review synthesizes existing information on brucellosis, medicinal plants, and their therapeutic applications. A comprehensive search was conducted using Google Scholar, SID, Magiran, and Scopus. The searches employed keywords such as “brucellosis,” “Malta fever,” “traditional medicine,” “medicinal plants,” and “treatment.”

Initially, the retrieved articles were screened for relevance, and studies unrelated to the scope of this review were excluded. Only articles directly addressing the topic were selected for detailed analysis (14-30).

The results of the relevant studies were then systematically and comprehensively reviewed to provide evidence-based insights into the role of medicinal plants in brucellosis management.

A flowchart illustrating the search strategy, as well as the articles and sources included in this review, is presented in Figure 1.

RESULTS

The analysis revealed that numerous medicinal plants within the Iranian flora exhibit significant potential in the management of brucellosis. Notable examples include *Zingiber officinale* Roscoe, *Mentha piperita* L., *Cinnamomum verum* J. Presl, *Thymus vulgaris* L., *Salvia officinalis* L., *Origanum vulgare* L., *Matricaria chamomilla* L., *Aloe vera* L., *Cuminum cyminum* L., *Alcea rosea* L., *Achillea millefolium* L., *Calendula officinalis* L., *Allium cepa* L., *Anethum graveolens* L., *Apium graveolens* L., *Allium sativum* L., *Coriandrum sativum* L., *Cucurbita* spp. L., *Rosa canina* L., *Elaeagnus angustifolia* L., *Scrophularia deserti* Eig, *Berberis vulgaris* L., *Ziziphora tenuior* L., and *Alhagi maurorum* Medik (Table 1).

These species have demonstrated therapeutic potential in traditional medicine, largely attributed to their antibacterial, anti-inflammatory, and immunomodulatory properties, which may contribute to their effectiveness in managing brucellosis.

Table 2 summarizes essential information on the selected medicinal plants, including the plant parts utilized, principal bioactive compounds, growth habits, life cycle, and natural habitat, providing a valuable reference for investigating their pharmacological and ecological characteristics.

The analysis reveals that the Lamiaceae family, with six occurrences (24%), is the most frequently represented among the listed plant families. This prominence highlights its significance within this compilation. The Asteraceae and Apiaceae families follow, each with four occurrences (16%), while other families are mentioned sporadically, with one or two occurrences each. These findings underscore the importance of the Lamiaceae, Asteraceae, and Apiaceae families as key targets for botanical and pharmaceutical research.

Most selected medicinal plants utilize leaves and flowers as primary sources of secondary bioactive compounds, including flavonoids and essential oils. This reflects an ecological strategy focused on chemical defense and efficient light-energy capture. Underground organs, such as rhizomes, bulbs, and roots, serve both as reservoirs of bioactive compounds and as means for regeneration under adverse environmental conditions. Seeds and fruits, in contrast, contain lipid-soluble compounds and vitamins, fulfilling protective and reproductive roles. The use of different or multiple organs within a single species demonstrates a comprehensive medicinal strategy and a diverse repertoire of secondary metabolites, aligned with the plant’s ecological adaptations and life cycle requirements.

Most selected species exhibit perennial life cycles, enabling continuous regeneration and year-round production of bioactive compounds via rhizomes, bulbs, or bush-like structures an adaptation particularly suited to arid and semi-arid environments. Annual

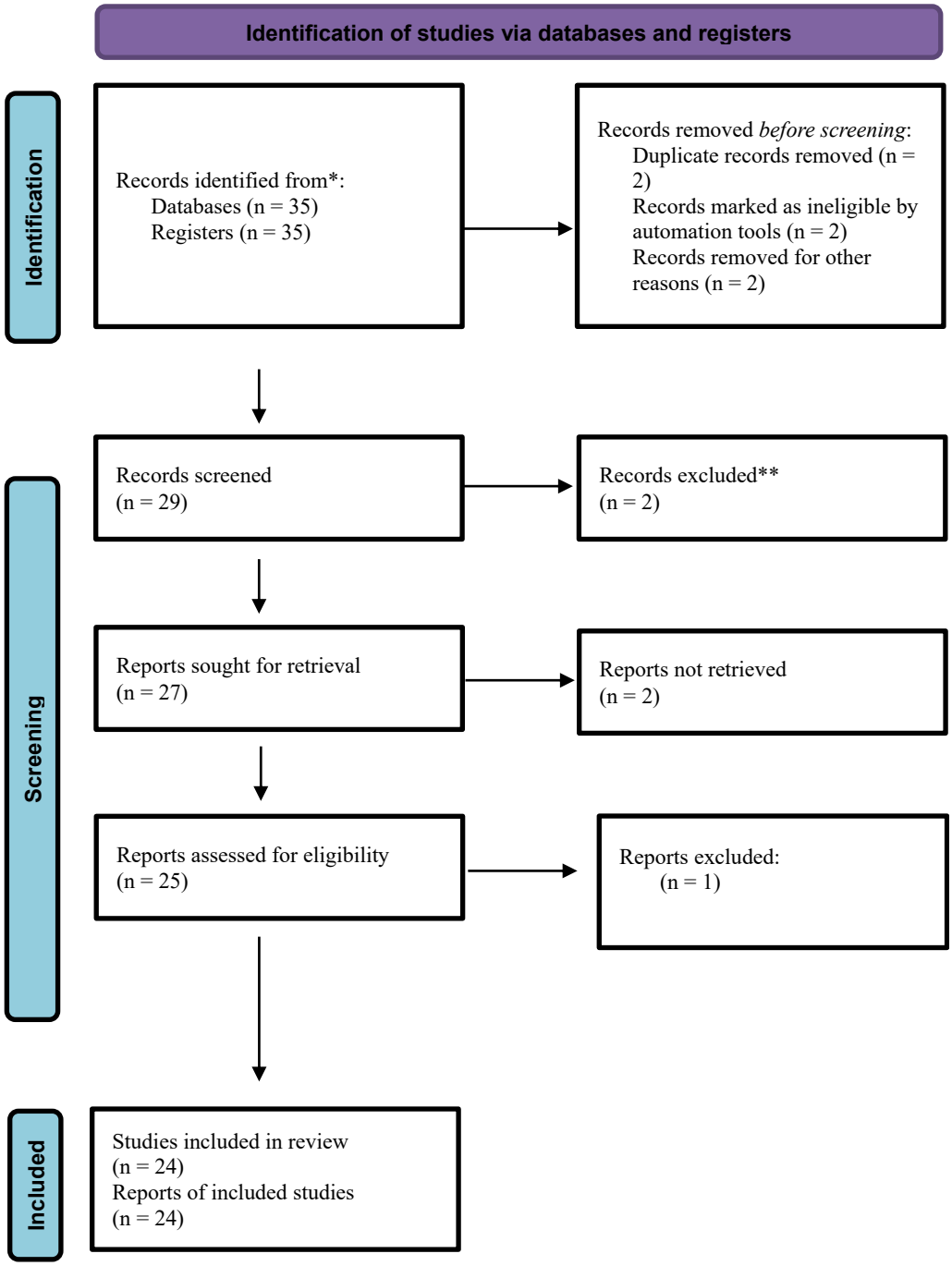

Fig 1. Flowchart of Search Strategy

plants, with their shorter life cycles, allocate energy primarily to seed production and secondary metabolite synthesis, making the timing of organ harvest critical. Evergreen and shrub species can continuously produce leaves, flowers, and fruits. This diversity of growth strategies reflects ecological adaptations and ensures optimal utilization of medicinal metabolites.

The selected medicinal plants are adapted to a wide range of habitats, from humid tropical regions to deserts and dry, calcareous soils. Habitat conditions directly influence secondary metabolite production and the medicinal potential of specific plant organs. Tropical species in moist soils predominantly produce underground organs and leaves rich in bioactive metabolites, whereas species from arid, sun-exposed environments have adapted to water scarcity through small or thick leaves, spiny stems, and storage of water and active compounds. This ecological diversity illustrates the close relationship between habitat adaptation and the synthesis and accumulation of medicinal compounds.

Functional analysis indicates that antibacterial

Table 1. Medicinal Plants Native to Iran Effective in the Treatment of Brucellosis in Traditional Medicine.

English Name	Persian Name	Scientific Name	Plant Family	Mechanism / Activity (14-30)
Ginger	Zangabil	*Zingiber officinale* Roscoe	Zingiberaceae	Anti-inflammatory, Immune-boosting
Mint	Naena	*Mentha piperita* L.	Lamiaceae	Antimicrobial, Improves digestive function
Cinnamon	Darchin	*Cinnamomum verum* J. Presl	Lauraceae	Antibacterial
Thyme	Avishan baghi	*Thymus vulgaris* L.	Lamiaceae	Contains thymol; antibacterial
Sage	Maryam goli	*Salvia officinalis* L.	Lamiaceae	Antioxidant, Immune-boosting
Oregano	Pouneh kouhi	*Origanum vulgare* L.	Lamiaceae	Contains carvacrol and thymol; antibacteria
Chamomile	Babouneh	*Matricaria chamomilla* L.	Asteraceae	Anti-inflammatory, Reduces fever and pain
Aloe vera	Aloe vera	*Aloe vera* L.	Asphodelaceae	Wound healing, Antimicrobial
Cumin	Zireh sabz	*Cuminum cyminum* L.	Apiaceae	Antioxidant, Antibacterial
Hollyhock	Khatmi	*Alcea rosea* L.	Malvaceae	Soothing, Anti-inflammatory
Yarrow	Boumadaran	*Achillea millefolium* L.	Asteraceae	Antibacterial, Immune-boosting
Calendula	Hamishe bahar	*Calendula officinalis* L.	Asteraceae	Anti-inflammatory, Antibacterial
Onion	Piaz	*Allium cepa* L.	Amaryllidaceae	Contains quercetin; antibacterial
Dill	Shevid	*Anethum graveolens* L.	Apiaceae	Antibacterial
Celery	Karafs	*Apium graveolens* L.	Apiaceae	Anti-inflammatory, Antioxidant
Garlic	Sir	*Allium sativum* L.	Amaryllidaceae	Contains allicin; strong antibacterial
Coriander	Geshniz	*Coriandrum sativum* L.	Apiaceae	Antioxidant, Antibacterial
Squash	Kadou	*Cucurbita* spp. L.	Cucurbitaceae	Immune-boosting via antioxidant activity
Rosehip	Nastaran	*Rosa canina* L.	Rosaceae	Immune-boosting; rich in vitamin C
Russian Olive	Senjed	*Elaeagnus angustifolia* L.	Elaeagnaceae	Antioxidant, Anti-inflammatory
Snapdragon	Gole meymoni biabani	*Scrophularia deserti* Eig	Plantaginaceae	Soothing properties
Barberry	Zereshk kouhi	*Berberis vulgaris* L.	Berberidaceae	Antibacterial
Ziziphora	Kakouti	*Ziziphora tenuior* L.	Lamiaceae	Antibacterial, Immune-boosting
Camelthorn	Kharshotor	*Alhagi maurorum* Medik.	Fabaceae	Antimicrobial effects

properties, with eight mentions (32%), are the most frequent therapeutic attribute among the selected plants. Anti-inflammatory properties follow with six occurrences (24%), while antioxidant and immune-boosting activities, each with five occurrences (20%), are also notable. These findings highlight the primary importance of antibacterial and anti-inflammatory effects, alongside the considerable potential of antioxidant and immunomodulatory properties. Collectively, these bioactivities are highly relevant for the development of herbal and pharmaceutical products aimed at disease prevention and treatment.

DISCUSSION

The therapeutic potential of *Zingiber officinale* is largely attributed to gingerol and shogaol, which exert antioxidant, anti-inflammatory, and immunomodulatory effects that may benefit patients with brucellosis (31). Mint (*Mentha piperita* L., Lamiaceae) exhibits antimicrobial and digestive-supportive effects. Consumption of mint may alleviate gastrointestinal symptoms commonly observed in brucellosis and also contributes to immune enhancement and relief of muscle pain associated with the disease (32). Cinnamon (*Cinnamomum verum* J. Presl, Lauraceae) contains cinnamaldehyde,

Table 2. Phytochemical Profile and Ecological Characteristics of Selected Medicinal Plants.

Medicinal Plant	Plant Part Used	Active Compounds	Growth and Life Cycle	Ecology / Habitat
***Zingiber officinale* Roscoe**	Rhizome	Gingerol, Shogaol	Perennial, rhizomatous	Tropical and subtropica regions, moist soils
***Mentha piperita* L.**	Leaves	Menthol, Menthone	Perennial, creeping stems	Moist soils, full sun
***Cinnamomum verum* J. Presl**	Bark	Cinnamaldehyde	Evergreen, small tree	Humid tropical regions
***Thymus vulgaris* L.**	Leaves, flowers	Thymol, Carvacrol	Perennial, bushy	Dry soils, full sun
***Salvia officinalis* L.**	Leaves, flowers	Salvin, Rosmarinic acid	Perennial, bushy	Dry, calcareous soils, full sun
***Origanum vulgare* L.**	Leaves, flowers	Carvacrol, Thymol	Perennial, bushy	Dry, sunny habitats
***Matricaria chamomilla* L.**	Flowers	Apigenin, Chamazulene	Annual	Sandy soils, full sun
***Aloe vera* L.**	Leaves	Aloin, Aloeic acid	Perennial, succulent	Arid and semi-arid regions
***Cuminum cyminum* L.**	Seeds	Cumin aldehyde	Annual	Light soils, full sun
***Alcea rosea* L.**	Flowers, leaves	Anthocyanins, Mucilage	Biennial or perennial	Rich soils, full sun
***Achillea millefolium* L.**	Leaves, flowers	Antol, Camphor	Perennial, bushy	Meadows, semi-moist soils
***Calendula officinalis* L.**	Flowers	Carotenoids, Flavonoids	Annual	Rich soils, full sun
***Allium cepa* L.**	Bulb	Flavonoids, Sulfur compounds	Biennial	Fertile soils, full sun
***Anethum graveolens* L.**	Leaves, seeds	Carvone, Limonene	Annual	Sandy soils, full sun
***Apium graveolens* L.**	Leaves, stems, seeds	Apiole, Limonene	Biennial	Moist soils, full sun
***Allium sativum* L.**	Bulb	Allicin	Perennial, bulbous	Fertile soils, full sun
***Coriandrum sativum* L.**	Leaves, seeds	Limonene, Linalool	Annual	Dry soils, full sun
***Cucurbita* spp. L.**	Fruit, seeds	Cucurbitin, Carotenoids	Annual	Fertile soils, full sun
***Rosa canina* L.**	Fruit, flowers	Vitamin C, Tannins	Perennial, shrub	Light soils, full sun
***Elaeagnus angustifolia* L.**	Fruit	Flavonoids, Tannins	Shrub	Dry and semi-arid soils
***Scrophularia deserti* Eig**	Leaves, flowers	Flavonoids, Saponins	Perennial	Arid and semi-arid regions
***Berberis vulgaris* L.**	Fruit, roots	Berberine, Alkaloids	Shrub	Dry soils, full sun

a compound with strong antibacterial activity, making it effective against microbes responsible for brucellosis and other bacterial infections (33). Thyme (*Thymus vulgaris* L., Lamiaceae), rich in thymol, demonstrates notable antibacterial and anti-inflammatory effects, particularly relevant for controlling infections such

as brucellosis (34). Similarly, oregano (*Origanum vulgare* L., Lamiaceae), containing carvacrol and thymol, exhibits antibacterial and anti-inflammatory properties, strengthens immune function, and aids in combating infections (35). Chamomile (*Matricaria chamomilla* L., Asteraceae) is recognized for its natural anti-inflammatory and analgesic activities. It can reduce fever and pain and is particularly useful in alleviating acute symptoms of brucellosis (36). Aloe vera (*Aloe vera* L., Asphodelaceae) possesses antimicrobial and wound-healing properties, making it effective in treating inflammation and tissue damage associated with chronic infections such as brucellosis (37). Garlic (*Allium sativum* L.) contains allicin, which provides strong antibacterial effects and enhances immune defenses against brucellosis-causing bacteria (38). Celery (*Apium graveolens* L., Apiaceae) exhibits both anti-inflammatory and antioxidant properties, supporting immune function and reducing bacterial inflammation (39). Thyme (*Thymus vulgaris* L., Lamiaceae) also demonstrates antibacterial and immunomodulatory effects, making it valuable in treating various infectious and inflammatory conditions, including brucellosis (40). Wild barberry (*Berberis vulgaris* L., Berberidaceae) possesses antibacterial activity and can aid in controlling brucellosis-related bacterial infections, improving overall disease outcomes (41). The bioactive and secondary metabolites, along with the antioxidant compounds present in plants, play a central role in mediating their antimicrobial effects (42-47).

CONCLUSION

This review has several limitations, primarily the limited availability of clinical evidence, as most published data originate from traditional medicine, in vitro studies, and animal models. Iranian medicinal plants with antibacterial, anti-inflammatory, immunomodulatory, and antioxidant activities may offer complementary benefits in brucellosis management. Nevertheless, their clinical application should be evidence-based and used alongside standard therapy under medical supervision. Well-designed clinical trials are needed to confirm their efficacy, safety, and place in future treatment strategies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

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Exploratory Transcriptomic Profiling of Three RNA-Seq Samples Reveals Heterogeneity in KRAS-Associated Signaling and Epithelial Lineage Programs

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ABSTRACT

KRAS-driven lung adenocarcinoma is molecularly heterogeneous; however, evaluation of mutation-associated programs requires matched mutation and clinical metadata. Here, three GDC STAR-count RNA-sequencing files annotated with GENCODE v36 were analyzed to determine which transcriptomic conclusions could be supported from the available data alone. Protein-coding TPM values were used for library-level quality assessment, transcriptome-wide correlation, principal component analysis, sample-specific expression contrasts, and exploratory scoring of curated KRAS/MAPK, PI3K–AKT–mTOR, cell-cycle, epithelial-lineage, epithelial–mesenchymal transition, hypoxia, inflammatory, interferon, apoptosis, and NRF2-related gene sets. The samples contained 24.3–49.5 million assigned reads and 12,808–14,574 protein-coding genes with TPM ≥1. Pairwise transcriptome correlations ranged from r=0.745 to 0.825. Marked biological heterogeneity was observed. Sample 1 showed a secretory epithelial profile characterized by PAEP, CEACAM5, CEACAM6, BPIFA1, and EPCAM. Sample 2 displayed a keratinizing/squamous-like program dominated by KRT5, KRT6A, KRT14, KRT17, SPRR family genes, and elevated NRF2-associated and glycolytic scores. Sample 3 exhibited a strong alveolar-lineage and immune-associated profile, with high SFTPA1, SFTPA2, SFTPB, SFTPC, HLA-DRA, CD74, and inflammatory pathway scores. KRAS expression was detectable in all samples (16.33–30.09 TPM), but RNA expression alone could not establish KRAS mutation status. These findings demonstrate substantial lineage and pathway heterogeneity among the three transcriptomes and provide a hypothesis-generating framework for subsequent mutation-informed analysis. Mutation-stratified differential expression, prognostic modeling, and survival analysis were not performed because mutation and clinical outcome data were unavailable.

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INTRODUCTION

Lung adenocarcinoma (LUAD) is the most common histological subtype of non-small-cell lung cancer and is characterized by extensive genomic, transcriptomic, and phenotypic heterogeneity. Large-scale molecular profiling by The Cancer Genome Atlas demonstrated that LUAD contains recurrent alterations in receptor tyrosine kinase–RAS–RAF signaling, cell-cycle control, oxidative-stress regulation, chromatin remodeling, and lineage-determining transcriptional programs (1). This diversity has important biological and clinical consequences because tumors with

a similar histopathological diagnosis may differ markedly in cellular differentiation, metabolic state, immune contexture, and therapeutic vulnerability. Transcriptome profiling therefore provides a functional layer of information that complements genomic testing by revealing the cellular programs that are active in an individual tumor specimen.

KRAS is one of the most frequently altered oncogenic drivers in LUAD. The encoded small GTPase operates as a molecular switch that couples upstream growth-factor signals to several downstream effector networks, including RAF–MEK–ERK, PI3K–AKT–mTOR, RAL-GEF, and stress-adaptation pathways. Activating substitutions, most commonly affecting codon 12, impair normal GTP hydrolysis and favor persistent signaling that promotes proliferation, survival, metabolic reprogramming, invasion, and remodeling of the tumor microenvironment (2, 7). The clinical development of covalent KRAS G12C inhibitors has confirmed that mutant KRAS can be therapeutically targeted; however, incomplete responses and acquired resistance indicate that the biological state of a KRAS-driven tumor cannot be explained by the driver mutation alone (8).

A major source of heterogeneity in KRAS-associated LUAD is the presence of co-occurring genomic alterations. Mutations or losses involving TP53, STK11/LKB1, KEAP1, and CDKN2A/B define biologically distinct subsets with different inflammatory profiles, metabolic dependencies, differentiation states, and treatment responses (2, 9). In particular, STK11 loss can produce an immune-cold phenotype, whereas concurrent KEAP1–NRF2 pathway activation enhances antioxidant defense, redox homeostasis, and nutrient dependence in KRAS-mutant tumors (9, 10). These observations emphasize that pathway activity should be interpreted as the product of interacting genomic events and cellular context rather than as a direct readout of KRAS messenger RNA abundance.

Epithelial lineage identity is another central determinant of LUAD biology. Alveolar type II cells are an established cell of origin for experimental KRAS-driven lung adenocarcinoma, and surfactant genes such as SFTPA1, SFTPA2, SFTPB, and SFTPC provide transcriptional evidence of alveolar differentiation (11). The lineage transcription factor NKX2-1/TTF-1 regulates pulmonary identity and may function as a lineage-survival factor in differentiated LUAD, while loss or redistribution of its transcriptional program can accompany progression toward poorly differentiated, mucinous, gastric-like, or metastatic states (6, 12-14). Conversely, increased expression of basal keratins and small proline-rich proteins may indicate a basal or squamous-like differentiation program. Secretory epithelial markers, including CEACAM5, CEACAM6, EPCAM, and AGR2, represent a further state associated with epithelial differentiation, adhesion, secretory-protein processing, and potentially invasive behavior (15). Thus, differences in lineage composition can substantially modify the apparent output of oncogenic signaling pathways in bulk RNA-sequencing data.

The interpretation of bulk tumor RNA sequencing is additionally influenced by non-neoplastic stromal and immune cells. Expression of HLA class II genes, CD74, interferon-response genes, and leukocyte-associated transcripts may reflect immune infiltration, tumor-cell-intrinsic inflammatory signaling, or both. Without matched pathology, tumor-purity estimates, somatic-variant calls, and clinical metadata, such signals should be treated as biologically informative but not assigned to a specific cell population or prognostic category. Nevertheless, carefully constrained exploratory analysis can identify dominant epithelial states, stress-response programs, and immune-associated features that guide subsequent hypothesis-driven investigation.

In the present study, three GDC STAR-count RNA-sequencing files were analyzed to characterize their principal transcriptomic features and to determine which conclusions could be supported from gene-expression data alone. The analysis focused on library-level quality metrics, global transcriptional similarity, sample-enriched genes, epithelial and alveolar lineage markers, KRAS-associated signaling components, oxidative-stress programs, and immune-related expression. Because matched mutation and survival information was unavailable, the study was intentionally framed as an exploratory three-sample transcriptomic analysis rather than a mutation-stratified or prognostic investigation. This conservative design allowed biologically meaningful heterogeneity to be described while avoiding unsupported claims regarding KRAS genotype, differential expression, or clinical outcome.

MATERIALS AND METHODS

RNA-sequencing data and preprocessing

Three GDC gene-expression files generated by the STAR-count workflow were analyzed. Each file contained gene identifiers, gene symbols, gene biotypes, unstranded and stranded read counts, transcripts per million (TPM), fragments per kilobase per million, and upper-quartile-normalized FPKM values. The files were annotated against GENCODE v36, consistent with the GDC harmonized mRNA pipeline (3). The unstranded count column was used for library summaries, whereas TPM values were used for cross-sample descriptive analyses. Rows representing alignment summary categories were separated from gene-level records. Analyses were restricted to protein-coding genes, and duplicated gene symbols, where present, were aggregated.

Quality assessment and exploratory transcriptomic analysis

For each sample, assigned reads, unmapped reads, multimapping reads, reads without an annotated feature, ambiguous reads, the number of detected protein-coding genes, mitochondrial read fraction, and ribosomal read fraction were calculated. Expression values were transformed as log2(TPM+1). Pairwise Pearson and Spearman correlations were calculated across protein-coding genes. Principal component analysis was performed on the 1,000 most variable protein-coding genes after feature standardization.

Because the dataset contained only three unlabelled samples and no biological replicates per group, formal differential-expression testing and false-discovery-rate estimation were not performed.

Sample-specific genes and pathway scoring

For descriptive identification of sample-enriched transcripts, each sample was compared with the mean TPM of the other two samples using log2((TPM+0.1)/(mean TPM of the other samples+0.1)). Genes with TPM ≥5 were ranked by this descriptive ratio; these values were not treated as inferential fold changes. Exploratory pathway scores were calculated from curated gene sets representing KRAS/MAPK, PI3K–AKT–mTOR, cell cycle/E2F, epithelial–mesenchymal transition (EMT), epithelial and alveolar lineage, hypoxia/glycolysis, inflammatory/NF-κB, interferon/antigen presentation, apoptosis, and KEAP1–NRF2 oxidative-stress programs. For each gene, log2(TPM+1) values were standardized across the three samples, and pathway scores were defined as the mean standardized expression of genes in the corresponding set. These scores indicate relative activity only within this three-sample dataset and are not equivalent to formal GSEA or GSVA.

RESULTS

Library characteristics

The three libraries contained 49.5, 35.5, and 24.3 million assigned gene-level reads, respectively. The proportion of assigned reads among all STAR accounting categories ranged from 38.6% to 41.6%. Protein-coding genes with TPM ≥1 numbered 12,808, 14,516, and 14,574. Mitochondrial reads represented 1.77%, 1.52%, and 0.70% of assigned gene counts, indicating no marked mitochondrial-read excess (Table 1 and Figure 1).

Global transcriptomic similarity and separation

Pairwise Pearson correlations of log2-transformed protein-coding expression were 0.825 between Samples 1 and 2, 0.745 between Samples 1 and 3, and 0.822 between Samples 2 and 3. Spearman correlations showed the same pattern, indicating that Sample 1 and Sample 3 were the most transcriptionally divergent pair. Principal component analysis of the 1,000 most variable genes separated all three samples, with Sample 2 and Sample 3 occupying distinct expression states and Sample 1 forming a third independent profile (Figures 2 and 3).

Table 1. RNA-sequencing library summary

Sample	Assigned reads	Assigned (%)	PC genes TPM>0	PC genes TPM≥1	Mitochondrial (%)	KRAS TPM
Sample_1	49,496,117	41.6	17,503	12,808	1.77	16.33
Sample_2	35,507,173	38.8	17,977	14,516	1.52	30.09
Sample_3	24,300,711	38.6	17,847	14,574	0.70	23.16

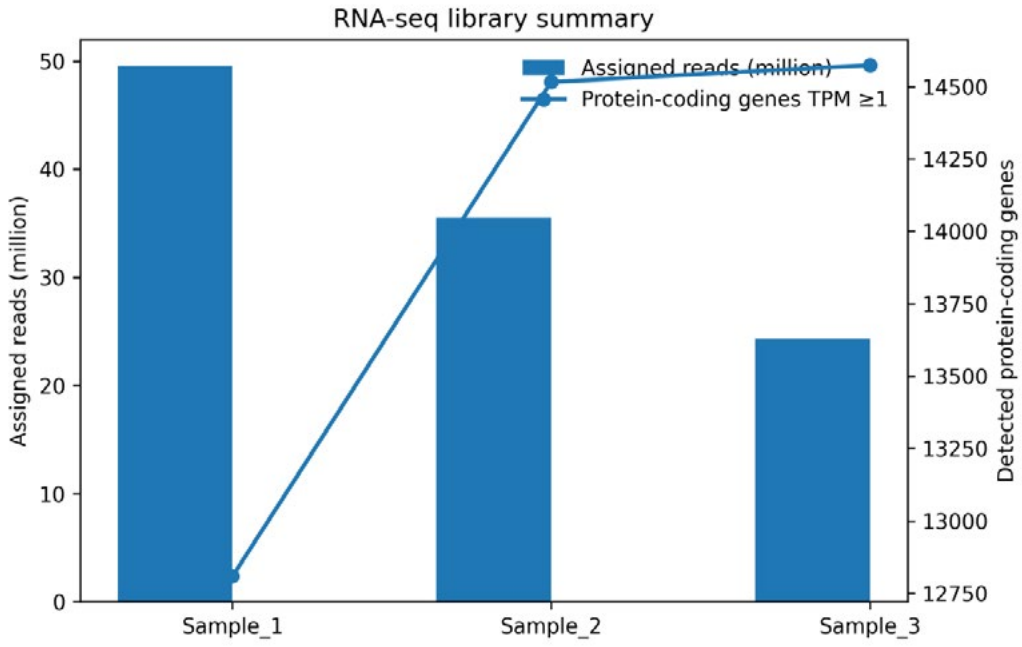

Fig 1. RNA-seq library summary. Bars show assigned gene-level reads; the line shows the number of protein-coding genes with TPM ≥1.

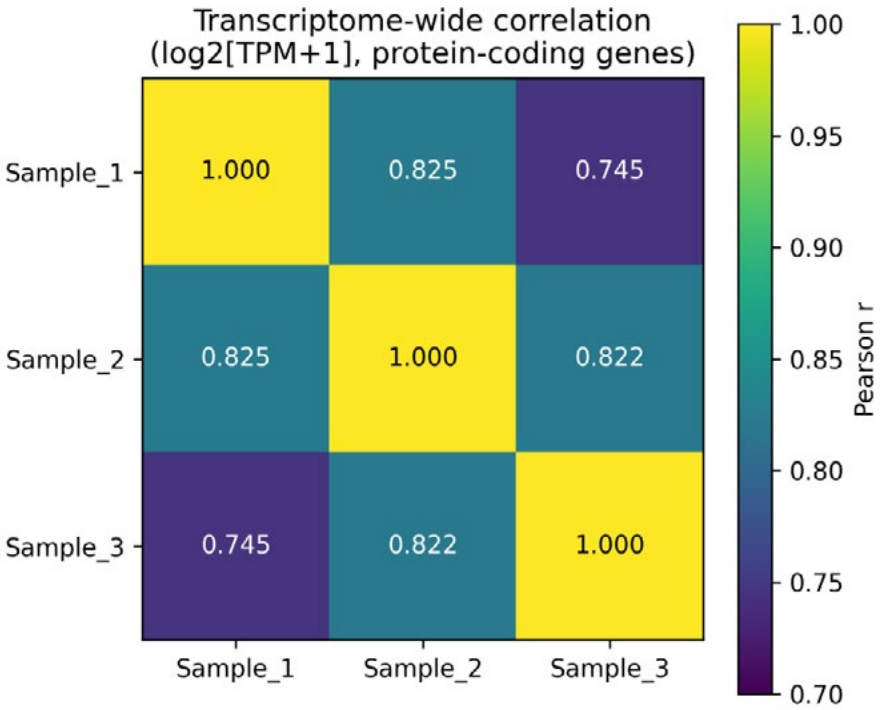

Fig2. Transcriptome-wide Pearson correlation based on log2(TPM+1) values for protein-coding genes.

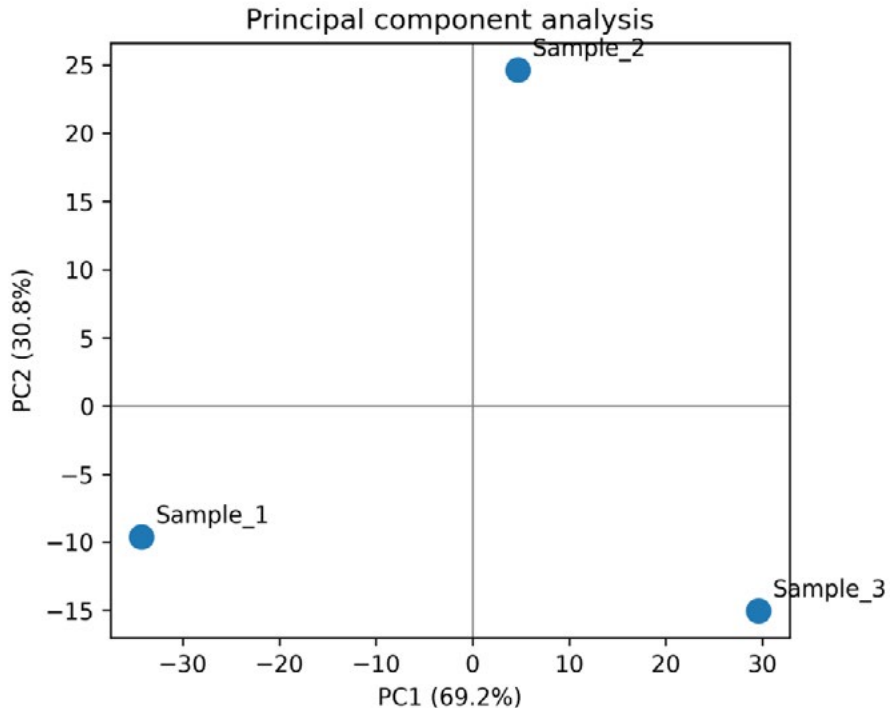

Fig 3. Principal component analysis of the 1,000 most variable protein-coding genes. The analysis is descriptive because only three samples were available.

Distinct epithelial and lineage-associated expression programs

Sample 1 was characterized by high expression of CEACAM6, CEACAM5, KRT18, AGR2, BPIFA1, EPCAM, and KRT19, together with marked sample-specific expression of PAEP, COL2A1, SEPTIN14, CYP1A1, and several secretory epithelial transcripts. This profile was consistent with a differentiated secretory epithelial state. Sample 2 showed a striking keratinizing program, with KRT5, KRT6A, KRT14, KRT17, KRT16, DSG3, SFN, and multiple small proline-rich proteins among its dominant or most sample-enriched genes. Sample 3 showed a strong alveolar-associated program, with SFTPA1, SFTPA2, SFTPB, and SFTPC among the most abundant transcripts, accompanied by HLA-DRA, HLA-DRB1,

CD74, B2M, and JCHAIN. These profiles indicate pronounced differences in epithelial differentiation and immune-cell or inflammatory contributions among the samples.

Exploratory KRAS-associated pathway patterns

KRAS transcript expression was detectable in all samples and was highest in Sample 2 (30.09 TPM), followed by Sample 3 (23.16 TPM) and Sample 1 (16.33 TPM). However, transcript abundance is not a surrogate for mutation status. Relative pathway scoring showed that Sample 2 had the highest PI3K–AKT–mTOR, hypoxia/glycolysis, interferon/antigen-presentation, apoptosis, and KEAP1–NRF2 oxidative-stress scores. Sample 3 had the highest KRAS/MAPK, EMT, epithelial/alveolar-lineage, and inflammatory/NF-κB scores, whereas its cell-cycle score was substantially lower than those of Samples 1 and 2. Sample 1 showed comparatively higher proliferation than Sample 3 but lower inflammatory, interferon, EMT, and oxidative-stress scores (Table 3 and Figure 5).

Scores are mean gene-wise z scores and indicate only relative activity within this dataset.

DISCUSSION

The present exploratory analysis shows that the three RNA-sequencing profiles represent biologically distinct transcriptional states despite retaining moderate-to-high transcriptome-wide correlations. The dominant source of variation was not the abundance of KRAS transcripts themselves, but the combination of epithelial lineage, differentiation status, stress-response activity, and immune-associated expression. This observation is consistent with the broader molecular heterogeneity of lung adenocarcinoma, in which tumors sharing a histological diagnosis may differ substantially in cell of origin, differentiation program, co-occurring molecular alterations, and microenvironmental composition (1). These factors are particularly relevant to studies of RAS signaling because the transcriptional consequences of pathway activation depend strongly on cellular context and cannot be interpreted independently of lineage identity (1, 2, 9, 10).

Sample 1 was characterized by a secretory epithelial program marked by CEACAM5, CEACAM6, AGR2, EPCAM, KRT18, and KRT19, together with pronounced PAEP expression. CEACAM5 and CEACAM6 are frequently associated with epithelial tumor differentiation, cell adhesion, and invasive behavior (15), whereas AGR2 is an endoplasmic-reticulum protein linked to secretory activity and maintenance of epithelial tumor phenotypes. The coordinated expression of these genes suggests a differentiated, secretory carcinoma-like state rather than a highly mesenchymal phenotype. The presence of EPCAM and keratin 18/19 further supports preservation of epithelial identity. However, because no histopathological or genomic metadata were available, this profile should be interpreted as a transcriptional phenotype and not as evidence for a specific LUAD subtype or molecular driver.

Sample 2 displayed the most distinctive basal and keratinizing program, with high expression of KRT5, KRT6A, KRT14, KRT17, and multiple small proline-rich proteins. This combination is more typical of basal or squamous differentiation than of a classical surfactant-producing LUAD phenotype. Several explanations are possible, including a poorly differentiated or transdifferentiated adenocarcinoma, mixed histology, sampling of a squamous component, or inaccurate histological assignment. Bulk RNA-sequencing alone cannot distinguish among these possibilities. Nevertheless, the finding is important because inclusion of such a sample in a small mutation-stratified analysis could generate apparently strong differential-expression signals that actually reflect lineage imbalance rather than KRAS mutation status. Histological review and expression of lineage markers such as NKX2-1, NAPSA, TP63, and KRT5 should therefore be considered when constructing mutation-

Table 2. Top sample-enriched protein-coding transcripts

Rank	Sample 1	Sample 2	Sample 3
1	PAEP (2879.4 TPM)	SPRR1A (2432.5 TPM)	SFTPC (12371.1 TPM)
2	SEPTIN14 (513.5 TPM)	KRT6A (3312.0 TPM)	PGC (937.4 TPM)
3	COL2A1 (610.2 TPM)	KRT14 (3085.7 TPM)	SFTPA1 (9524.9 TPM)
4	FGB (191.4 TPM)	KRT5 (3307.0 TPM)	SFTPB (4274.9 TPM)
5	MAGEA12 (94.5 TPM)	SPRR1B (1241.9 TPM)	SFTA2 (133.8 TPM)
6	OBP2A (127.9 TPM)	SPRR3 (638.6 TPM)	MT1M (537.7 TPM)
7	BPIFA2 (45.8 TPM)	SPRR2A (613.9 TPM)	HLA-DQA2 (236.5 TPM)
8	SPAG11B (27.3 TPM)	DSG3 (717.6 TPM)	PEBP4 (142.6 TPM)
9	CSAG1 (59.4 TPM)	SPRR2E (439.9 TPM)	PLA2G1B (55.3 TPM)
10	CYP1A1 (237.0 TPM)	KRT16 (617.8 TPM)	HAS1 (76.7 TPM)

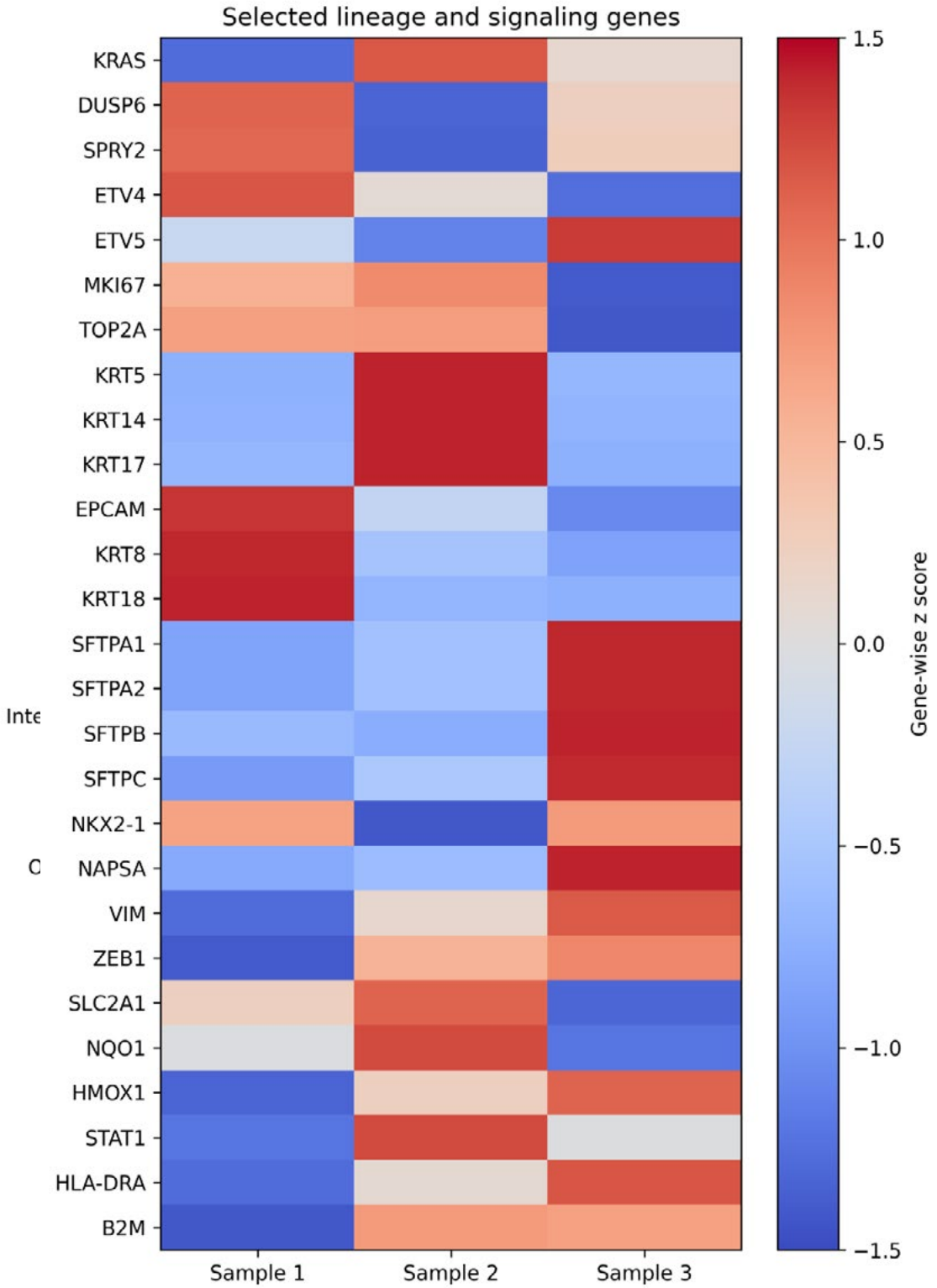

Fig4. Relative expression of selected lineage, KRAS-associated signaling, proliferation, stress-response, and immune genes. Values are gene-wise z scores across the three samples.

comparison cohorts.

Sample 2 also showed the highest relative NRF2-associated and glycolytic scores. This pattern was supported by increased expression of oxidative-stress and detoxification genes, including NQO1, members of the AKR1C family, and SLC7A11. Activation of this transcriptional program can promote adaptation to reactive oxygen species, maintenance of redox balance, and metabolic survival under stressful conditions. In LUAD, similar expression patterns may occur in tumors with KEAP1 or NFE2L2 pathway alterations, but they can also be induced by non-genetic oxidative

Table 3. Relative pathway scores across the three samples

Pathway	Sample 1	Sample 2	Sample 3	Genes
KRAS/MAPK signaling	-0.34	-0.12	0.45	20
PI3K–AKT–mTOR	-0.63	0.55	0.08	18
Cell cycle/E2F	0.61	0.76	-1.37	19
EMT	-0.84	0.18	0.67	16
Epithelial/LUAD markers	0.14	-0.64	0.51	13
Hypoxia/glycolysis	-0.33	0.69	-0.36	13
Inflammatory/NF-kB	-0.84	-0.44	1.29	13
Interferon/antigen presentation	-1.32	0.90	0.42	17
Apoptosis	-0.84	0.55	0.30	14
Oxidative stress/KEAP1-NRF2	-0.48	1.13	-0.64	13

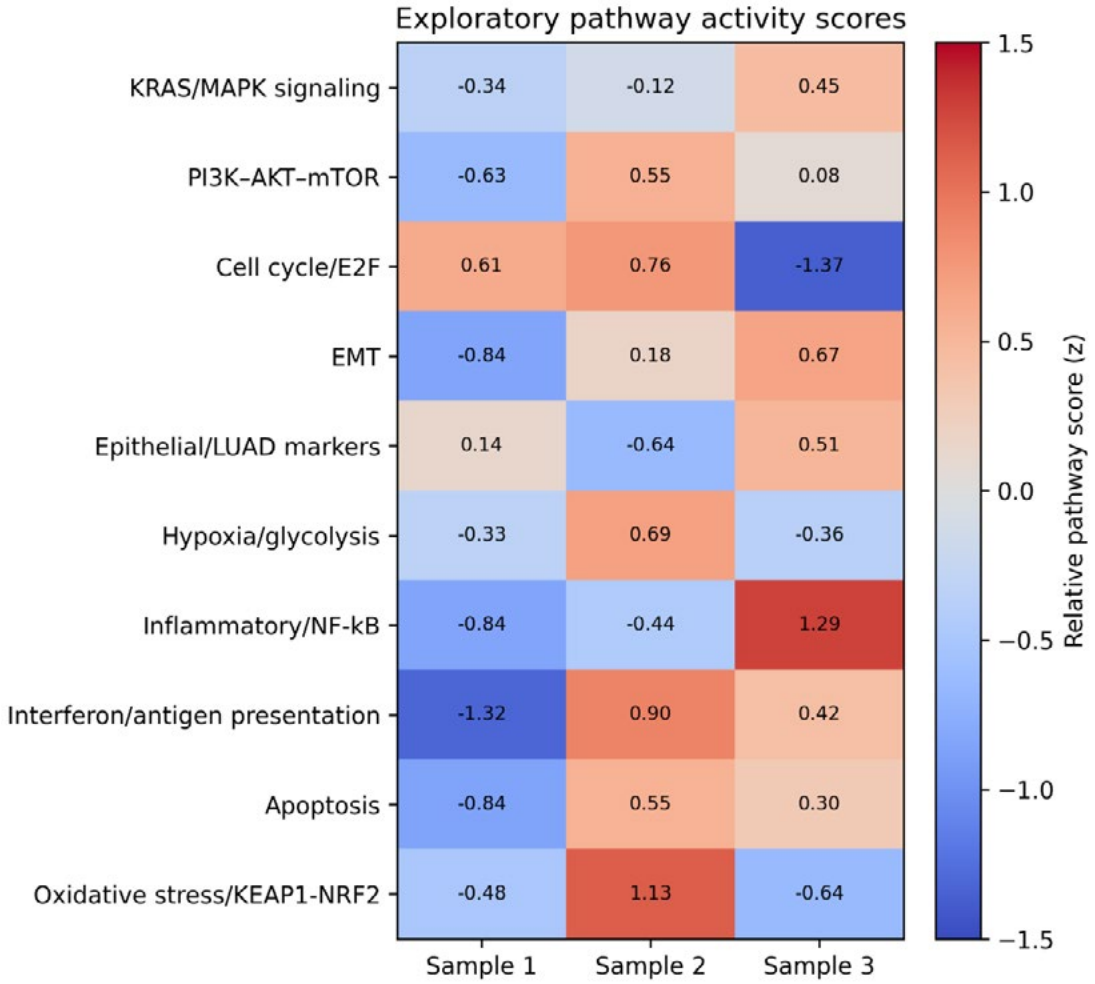

Fig5. Exploratory pathway activity scores. Red indicates relatively higher and blue relatively lower standardized expression within the three-sample dataset.

stress and metabolic remodeling. Consequently, the current findings indicate an NRF2-like transcriptional state but do not establish the presence of a KEAP1/NFE2L2 mutation. This distinction is critical because pathway activity inferred from RNA expression is a functional readout, whereas mutation status is a genomic classification, and the two are related but not interchangeable.

Sample 3 exhibited a markedly different phenotype characterized by strong expression of the surfactant genes SFTPA1, SFTPA2, SFTPB, and SFTPC. These genes are hallmarks of alveolar type II cell differentiation and support an alveolar lineage state. Maintenance of alveolar identity can influence oncogenic signaling,

tumor differentiation, and therapeutic dependencies. Experimental and clinical studies have shown that lineage-defining transcription factors, including NKX2-1 and FOXA-family factors, can modulate the biological output of oncogenic pathways and preserve distinct LUAD cell states (6, 11-14, 16). The surfactant-rich profile of Sample 3 therefore provides a plausible biological explanation for its separation from the keratinizing Sample 2, even before consideration of any underlying mutation differences.

In addition to alveolar markers, Sample 3 showed increased expression of HLA class II-related genes, CD74, B2M, and other immune-associated transcripts. This pattern may reflect greater infiltration by antigen-presenting immune cells, interferon-responsive tumor cells, or a combination of both. Because the analyzed files were derived from bulk tissue, expression originating from malignant epithelial cells cannot be separated from stromal or immune-cell expression without deconvolution or single-cell data. Nevertheless, the coordinated elevation of antigen-presentation genes and inflammatory pathway scores suggests that Sample 3 had a more immune-active transcriptional environment than the other two samples. In a larger cohort, this phenotype would warrant evaluation using immune-deconvolution methods and comparison with tumor purity, interferon signaling, and immune-checkpoint expression.

KRAS transcripts were detected in all three samples, with the highest TPM value in Sample 2. This variation should not be interpreted as evidence of KRAS mutation or oncogenic activity. Activating KRAS mutations alter the biochemical state and downstream signaling of the protein and do not necessarily cause a proportional increase in KRAS mRNA abundance. Conversely, elevated KRAS expression may occur in wild-type tumors. Similarly, the relative KRAS/MAPK pathway score observed in Sample 3 is not specific to mutant KRAS, because MAPK-responsive genes can be activated by EGFR, ERBB2, MET, BRAF, NF1 loss, inflammatory signaling, and other upstream mechanisms. The present analysis therefore distinguishes between KRAS expression, RAS/MAPK-related transcriptional output, and confirmed KRAS mutation status; only the first two can be evaluated from the available files.

The data also illustrate why pathway-level interpretation may be more informative than inspection of a single oncogene transcript. The three samples differed simultaneously in epithelial differentiation, oxidative-stress adaptation, glycolysis, inflammatory signaling, and antigen-presentation programs. Such coordinated changes can alter tumor behavior and may modify response to targeted or immune-based therapies even when the initiating genomic alteration is shared. In larger mutation-annotated LUAD cohorts, combining somatic-variant data with transcriptomic pathway scores could help distinguish biologically different KRAS-mutant subgroups, including tumors dominated by epithelial lineage programs, oxidative-stress adaptation, or immune activation. This strategy would be more robust than defining tumors solely by KRAS mRNA abundance.

From a methodological perspective, the moderate-to-high global correlations observed among the samples indicate that all three retained a common core human epithelial transcriptome, whereas PCA and marker-gene analysis captured biologically meaningful differences in a smaller subset of highly variable genes. This pattern is expected in bulk tumor transcriptomics: most housekeeping and essential cellular genes remain broadly correlated, while lineage markers, immune genes, metabolic programs, and stress-response pathways drive sample separation. The concordance between PCA, sample-enriched genes, and curated pathway scores increases confidence that the observed distinctions are not attributable to a single isolated transcript. However, because pathway scores were calculated as relative standardized means across only three samples, they should be viewed as descriptive indicators rather than estimates of absolute pathway activation.

Collectively, these findings support a model in which transcriptional heterogeneity among the three specimens is shaped primarily by epithelial lineage and microenvironmental context. Sample 1 represents a secretory epithelial state, Sample 2 a basal/keratinizing and oxidative-stress-adapted state, and Sample 3 an alveolar and immune-associated state. These profiles generate testable hypotheses for subsequent work. Mutation sequencing could determine whether the samples carry KRAS or other RAS-pathway alterations; histopathological reassessment could clarify the basal/squamous-like phenotype of Sample 2; and immune deconvolution or immunohistochemistry could evaluate the antigen-presentation signature of Sample 3. A larger cohort with matched mutation, clinical, and survival data would then be required to identify statistically supported KRAS-associated hub genes and determine whether any of the observed programs have prognostic significance.

Limitations

The study included only three samples without known biological grouping, mutation status, histopathological annotation, treatment information, or survival data. Accordingly, formal differential-expression testing, multiple-testing correction, mutation-stratified comparisons, protein–protein interaction network inference, hub-gene selection, Kaplan–Meier analysis, and Cox regression were not statistically justified and were not performed. The curated pathway scores were exploratory and were based on relative standardized expression rather than formal enrichment against a reference cohort. Potential differences in tumor purity and stromal or immune-cell content could also contribute to the observed patterns.

CONCLUSION

Analysis of the three available STAR-count RNA-sequencing files identified substantial transcriptomic heterogeneity. Sample 1 displayed a secretory epithelial program, Sample 2 a keratinizing/squamous-like and oxidative-stress-associated program, and Sample 3 an alveolar and inflammatory program. KRAS and multiple downstream signaling genes were expressed in all samples, but neither KRAS mutation status nor prognostic relevance could be determined. These results

provide a defensible exploratory manuscript based solely on the available data and identify the metadata required for a subsequent mutation-informed LUAD study.

Data Availability

The analysis was based on three GDC STAR-count gene-expression files supplied for the study. Derived summary tables and figures were generated from the unstranded count and TPM columns.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this study.

Consent for Publication

Not applicable. This study did not involve the collection of identifiable personal information, and no individual-level identifiable data are presented in this manuscript.

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Ethical Considerations

Ethical approval was not required for this study because the analysis was based exclusively on previously generated RNA-sequencing data obtained from the Genomic Data Commons (GDC). No human participants were prospectively recruited, no intervention was performed, and no identifiable personal information was collected or analyze.

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Beyond Dysbiosis: Multi-Omics, Personalized Interventions, and the Vaginal Microbiome in Female Reproductive Health

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ABSTRACT

The vaginal microbiome is a dynamic mucosal ecosystem that plays a crucial role in female reproductive health. Unlike the gut microbiome, where high diversity is often considered beneficial, a healthy vaginal microbiome in reproductive-age women is commonly recognized by low microbial diversity and dominance of Lactobacillus species, particularly Lactobacillus crispatus. These communities help maintain an acidic vaginal environment, inhibit pathogen growth, support epithelial barrier integrity, and regulate mucosal immune responses. Growing evidence links vaginal microbial composition and function to fertility, assisted reproductive technology outcomes, pregnancy complications, and gynecological disorders. Lactobacillus-dominant communities are generally associated with favorable reproductive outcomes, whereas high-diversity anaerobe-rich communities have been implicated in bacterial vaginosis, pelvic inflammatory disease, HPV persistence, cervical dysplasia, recurrent infections, preterm birth, and potentially impaired fertility. However, the traditional concept of dysbiosis is insufficient to explain the biological complexity of vaginal microbial ecosystems. Similar taxonomic profiles may differ in microbial gene content, metabolic activity, biofilm formation, inflammatory potential, ecological stability, and clinical impact. Therefore, multi-omics approaches, including metagenomics, metatranscriptomics, metabolomics, proteomics, and host-response profiling, provide a more comprehensive framework for understanding host–microbiome interactions. This review summarizes current evidence on the vaginal microbiome in fertility, pregnancy outcomes, and gynecological disorders, while emphasizing the transition from descriptive dysbiosis models toward functional, ecological, and personalized perspectives. Emerging strategies such as microbiome-based biomarkers, targeted probiotics, live biotherapeutics, vaginal microbiome transplantation, and AI-driven prediction models may support future precision gynecology. Nevertheless, clinical translation requires standardized methods, longitudinal studies, diverse cohorts, validated biomarkers, and careful ethical oversight.

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INTRODUCTION

The human microbiome has emerged as a central determinant of health and disease, reshaping our understanding of host physiology beyond the classical pathogen-centered model. Large-scale initiatives such as the Human Microbiome Project demonstrated that micro-

bial communities vary substantially across body sites and individuals, with the vagina representing one of the most distinctive microbial ecosystems in the human body (1). Unlike the gut microbiome, where high microbial diversity is often interpreted as a marker of ecological resilience, the vaginal microbiome in reproductive-age women is frequently characterized by relatively low bacterial diversity and dominance of *Lactobacillus species* (2). This ecological configuration is considered functionally important because lactobacilli contribute to vaginal acidification, production of antimicrobial metabolites, competitive exclusion of pathogens, and regulation of mucosal immune responses (3, 4).

One of the foundational studies in the field classified vaginal bacterial communities of reproductive-age women into major community state types, or CSTs, including states dominated by *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri*, or *Lactobacillus jensenii*, as well as a high-diversity state enriched with anaerobic bacteria (2). This framework has been widely used to interpret vaginal microbial ecology, although subsequent studies have shown that vaginal communities are dynamic rather than fixed. Longitudinal analyses revealed that some vaginal microbiota profiles remain relatively stable over time, whereas others shift rapidly in association with menstruation, sexual activity, hormonal fluctuations, antibiotic exposure, and other host or environmental factors (5). Therefore, vaginal health cannot be understood only by identifying the presence or absence of specific taxa; rather, it requires attention to temporal stability, microbial function, host context, and ecological resilience.

The protective role of *Lactobacillus*-dominant vaginal communities is closely linked to lactic acid production and maintenance of a low vaginal pH. Experimental and clinical studies have shown that vaginal lactobacilli can acidify the vaginal environment to levels that restrict the growth of many reproductive tract pathogens (6). Lactic acid, particularly under acidic conditions, has also been shown to possess microbicidal and immunomodulatory properties, suggesting that its biological relevance extends beyond pH reduction alone (3). Among lactobacilli, L. crispatus is often associated with a more stable and protective vaginal ecosystem, whereas L. iners may represent a more transitional state because it is frequently detected in both healthy and dysbiotic conditions (4, 7). This distinction is important because it challenges the simplified assumption that all *Lactobacillus*-dominant communities have equivalent biological functions.

Vaginal dysbiosis, classically represented by bacterial vaginosis, is characterized by depletion of protective lactobacilli and expansion of diverse anaerobic bacteria such as Gardnerella, Prevotella, Atopobium/Fannyhessea, Sneathia, and other bacterial vaginosis-associated organisms (8). Clinically, bacterial vaginosis is important not only because of symptoms such as discharge, odor, and discomfort, but also because it is associated with increased susceptibility to sexually transmitted infections, pelvic inflammatory disease, and adverse pregnancy outcomes (8, 9). The Centers for Disease Control and Prevention indicate that symptomatic bacterial vaginosis in pregnancy correlates with premature rupture of membranes, preterm delivery, intra-amniotic infection, and postpartum endometritis (9). These associations place the vaginal microbiome at the intersection of gynecology, obstetrics, infectious disease, and reproductive medicine.

Mechanistically, the vaginal microbiome may influence reproductive health through several interconnected pathways, including epithelial barrier integrity, mucosal immune activation, metabolic signaling, and pathogen exclusion. Anahtar et al. showed that cervicovaginal bacterial communities can strongly modulate host inflammatory responses in the female genital tract, with high-diversity communities correlating with elevated pro-inflammatory cytokines and immune activation (10). Such findings provide biological plausibility for the association between vaginal dysbiosis and reproductive tract disorders. Inflammation induced by dysbiotic communities may impair epithelial defense, alter cervical mucus properties, facilitate ascending infection, and disrupt immune tolerance required for implantation and pregnancy maintenance.

The vaginal microbiome is increasingly recognized as a clinically relevant factor in fertility, pregnancy, and gynecological health. In reproductive medicine, observational studies suggest that vaginal and endometrial microbial profiles may be associated with implantation, clinical pregnancy, miscarriage, and live birth outcomes after IVF/ICSI (11-13). Although causality remains uncertain, these findings indicate that the lower female reproductive tract microbiome may serve as a potential biomarker of reproductive success.

During pregnancy, vaginal microbial communities often become more stable and more *Lactobacillus*-dominant, but deviations from this pattern have been associated with adverse outcomes. Multi-omics studies have linked reduced L. crispatus and enrichment of bacterial vaginosis-associated taxa, such as BVAB1 and Sneathia amnii, with preterm birth risk (14, 15). Beyond fertility and pregnancy, vaginal dysbiosis has also been implicated in recurrent bacterial vaginosis, vulvovaginal candidiasis, pelvic inflammatory disease, HPV persistence, cervical disease, endometriosis, and polycystic ovary syndrome, although evidence for some conditions remains largely observational (16-19).

However, the concept of dysbiosis alone is insufficient to explain the complexity of host–microbiome interactions. Taxonomic profiling does not fully cap-

ture microbial function, metabolic activity, host immune responses, or treatment susceptibility. Therefore, multi-omics approaches, including metagenomics, metatranscriptomics, metabolomics, proteomics, and host transcriptomics, provide a more comprehensive framework for understanding vaginal microbial ecology (15, 20, 21). These advances are also supporting the development of personalized interventions, such as targeted probiotics, microbiome-based biomarkers, and experimental approaches including vaginal microbiome transplantation (22-24). Accordingly, this review synthesizes current evidence on the vaginal microbiome in female reproductive health, with a focus on multi-omics, precision diagnostics, and personalized microbiome-targeted strategies.

Composition and Dynamics of the Vaginal Microbiome

The vaginal microbiome is a unique mucosal environment mostly consisting of bacteria, accompanied by lesser quantities of fungus, viruses, archaea, and bacteriophages. In women of reproductive age, this environment is often characterized by diminished bacterial diversity and a predominance of Lactobacillus species, a pattern that distinguishes the vagina from many other human microbial habitats such as the gut, where high diversity is generally considered beneficial (2, 25). The dominance of lactobacilli is biologically important because these organisms contribute to vaginal acidification, competitive exclusion of pathogens, production of antimicrobial compounds, and modulation of local immune responses (26).

A widely used framework for describing vaginal bacterial communities is the classification into community state types, or CSTs. In the landmark study by Ravel et al., vaginal microbial communities from asymptomatic reproductive-age women clustered into five major CSTs: CST I dominated by Lactobacillus crispatus, CST II by Lactobacillus gasseri, CST III by Lactobacillus iners, CST V by Lactobacillus jensenii, and CST IV characterized by reduced lactobacilli and enrichment of diverse anaerobic bacteria (2). Importantly, the distribution of these CSTs varies across populations. Ravel et al. reported that Lactobacillus-dominant communities were more common among Asian and White women than among Hispanic and Black women, who more frequently had higher-diversity communities and higher vaginal pH values (2). These findings indicate that the concept of a “normal” vaginal microbiome should be interpreted cautiously and within demographic, hormonal, and ecological context. The major vaginal community state types and their clinical interpretations are summarized in Table 1.

Among lactobacilli, L. crispatus is often considered one of the most protective species because it is associated with low vaginal pH, high lactic acid production, and relatively stable microbial communities (27). In contrast, L. iners occupies a more ambiguous ecological position. Although it is highly prevalent in the vagina and can dominate apparently healthy communities, it is also frequently detected during transitions between eubiosis and dysbiosis and after treatment for bacterial vaginosis (28). This dual behavior may be partly explained by its smaller genome, limited biosynthetic capacity, and ability to persist in fluctuating vaginal environments (28). Therefore, not all Lactobacillus-dominated states should be considered functionally equivalent.

The safeguarding characteristics of lactobacilli are intimately associated with estrogen-dependent vaginal

Table 1. Major vaginal community state types and their clinical interpretation.

Community state type	Dominant microbial pattern	General ecological interpretation	Potential clinical relevance
CST I	Lactobacillus crispatus	Stable, low-diversity, lactic acid-rich state	Often associated with vaginal health and lower dysbiosis risk
CST II	Lactobacillus gasseri	Lactobacilli-dominant state	Generally considered protective, but less frequently dominant
CST III	Lactobacillus iners	Flexible or transitional lactobacilli-dominant state	May occur in both health and dysbiosis-associated transitions
CST V	Lactobacillus jensenii	Low-diversity lactobacilli-dominant state	Usually associated with low pH and vaginal stability
CST IV	Gardnerella, Prevotella, Fannyhessea/Atopobium, Sneathia, anaerobes	High-diversity, low-lactobacilli state	Associated with BV, inflammation, higher pH, and infection susceptibility

physiology. Estrogen promotes the development of the vaginal epithelium and the buildup of glycogen, which supplies substrates that sustain lactobacilli and lactic acid synthesis (26, 29). The resulting acidic environment, commonly around pH 3.5–4.5 in many reproductive-age women, restricts the growth of numerous opportunistic and sexually transmitted pathogens (25, 29). This relationship can be summarized as:

Estrogen↑→Glycogen↑→Lactobacillus↑→Lactic Acid↑→pH↓→Pathogen Growth↓

However, vaginal microbial composition is not static. Longitudinal studies have shown that some women maintain stable Lactobacillus-dominated communities over time, whereas others experience frequent transitions between CSTs (5). In a 16-week longitudinal study, Gajer et al. demonstrated that vaginal microbial communities may change markedly over short periods, with some CSTs showing greater temporal instability than others (5). These findings emphasize that single time-point sampling may not fully capture vaginal microbial ecology, especially in women with fluctuating or transitional microbiota.

Numerous internal and external variables influence the structure and behavior of the vaginal microbiome. The menstrual cycle is one of the most important physiological drivers. Daily sampling studies have shown that menstruation is often associated with increased microbial diversity, decreased relative abundance of lactobacilli, and higher rates of community change (30). Menstrual blood can transiently increase vaginal pH and introduce substrates such as iron, creating conditions that may favor non-Lactobacillus organisms. After menstruation, many women return to a more lactobacilli-dominant state, although the speed and completeness of recovery vary among individuals (30). Key physiological, behavioral, and environmental factors influencing vaginal microbiome dynamics are presented in Table 2.

Pregnancy represents another major physiological state associated with vaginal microbial remodeling. In normal pregnancy, the vaginal microbiota often becomes more stable and more strongly dominated by Lactobacillus species compared with the non-pregnant state. Romero et al. reported that pregnant women commonly maintained Lactobacillus-dominated CSTs throughout gestation, with higher stability than non-pregnant women (31). This stability may reflect elevated estrogen levels, increased glycogen deposition, and immune adaptations that support reproductive tolerance. Nevertheless, pregnancy-associated microbial patterns vary across individuals and populations, and deviations from stable Lactobacillus dominance have been linked in later sections to adverse obstetric outcomes.

At the other end of the reproductive lifespan, menopause is commonly associated with reduced estrogen, decreased epithelial glycogen, increased vaginal pH, and lower abundance of lactobacilli (32). These changes may contribute to genitourinary syndrome of menopause, recurrent urinary symptoms, vaginal dryness, ir-

Table 2. Major factors influencing vaginal microbiome dynamics.

Factor	Typical microbial effect	Biological explanation
Estrogen	Increased lactobacilli, lower pH	Promotes epithelial glycogen and lactic acid production
Menstruation	Reduced lactobacilli, increased diversity	Menstrual blood raises pH and alters nutrient availability
Pregnancy	Greater stability and lactobacilli dominance	High estrogen and pregnancy-related immune adaptation
Menopause	Reduced lactobacilli, increased pH	Estrogen decline reduces glycogen availability
Sexual activity	Variable; may increase diversity	Semen and partner-associated microbes may alter pH and composition
Antibiotics	Reduced microbial stability	May deplete lactobacilli and allow recolonization by anaerobes
Hormonal contraception	Often more lactobacilli-dominant	Hormonal effects may reduce BV risk in some populations
Copper IUD	May increase BV-associated diversity in some studies	Possible effects on bleeding patterns and local inflammation
Douching/intimate hygiene products	Increased dysbiosis risk	Disruption of pH, mucus, and resident lactobacilli

ritation, and increased susceptibility to infections (33). Hormone therapy can partially restore lactobacilli and reduce vaginal pH in some postmenopausal women, supporting the role of estrogen in maintaining the vaginal microbial environment (32).

Behavioral and environmental factors also contribute to vaginal microbiome variability. Sexual activity, semen exposure, condom use, douching, smoking, intimate hygiene practices, and antibiotic exposure may alter vaginal pH, introduce new microbes, or disrupt lactobacilli-dominant states. Reviews of behavioral influences suggest that practices such as vaginal douching are associated with dysbiosis and bacterial vaginosis, while condom use may help maintain lactobacilli dominance by reducing exposure to alkaline semen and partner-associated microbes (34). Contraceptive methods may also influence vaginal microbial ecology. Hormonal contraception has often been associated with a lower risk of bacterial vaginosis and more lactobacilli-dominant profiles, whereas copper intrauterine devices have been linked in some studies to increased bacterial vaginosis risk and higher microbial diversity (35, 36). However, contraceptive effects are not uniform and may vary by formulation, route, duration of use, and host factors.

Non-Lactobacillus-dominant communities are typically more diverse and enriched with anaerobic and facultative anaerobic bacteria, including Gardnerella, Prevotella, Fannyhessea/Atopobium, Sneathia, Mobiluncus, Megasphaera, and microorganisms linked to bacterial vaginosis (37). These communities are often grouped under CST IV and are commonly associated with bacterial vaginosis-like states, higher vaginal pH, inflammation, epithelial barrier disruption, and increased susceptibility to reproductive tract infections (4, 37). Nevertheless, high-diversity communities are not always symptomatic, and their clinical meaning may differ across populations. This reinforces the need to distinguish taxonomic composition from microbial function and host response.

From an ecological perspective, the vaginal microbiome can be understood in terms of stability, resilience, and transition. Stability refers to the maintenance of a microbial state over time, resilience refers to the ability to return to a previous state after disturbance, and transition refers to movement from one community configuration to another. For example, a stable L. crispatus-dominant community may resist perturbation after menstruation or sexual exposure, whereas an L. iners-dominant or CST IV community may be more likely to transition toward dysbiosis or recurrence after treatment (5, 28). This ecological interpretation is particularly important for the theme of this review because it moves beyond a simple "healthy versus dysbiotic" model and highlights the dynamic behavior of microbial communities.

Overall, the composition of the vaginal microbiome is determined by the interaction of microbial taxa, host hormones, epithelial metabolism, immune responses, behavior, reproductive stage, and environmental exposures. Although Lactobacillus dominance, particularly L. crispatus, is generally associated with vaginal health, a complete understanding of vaginal microbial ecology requires consideration of temporal dynamics, population diversity, microbial function, and host context. This dynamic framework provides the foundation for multi-omics approaches, personalized diagnostics, and microbiome-targeted interventions discussed in later sections.

Moving Beyond Dysbiosis: Functional and Ecological Perspectives

The term dysbiosis has been widely used to describe vaginal microbial imbalance, particularly the depletion of Lactobacillus species and enrichment of anaerobic bacteria associated with bacterial vaginosis. Although this concept remains clinically useful, it is often too broad to explain the biological complexity of the vaginal ecosystem. A taxonomic shift alone does not reveal whether microbes are metabolically active, whether they induce inflammation, whether they form biofilms, or whether the host can tolerate or recover from that state (4, 5). Recent reviews, therefore, emphasize the need to move from a purely compositional definition of dysbiosis toward a more functional and ecological interpretation of vaginal microbiota. This conceptual transition from a taxonomic dysbiosis model to a functional–ecological framework is illustrated in Figure 1.

A functional perspective asks not only "which bacteria are present?" but also "what are they doing?". For example, two women may both have Lactobacillus-dominant microbiota, but their communities may differ in lactic acid production, antimicrobial activity, epithelial adhesion, immune modulation, and resilience after disturbance. Lactobacillus crispatus is often linked to a more stable, lactic acid-rich, low-pH environment, whereas Lactobacillus iners may occupy a more transitional ecological niche and is frequently observed in both healthy and dysbiotic states (28, 38). This distinction shows that even similar taxonomic profiles may have different biological meanings.

Conversely, anaerobe-rich communities are not merely "imbalanced" versions of healthy microbiota; they may represent functionally distinct ecosystems. In bacterial vaginosis, organisms such as Gardnerella, Prevotella, Sneathia, Fannyhessea/Atopobium, and Megasphaera can contribute to higher pH, production of amines and other metabolites, epithelial disruption, and inflammatory signaling (20, 39). Some Gardnerella-associated communities may also form polymicrobial biofilms that protect bacteria from host defenses and antimicrobial treatment, helping to explain recur-

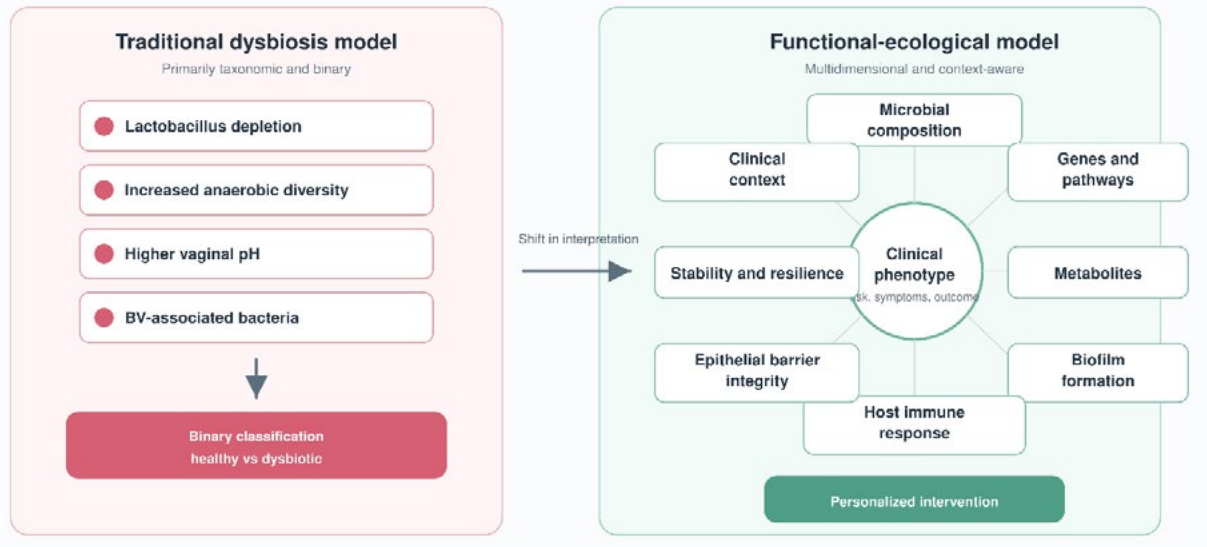

Figure 1. Moving beyond dysbiosis in vaginal microbiome research. Traditional models define vaginal dysbiosis mainly by taxonomic changes, including depletion of Lactobacillus species and enrichment of anaerobic bacteria. A functional–ecological framework integrates microbial composition with gene content, metabolic activity, biofilm formation, host immune responses, epithelial barrier integrity, ecological stability, and clinical context. This multidimensional approach may improve risk stratification, biomarker discovery, and personalized microbiome-targeted interventions.

rence after standard therapy (40).

Host response is another key reason why dysbiosis should not be interpreted only as a microbial pattern. Anahtar et al. showed that cervicovaginal bacterial communities can strongly modulate local inflammatory responses, with high-diversity communities associated with increased genital inflammation (10). This suggests that clinical risk may depend on the interaction between microbial composition and host immunity rather than microbial composition alone. Therefore, a vaginal microbiome profile should ideally be interpreted together with inflammatory markers, epithelial barrier status, hormonal state, and clinical phenotype.

Metabolomic studies further support this functional view. Srinivasan et al. demonstrated that bacterial vaginosis is associated with strong metabolic signatures involving amino acid, carbohydrate, and lipid metabolism (20). Similarly, multi-platform metabolomics studies identified metabolites associated with bacterial diversity and clinical BV, suggesting that metabolic biomarkers may improve diagnosis and risk stratification beyond conventional microscopy or 16S rRNA profiling (41). These findings indicate that vaginal health is shaped by microbial activity and biochemical output, not simply by the presence or absence of specific taxa.

From an ecological perspective, the vaginal microbiome should be understood in terms of stability, resilience, resistance, and transition. Stability refers to the persistence of a microbial state over time; resistance refers to the ability to withstand disturbance; resilience refers to recovery after disturbance; and transition refers to movement between community states. Longitudinal studies have shown that vaginal communities can shift rapidly over short periods and that these shifts may be influenced by menstruation, sexual activity, bacterial composition, and other host factors (42). Thus, a single cross-sectional sample may not fully represent a woman's vaginal microbial ecology.

The development of gene-centered and multi-omics resources has accelerated this transition from descriptive microbiome research to functional ecology. The VIRGO catalog provided a vaginal microbiome-specific gene reference containing approximately 0.95 million non-redundant genes, enabling more detailed metagenomic and metatranscriptomic analyses (43). More recently, VIRGO2 expanded this framework to more than 1.7 million annotated genes, aiming to improve understanding of vaginal microbial function, ecology, and strain-level variation (44). Such tools allow researchers to investigate microbial pathways, virulence potential, metabolic capacity, and host–microbe interactions with greater precision.

Overall, moving beyond dysbiosis means shifting from a binary model of "healthy versus abnormal" microbiota toward a multidimensional model that integrates microbial composition, functional capacity, metabolic activity, host immunity, ecological stability, and clinical context. This approach is particularly important for precision gynecology, where the same taxonomic community may have different implications depending on reproductive stage, symptoms, immune response, recurrence risk, and fertility goals.

Multi-Omics Approaches in Vaginal Microbiome Research

Traditional vaginal microbiome studies have largely relied on culture-based methods, microscopy, Nugent

scoring, Amsel criteria, and later 16S rRNA gene sequencing. Although these approaches have been essential for defining microbial composition and community state types, they provide limited information about microbial function, gene expression, metabolite production, host response, and strain-level variation. Multi-omics approaches address these limitations by integrating complementary molecular layers, including metagenomics, metatranscriptomics, metabolomics, metaproteomics, and host immune or transcriptomic profiling. This shift is central to understanding the vaginal microbiome beyond dysbiosis, because similar taxonomic profiles may differ substantially in biological activity and clinical impact (15, 43, 44).

16S rRNA Sequencing and Shotgun Metagenomics

16S rRNA gene sequencing remains widely used because it is relatively cost-effective and suitable for profiling bacterial community composition. It can identify dominant taxa and classify samples into CSTs, but it often has limited species- or strain-level resolution and provides only indirect functional inference. In contrast, shotgun metagenomics sequences total microbial DNA, allowing more detailed assessment of microbial genes, functional pathways, virulence potential, antimicrobial resistance genes, and strain-level diversity (45, 46). A major advance in this field was the development of VIRGO, a vaginal microbiome-specific non-redundant gene catalog containing approximately 0.95 million genes (43). More recently, VIRGO2 expanded this resource to more than 1.7 million annotated genes and improved representation of bacteria, fungi, viruses, and phages from vaginal samples (44). These gene-centered resources enable more precise functional profiling of vaginal metagenomic and metatranscriptomic datasets.

Metatranscriptomics

Metatranscriptomics analyzes microbial RNA and therefore identifies which genes are actively expressed in vivo. This is particularly valuable because microbial abundance does not always reflect microbial activity. For example, integrative metagenomic and metatranscriptomic analyses have shown that the transcriptional activity of Lactobacillus iners, Lactobacillus crispatus, and Gardnerella vaginalis can vary depending on the surrounding microbial community (47, 48).

Metatranscriptomic studies have also revealed functional differences between health-associated and bacterial vaginosis-associated communities. A recent meta-analysis of vaginal metatranscriptomes identified functional BV subgroups characterized by pathways related to motility, chemotaxis, biofilm formation, epithelial barrier disruption, and resistance to host antimicrobial peptides (49). These findings suggest that transcriptomic states may provide more clinically meaningful information than taxonomy alone.

Metabolomics

Metabolomics measures small molecules produced by microbes, host cells, or their interactions. This layer is especially relevant in the vagina because metabolites such as lactic acid, amines, short-chain fatty acids, amino acid catabolites, and lipid mediators can influence pH, inflammation, odor, epithelial integrity, and pathogen growth (50). Srinivasan et al. demonstrated that bacterial vaginosis correlates with pronounced metabolic signatures in amino acid, carbohydrate, and lipid metabolism, with distinct metabolite profiles seen between women with and without BV (20). In a similar vein, McMillan et al. used a multi-platform metabolomics strategy and discovered a robust correlation between the vaginal metabolome and bacterial diversity; metabolites including 2-hydroxyisovalerate and γ-hydroxybutyrate were associated with high-diversity BV-related conditions (51). These studies demonstrate that metabolomics can identify functional biomarkers that may improve diagnosis and risk stratification beyond compositional profiling.

Metaproteomics and Host-Response Profiling

Metaproteomics provides information about proteins expressed by both microbes and host cells. Although technically more challenging than DNA-based approaches, it can help identify active microbial pathways, host inflammatory proteins, antimicrobial peptides, and epithelial response markers. Studies using cervicovaginal or residual Pap test samples suggest that metaproteomics can complement sequencing by linking microbial composition to expressed proteins and functional activity (52).

Data Integration and Clinical Translation

The major strength of multi-omics is its ability to connect multiple biological layers:

Taxa→Genes→Transcripts→Proteins→Metabolites→Host Response→Clinical Phenotype

This integrated framework can help identify microbial biomarkers for bacterial vaginosis recurrence, preterm birth risk, IVF outcomes, HPV persistence, and response to microbiome-targeted interventions. Nonetheless, the clinical translation is constrained by restricted sample numbers, varied research designs, disparate sequencing technologies, contamination hazards, batch effects, high-dimensional data, and the need for standardized bioinformatic workflows. Future studies should prioritize longitudinal sampling, diverse populations, harmonized metadata, and validation of multi-omics biomarkers in independent cohorts. A multi-omics framework can link microbial composition, functional activity, host response, and clinical phenotype to support mechanism-based interpretation

of vaginal microbial states (Figure 2).

Overall, multi-omics approaches provide a functional and mechanistic framework for vaginal microbiome research. Rather than asking only which microbes are present, they allow researchers to determine what microbial communities can do, what they are actively expressing, what metabolites they produce, how the host responds, and how these interactions influence reproductive health.

Host–Microbiome Interactions and Mechanistic Pathways

The vaginal microbiome influences female reproductive health through continuous interactions with the mucosal epithelium, local immune system, hormones, metabolites, and reproductive tract physiology. These interactions help determine whether a microbial community remains protective, becomes inflammatory, or contributes to disease susceptibility. Therefore, vaginal microbial states should be interpreted not only by taxonomic composition but also by their functional effects on the host environment (3, 4, 53).

A central protective mechanism of Lactobacillus-dominant communities is the maintenance of an acidic vaginal environment. Vaginal lactobacilli metabolize glycogen-derived substrates and produce lactic acid, which lowers vaginal pH and restricts the growth of many opportunistic and sexually transmitted pathogens. Lactic acid also has direct antimicrobial and immune-modulatory effects, suggesting that its role extends beyond simple acidification (54). In particular, Lactobacillus crispatus is often associated with strong lactic acid production, lower pH, and greater ecological stability, whereas Lactobacillus iners may be more permissive to transition toward dysbiosis (4).

The epithelial barrier is another key site of host–microbiome interaction. A healthy vaginal epithelium provides a physical and biochemical barrier through tight junctions, mucus, antimicrobial peptides, and immune mediators. Experimental studies indicate that physiological concentrations of lactic acid can enhance cervicovaginal epithelial barrier integrity and increase the expression of junction-associated molecules (55). In contrast, dysbiotic communities enriched with anaerobes may compromise barrier function through inflammatory metabolites, mucin degradation, and enzymes such as sialidases and mucinases (39, 56). These processes may facilitate pathogen adherence, epithelial exfoliation, and ascending infection.

Local immune modulation is a major pathway linking the vaginal microbiome to reproductive outcomes. Anahtar et al. demonstrated that cervicovaginal bacterial communities are strong modulators of genital tract inflammation, with high-diversity communities associated with increased pro-inflammatory cytokines and immune activation (10). Such inflammation may increase vulnerability to sexually transmitted diseases, impair mucosal tolerance, and contribute to adverse reproductive outcomes. Conversely, lactobacilli-dominant communities are generally associated with lower inflammatory tone and a more protective mucosal environment (53, 55).

Microbial metabolites provide an important biochemical bridge between vaginal microbes and host tissues. In eubiotic states, lactic acid predominates and supports antimicrobial defense, epithelial protection, and immune regulation. In bacterial vaginosis-associated states, lactic acid is often depleted, while short-chain fatty acids, amines, succinate, and other metabolites increase (57). These metabolic shifts can affect

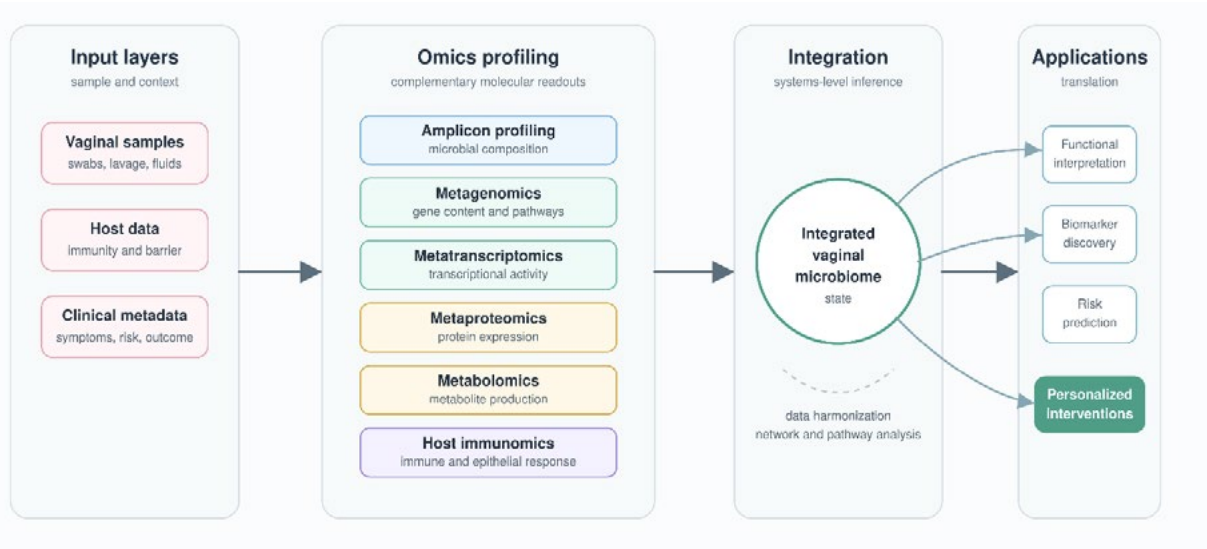

Figure 2. Multi-omics approaches in vaginal microbiome research. Multi-omics integration connects microbial composition with gene content, transcriptional activity, protein expression, metabolite production, host immune responses, and clinical phenotypes. This framework enables a more functional interpretation of vaginal microbial states and supports biomarker discovery, risk prediction, and personalized interventions.

vaginal odor, pH, epithelial integrity, cytokine production, and pathogen growth. Therefore, metabolite profiles may be more directly linked to biological activity than microbial composition alone (57).

Biofilm formation represents another important mechanism, especially in recurrent bacterial vaginosis. Gardnerella species and other BV-associated bacteria can adhere to vaginal epithelial cells and form polymicrobial biofilms, which may protect bacteria from host defenses and reduce antibiotic penetration (58, 59). This helps explain why bacterial vaginosis frequently recurs after standard antimicrobial therapy. However, Gardnerella is not uniformly pathogenic; recent work shows species- and strain-level heterogeneity in virulence potential, suggesting that clinical risk depends on microbial function, community context, and host response (60).

These pathways are particularly relevant to fertility and pregnancy. Dysbiosis-associated inflammation, impaired epithelial barrier integrity, altered metabolites, and ascending infection may influence sperm survival, embryo implantation, cervical remodeling, membrane integrity, and preterm birth risk. Recent pregnancy-focused studies and reviews suggest that vaginal microbiota can influence pregnancy outcomes through immune modulation, epithelial integrity, and inflammatory pathways, although the strength of these associations varies across populations and study designs (14, 61).

Overall, host–microbiome interactions in the vagina operate through interconnected mechanisms: acidification, antimicrobial activity, epithelial barrier maintenance, immune regulation, metabolic signaling, biofilm formation, and ecological stability. These mechanisms explain why similar taxonomic profiles may lead to different clinical outcomes and why functional biomarkers are increasingly important for precision gynecology and reproductive medicine.

Vaginal Microbiome and Fertility

The vaginal microbiome may influence fertility through its effects on the cervicovaginal environment, sperm survival, mucosal inflammation, ascending microbial exposure, endometrial receptivity, and implantation. In general, Lactobacillus-dominant communities, particularly those enriched with Lactobacillus crispatus, are considered more favorable for reproductive health, whereas high-diversity anaerobe-rich communities and bacterial vaginosis-associated dysbiosis have been linked to poorer reproductive outcomes (62-64).

Evidence from both natural conception and assisted reproduction suggests a potential relationship between vaginal microbial composition and fertility. In a prospective study of pregnancy attempts, persistent bacterial vaginosis was associated with reduced fecundability, indicating that vaginal dysbiosis may negatively affect the probability of conception in some populations (63). In infertility and IVF cohorts, abnormal vaginal microbiota has been associated with lower clinical pregnancy rates and higher risk of early pregnancy loss, although findings are not fully consistent across studies (11, 65, 66).

In assisted reproductive technology, the vaginal microbiome has gained attention as a possible predictor of IVF/ICSI outcomes. Koedooder et al. reported that vaginal microbiome profiling before IVF or IVF-ICSI could stratify the probability of pregnancy, supporting its potential use as a reproductive biomarker (11). Similarly, a meta-analysis by Singer et al. found that abnormal vaginal microbiota was associated with lower rates of early pregnancy development after IVF, although methodological heterogeneity remained a major limitation (65). Additional ART research has shown that Lactobacillus-dominant vaginal microbiome could correlate with enhanced implantation and pregnancy outcomes (67).

The endometrial microbiome may also be relevant because embryo implantation occurs in the uterine cavity. Moreno et al. proposed that a non-Lactobacillus-dominant endometrial microbiota may be associated with implantation failure and poorer reproductive outcomes (12). However, the existence, composition, and clinical relevance of the low-biomass endometrial microbiome remain debated because of contamination risk, sampling differences, and methodological variability (68, 69). Therefore, vaginal microbiome findings should not be directly extrapolated to the endometrium without careful interpretation.

Several mechanisms may explain the association between vaginal dysbiosis and reduced fertility. Dysbiotic communities may increase vaginal pH, reduce lactic acid, promote local inflammation, alter cervical mucus, facilitate pathogen ascension, and impair immune tolerance required for implantation. In contrast, Lactobacillus-dominant communities may support a low-inflammatory, acidic, and pathogen-resistant environment that is more compatible with conception and embryo implantation (70, 71).

Despite growing interest, vaginal microbiome testing is not yet a routine standard in fertility care. Most available studies are observational; sample sizes are often small, and definitions of “favorable” or “unfavorable” microbiota vary across studies. Future research should prioritize longitudinal sampling before and during fertility treatment, standardized sequencing and diagnostic methods, integration with endometrial and immune markers, and interventional trials testing whether microbiome modulation can improve live birth outcomes (72).

Overall, current evidence suggests that the vaginal microbiome may act as both a biomarker and a pos-

sible modifiable factor in fertility. However, stronger causal evidence is needed before microbiome-based diagnostics or interventions can be routinely integrated into reproductive medicine.

Vaginal Microbiome and Pregnancy Outcomes

Pregnancy is associated with marked hormonal, immunological, and epithelial changes that influence the vaginal microbiome. In uncomplicated pregnancies, vaginal microbial communities often become more stable and more strongly dominated by Lactobacillus species compared with the non-pregnant state (31). This stability is thought to support maternal–fetal health by maintaining low vaginal pH, limiting pathogen overgrowth, and reducing the risk of ascending infection.

The most compelling evidence connects changes in the vaginal microbiota with premature delivery. Numerous longitudinal investigations have shown that diminished Lactobacillus prevalence and heightened concentrations of anaerobic or bacterial vaginosis-related taxa could correlate with spontaneous premature delivery (14, 31, 73). In a significant multi-omics investigation, Fettweis et al. found that women who had preterm delivery exhibited reduced vaginal concentrations of Lactobacillus crispatus and elevated levels of BVAB1, Sneathia amnii, TM7-H1, and other Prevotella species (14). Kindinger et al. similarly discovered that the prevalence of Lactobacillus iners at 16 weeks of gestation correlated with a shortened cervix and premature delivery, whereas dominance of L. crispatus was indicative of full-term birth (73).

Vaginal dysbiosis may contribute to adverse pregnancy outcomes through multiple mechanisms, including increased vaginal pH, reduced lactic acid, epithelial barrier disruption, local inflammation, biofilm formation, and ascending infection. These pathways may promote cervical remodeling, weakening of fetal membranes, intra-amniotic inflammation, and premature rupture of membranes (9, 74, 75). The CDC further observes that symptomatic bacterial vaginosis in pregnancy is linked to premature rupture of membranes, preterm delivery, intra-amniotic infection, and postpartum endometritis (9).

Beyond preterm birth, altered vaginal microbiota has been investigated in relation to PPROM, chorioamnionitis, miscarriage, low birth weight, and neonatal outcomes. However, the evidence is less consistent for these outcomes than for preterm birth, partly because studies differ in sampling time, sequencing methods, population characteristics, diagnostic criteria, and adjustment for confounders. Importantly, microbiome-associated pregnancy risk may also vary by ancestry, geography, socioeconomic context, prior obstetric history, and host immune response (76).

Overall, a stable Lactobacillus-dominant vaginal microbiome, particularly one enriched with L. crispatus, appears to be associated with more favorable pregnancy outcomes, whereas high-diversity anaerobe-rich communities may indicate increased obstetric risk. Nevertheless, vaginal microbiome profiling is not yet a routine clinical tool for predicting pregnancy complications. Future studies should combine longitudinal microbiome sampling with metagenomics, metabolomics, cytokine profiling, cervical length measurement, and clinical risk factors to improve prediction and prevention of adverse pregnancy outcomes.

Table 3. Key gynecological disorders associated with vaginal microbiome alterations.

Disorder	Common microbiome-associated pattern	Possible mechanism	Strength of evidence
Bacterial vaginosis	Low Lactobacillus, high anaerobic diversity	Higher pH, biofilm, amines, inflammation	Strong
Vulvovaginal candidiasis	Variable bacterial profile; fungal overgrowth	Host immune response, fungal adhesion, microbial interactions	Moderate
Pelvic inflammatory disease	BV-like anaerobic communities	Ascending infection, epithelial disruption, inflammation	Moderate
HPV persistence / cervical dysplasia	High diversity, L. iners or non-Lactobacillus dominance	Inflammation, impaired antiviral immunity, barrier disruption	Moderate to strong
Endometriosis	Altered vaginal/gut/reproductive tract microbiota	Inflammation, immune dysregulation, estrogen-related pathways	Emerging
PCOS	Possible vaginal and gut dysbiosis	Metabolic inflammation, endocrine–immune interactions	Emerging

Vaginal Microbiome and Gynecological Disorders

The vaginal microbiome is closely linked to several gynecological disorders, particularly conditions involving infection, inflammation, epithelial barrier disruption, and altered mucosal immunity. Although not all associations are causal, a recurring pattern across many disorders is the depletion of protective Lactobacillus species and enrichment of anaerobic or pathobiont-dominated communities (8).

Bacterial vaginosis (BV) is the most established example of vaginal dysbiosis. It is characterized by reduced Lactobacillus abundance and expansion of anaerobic bacteria such as Gardnerella, Prevotella, Fannyhessea/Atopobium, Mobiluncus, Sneathia, and BV-associated bacteria (9). BV is clinically significant due to its correlation with vaginal symptoms, recurrence post-treatment, heightened vulnerability to sexually transmitted infections, pelvic inflammatory disease, and negative reproductive consequences. Mechanistically, BV-associated communities may increase vaginal pH, produce amines and short-chain fatty acids, form biofilms, degrade mucus, and promote local inflammation (8).

Vulvovaginal candidiasis (VVC) is another common vaginal disorder, but its relationship with the bacterial microbiome is more complex than BV. VVC is mainly caused by Candida species, especially Candida albicans, and may occur even in the presence of lactobacilli-dominant bacterial communities (77, 78). Therefore, VVC should not be interpreted simply as a bacterial dysbiosis. Instead, disease expression depends on fungal overgrowth, epithelial adhesion, host immune response, estrogen status, antibiotic exposure, and local microbial interactions. Recurrent VVC may involve altered bacterial–fungal interactions and metabolic changes, but stronger longitudinal and mechanistic studies are still needed (77).

The vaginal microbiome is also relevant to pelvic inflammatory disease (PID) and sexually transmitted infection susceptibility. BV-like communities may facilitate ascending infection by weakening epithelial and mucosal barriers, increasing inflammation, and reducing colonization resistance against pathogens (79, 80). Although classic PID pathogens include Chlamydia trachomatis and Neisseria gonorrhoeae, anaerobic bacteria and BV-associated taxa are increasingly recognized as contributors to polymicrobial upper genital tract infection (80).

A growing body of evidence links vaginal microbiota composition with human papillomavirus (HPV) infection, HPV persistence, cervical intraepithelial neoplasia, and cervical cancer risk. Systematic reviews and meta-analyses suggest that Lactobacillus crispatus-dominant communities are generally associated with lower HPV-related risk, whereas Lactobacillus iners-dominant, non-Lactobacillus-dominant, and high-diversity anaerobic communities are more often associated with HPV infection and cervical dysplasia (81-83). Possible mechanisms include altered vaginal pH, epithelial barrier disruption, inflammation, and impaired local antiviral immunity. However, causality remains uncertain, and vaginal microbiota should be considered a potential cofactor rather than a direct cause of cervical disease.

Emerging evidence also suggests possible associations between vaginal or reproductive tract microbiota and inflammatory or endocrine gynecological conditions such as endometriosis and polycystic ovary syndrome (PCOS). In endometriosis, microbiome alterations may interact with chronic inflammation, immune dysregulation, estrogen metabolism, and pelvic pain pathways (18, 84). In PCOS, studies suggest possible links between dysbiosis, hyperandrogenism, insulin resistance, and chronic low-grade inflammation, although most evidence currently comes from gut microbiome studies and limited vaginal microbiome data (19, 85). Therefore, these conditions should be discussed as promising but still developing areas of research.

Overall, vaginal microbiome alterations are most strongly established in BV and increasingly supported in HPV-related cervical disease, PID susceptibility, and recurrent vaginal infections. For endometriosis and PCOS, evidence remains preliminary and heterogeneous. Future research should distinguish correlation from causation, integrate bacterial, fungal, viral, metabolic, and immune data, and determine whether microbiome-targeted interventions can improve gynecological outcomes.

Personalized Microbiome-Based Interventions

Personalized interventions targeting the vaginal microbiome aim to move beyond uniform treatment strategies toward approaches guided by microbial composition, recurrence risk, symptoms, reproductive goals, and host factors. Current clinical management of bacterial vaginosis still relies mainly on antibiotics such as metronidazole or clindamycin, but recurrence remains common and optimal strategies for persistent or recurrent BV are still limited (8, 9). This has encouraged interest in microbiome-restorative approaches that not only suppress anaerobic bacteria but also promote durable recolonization by protective lactobacilli, particularly Lactobacillus crispatus.

Probiotics and live biotherapeutic products are among the most studied strategies. A randomized trial showed that L. crispatus CTV-05, known as Lactin-V, significantly reduced BV recurrence after vaginal metronidazole treatment compared with placebo, supporting the concept of strain-specific microbiome restoration (22). Meta-analytic evidence also suggests that probiotics may reduce BV recurrence, although effects

vary by strain, dose, route of administration, and study design (86). Therefore, probiotic therapy should not be treated as a single uniform intervention; its efficacy likely depends on selecting appropriate strains and matching them to the patient's baseline microbiome and clinical context.

More experimental strategies include vaginal microbiome transplantation and partner-based treatment. Vaginal microbiome transplantation has been reported in an exploratory study of women with intractable recurrent BV, showing potential benefit but requiring larger controlled trials, strict donor screening, and long-term safety evaluation before clinical adoption (23). More recently, a randomized trial found that treating male partners with combined oral and topical antimicrobial therapy reduced BV recurrence in women in monogamous heterosexual couples, highlighting the role of partner-associated microbial exchange (24). Overall, the future of vaginal microbiome intervention will likely involve stratified approaches that combine antibiotics, targeted live biotherapeutics, behavioral or partner-based strategies, and multi-omics biomarkers to predict treatment response and recurrence risk (87).

Microbiome-Based Biomarkers and AI-Driven Prediction

Microbiome-based biomarkers are increasingly being explored for risk stratification in reproductive and gynecological health. These biomarkers may include taxonomic markers such as community state types, Lactobacillus crispatus dominance, Lactobacillus iners dominance, or enrichment of BV-associated anaerobes, as well as functional markers such as microbial genes, metabolites, cytokines, and host-response profiles. In reproductive medicine, vaginal microbiome profiling before IVF/ICSI has been proposed as a potential predictor of pregnancy outcome (11). In bacterial vaginosis, metabolomic studies have identified distinct biochemical signatures involving amino acid, carbohydrate, lipid, and amine metabolism, suggesting that functional biomarkers may improve diagnosis beyond taxonomy alone (20, 51). Similarly, vaginal microbial patterns have been associated with HPV persistence and cervical dysplasia risk, supporting their potential value as adjunctive biomarkers in cervical disease research (81).

Artificial intelligence and machine learning can help integrate high-dimensional microbiome data with clinical, hormonal, immunological, and reproductive variables. Early machine-learning studies showed that vaginal microbial profiles could classify bacterial vaginosis-associated communities with high accuracy (88). In pregnancy research, models combining vaginal microbiota with clinical markers such as cervical length and white blood cell count have been used to predict preterm birth risk (89). The Microbiome Preterm Birth DREAM Challenge further demonstrated the potential of harmonized multi-cohort microbiome datasets for machine-learning prediction of preterm and early preterm birth (90). More recent deep-learning and IVF-focused models have begun integrating vaginal microbiome composition with inflammatory markers to improve the prediction of preterm birth or IVF success (91, 92).

Despite these advances, microbiome-based prediction is not yet ready for routine clinical use. Many models are limited by small cohorts, population heterogeneity, batch effects, differences in sequencing methods, inconsistent outcome definitions, and insufficient external validation. In addition, AI models must be interpretable and clinically actionable, not merely statistically accurate. Future biomarker studies should prioritize longitudinal sampling, multi-omics integration, standardized metadata, diverse populations, explainable AI, and prospective validation. Ultimately, microbiome-based biomarkers and AI-driven models may support precision gynecology by identifying women at higher risk of BV recurrence, IVF failure, preterm birth, or HPV persistence and by guiding personalized prevention or treatment strategies.

Challenges, Limitations, and Ethical Considerations

Despite rapid progress, vaginal microbiome research faces several methodological challenges. Differences in sampling site, menstrual or pregnancy timing, DNA extraction, sequencing platform, reference database, bioinformatic pipeline, and clinical definitions of dysbiosis or bacterial vaginosis can limit comparability across studies (93). Standardized reporting frameworks such as the STORMS checklist are therefore important for improving transparency, reproducibility, and cross-study interpretation (93). Another major issue is contamination, especially in low-biomass reproductive tract samples such as endometrial, placental, or upper genital tract specimens. Without appropriate negative controls, contamination-aware analysis, and biologically plausible interpretation, microbial signals in low-biomass samples may be overinterpreted (94, 95).

A second limitation is the difficulty of establishing causality. Many studies are cross-sectional or observational, making it unclear whether vaginal dysbiosis causes reproductive or gynecological disorders, results from them, or acts as a biomarker of another underlying process. Longitudinal sampling, mechanistic experiments, and interventional trials are needed to determine whether modifying the vaginal microbiome can improve outcomes such as IVF success, bacterial vaginosis recurrence, HPV persistence, or preterm birth. Multi-omics and AI-based models also face analytical challenges, including small sample sizes,

high-dimensional data, compositionality, sparsity, batch effects, overfitting, and limited external validation (96-98). Therefore, prediction models should be tested in independent and diverse cohorts before being translated into clinical practice.

Ethical considerations are equally important. Vaginal microbiome data are highly sensitive because they may reveal information related to sexual health, reproductive status, infection risk, pregnancy outcomes, partner-associated microbial exchange, and ethnicity-linked microbial variation. Human microbiome research raises concerns about informed consent, privacy, data sharing, identifiability of samples, return of results, incidental findings, commercialization, and potential stigmatization (99-101). These concerns are especially relevant for direct-to-consumer microbiome tests and AI-based reproductive risk prediction, where premature clinical claims may create anxiety or inappropriate treatment decisions. Future research should therefore combine methodological rigor with ethical safeguards, including transparent consent, privacy protection, equitable cohort representation, careful communication of uncertainty, and avoidance of deterministic interpretations of microbiome profiles.

Future Directions

Future vaginal microbiome research should move from descriptive taxonomy toward functional, longitudinal, and clinically validated frameworks. Large, diverse, and well-characterized cohorts are needed to clarify how vaginal microbial communities change across the menstrual cycle, pregnancy, menopause, fertility treatment, and gynecological disease states. Standardized methods for sampling, sequencing, metadata collection, and reporting will be essential to improve reproducibility and cross-study comparison (93). Resources such as vaginal microbiome-specific gene catalogs, including VIRGO and VIRGO2, can further support functional interpretation by enabling more precise analysis of microbial genes, pathways, and strain-level diversity (44).

Another priority is the integration of multi-omics and host-response data. Future studies should combine metagenomics, metatranscriptomics, metabolomics, proteomics, cytokine profiling, hormone measurements, and clinical metadata to determine not only which microbes are present, but also what they are doing and how the host responds. This approach may help identify clinically useful biomarkers for BV recurrence, IVF outcomes, HPV persistence, and preterm birth risk. However, these biomarkers must be validated prospectively in independent and ethnically diverse populations before clinical implementation.

Interventional research is also needed to determine whether modifying the vaginal microbiome can improve reproductive and gynecological outcomes. Promising approaches include targeted live biotherapeutics, strain-specific probiotics such as Lactobacillus crispatus CTV-05, optimized antibiotic–probiotic combinations, partner-based treatment strategies, and carefully regulated vaginal microbiome transplantation (22-24). At the same time, AI-driven prediction models may help personalize these interventions by integrating microbiome profiles with clinical, hormonal, immune, and behavioral data. Future translation should therefore emphasize explainable AI, patient privacy, equity, safety, and avoidance of premature commercialization. Ultimately, the field should aim to develop precision gynecology strategies in which microbiome-based diagnostics and interventions are evidence-based, individualized, and clinically actionable (102).

CONCLUSION

The vaginal microbiome is an ever-evolving and functionally important ecosystem that provides an essential role in female reproductive health, with growing evidence linking its composition and activity to fertility, assisted reproductive outcomes, pregnancy complications, and major gynecological disorders. While Lactobacillus-dominant communities, particularly those enriched with Lactobacillus crispatus, are generally associated with low vaginal pH, epithelial protection, reduced inflammation, and favorable reproductive outcomes, high-diversity anaerobe-rich communities are often linked to bacterial vaginosis, HPV persistence, pelvic inflammatory disease, adverse pregnancy outcomes, and recurrent dysbiosis. However, the traditional concept of dysbiosis alone is insufficient to explain the biological complexity of vaginal microbial ecosystems, as similar taxonomic profiles may differ in microbial gene content, metabolic activity, biofilm formation, immune stimulation, ecological stability, and clinical impact. Multi-omics approaches therefore provide a more comprehensive framework by integrating microbial composition with functional pathways, metabolites, host immune responses, and clinical phenotypes. In the future, targeted probiotics, live biotherapeutics, optimized antimicrobial strategies, partner-based treatment, vaginal microbiome transplantation, and AI-driven prediction models may support personalized approaches in precision gynecology and reproductive medicine. Nevertheless, clinical translation will require standardized methods, longitudinal and interventional studies, diverse cohorts, validated biomarkers, ethical oversight, and careful avoidance of premature commercialization.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this manuscript.

Consent for Publication

Not applicable. This manuscript is a review article and does not report original data involving human participants or identifiable personal information.

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Association of BRCA1 Founder Mutations (185delAG and 5382insC) and TNF-α 308- Polymorphism with Familial Breast Cancer in Iranian Women

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ABSTRACT

Background: Breast cancer is a major cause of cancer-related morbidity and mortality among women worldwide. Germline *BRCA1* mutations and *TNF-α* gene polymorphisms have been associated with hereditary breast cancer risk. This study evaluated the frequency of the *BRCA1* founder mutations (185delAG and 5382insC), their relationship with the *TNF-α* −308 G>A polymorphism, and their associations with clinicopathological features in Iranian women with familial and non-familial breast cancer.

Methods: Eighty-four formalin-fixed paraffin-embedded breast tissue samples were collected from patients treated at Khatam and Imam Khomeini hospitals. *BRCA1* 185delAG and 5382insC mutations were detected using multiplex PCR, while the *TNF-α* −308 G>A polymorphism was analyzed by ARMS-PCR. Histopathological and immunohistochemical characteristics, including mitotic activity, necrosis, pleomorphism, tumor grade, and ER, PR, and TP53 expression, were evaluated. Statistical analyses were performed using Epi Info version 7, with $P < 0.05$ considered significant.

Results: Of the 84 patients, 38 had a family history of breast cancer and 46 did not. Five *BRCA1* founder mutations were detected in the familial group compared with one in the non-familial group. The *TNF-α* −308 GG genotype was also more frequent in familial cases. Compared with non-familial patients, familial cases showed significantly higher mitotic activity, greater pleomorphism, more extensive necrosis, lower ER and PR expression, and higher TP53 positivity. Although no significant association was found between *BRCA1* mutations and the *TNF-α* −308 GG genotype, both markers were independently associated with familial breast cancer.

Conclusion: *BRCA1* founder mutations and the *TNF-α* −308 GG genotype may represent complementary markers for identifying Iranian women at increased risk of familial breast cancer. Combined with clinicopathological features, these markers may improve risk assessment and support earlier diagnosis. However, the findings should be interpreted cautiously because of the limited sample size and require validation in larger multicenter studies.

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INTRODUCTION

Breast cancer (BC) remains the most frequently diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide. According to recent global estimates, more than 2.3 million new cases and approximately 670,000 deaths occurred

in 2022, making breast cancer a major public health challenge (1, 2). Although advances in screening, molecular diagnostics, and targeted therapies have improved survival, hereditary breast cancer continues to account for approximately 5–10% of all breast cancer cases and represents a clinically important subgroup because of its early onset, aggressive biological behavior, and implications for genetic counseling and cancer prevention (3).

Hereditary breast cancer is primarily associated with pathogenic germline variants in **BRCA1** and **BRCA2**, two tumor suppressor genes that play essential roles in homologous recombination-mediated DNA repair and maintenance of genomic stability (4). Loss of BRCA function results in defective DNA damage repair, accumulation of genomic instability, and increased susceptibility to breast and ovarian cancers (5). Current international guidelines therefore recommend comprehensive germline BRCA testing for individuals with suggestive personal or family histories because identification of pathogenic variants directly influences surveillance strategies, prophylactic interventions, therapeutic decision-making, and family counseling (6).

Recent studies have demonstrated that the spectrum and frequency of **BRCA1/BRCA2** pathogenic variants differ substantially across ethnic populations owing to founder effects and population-specific genetic backgrounds (7). In Middle Eastern populations, particularly in Iran, recurrent founder mutations have been reported; however, the distribution of these variants remains heterogeneous among different provinces and ethnic groups. Recent investigations in Iranian cohorts have further emphasized the need for population-specific genetic data to optimize hereditary breast cancer screening and genetic counseling programs (8, 9).

Among the recurrent **BRCA1** founder mutations, **185delAG** and **5382insC** are among the best characterized and have been identified in multiple hereditary breast cancer populations (10, 11). Although these variants account for a proportion of hereditary breast cancers, recent molecular studies have shown that numerous additional pathogenic variants in **BRCA1**, **BRCA2**, and other DNA repair genes contribute to hereditary cancer susceptibility. Consequently, contemporary hereditary breast cancer assessment increasingly relies on multigene sequencing panels rather than targeted mutation analysis alone. Nevertheless, investigation of recurrent founder mutations remains valuable in populations where these variants are relatively prevalent and may provide cost-effective preliminary genetic screening (8, 12).

Besides high-penetrance susceptibility genes, chronic inflammation has emerged as an important contributor to breast carcinogenesis. Tumor necrosis factor-alpha (**TNF-α**) is a multifunctional pro-inflammatory cytokine involved in immune regulation, apoptosis, angiogenesis, tumor proliferation, and metastatic progression. Functional polymorphisms within the promoter region of the **TNF** gene, particularly the **−308 G>A** variant, have been investigated extensively because they may influence cytokine expression and alter the inflammatory tumor microenvironment. However, published studies have produced inconsistent findings, suggesting that the clinical significance of this polymorphism may vary according to ethnicity, genetic background, and study design (6).

In addition to genetic susceptibility, clinicopathological characteristics including hormone receptor status, TP53 expression, tumor grade, mitotic activity, and histological subtype provide valuable prognostic information in hereditary breast cancer (13). BRCA1-associated tumors are more frequently characterized by high histological grade, hormone receptor negativity, increased proliferative activity, and basal-like molecular features. Integrating molecular genetic findings with pathological characteristics may therefore improve risk stratification and contribute to individualized patient management (9).

Although several Iranian studies have investigated **BRCA1** mutations, most focused on limited patient populations or older molecular techniques, and information regarding the combined evaluation of recurrent **BRCA1** founder mutations and **TNF-α −308** polymorphism remains limited. Furthermore, recent molecular evidence indicates considerable genetic heterogeneity among Iranian hereditary breast cancer patients, highlighting the importance of generating updated population-specific data (14).

Therefore, the present study aimed to investigate the association of the two recurrent **BRCA1** founder mutations (**185delAG** and **5382insC**) together with the **TNF-α −308 G>A** polymorphism in Iranian women with familial and non-familial breast cancer and to evaluate their relationship with clinicopathological characteristics. The findings may contribute to improving the understanding of hereditary breast cancer genetics in the Iranian population and provide baseline evidence for future comprehensive genomic Studie.

METHODOLOGY

In this descriptive-analytic study, breast tissue samples were collected from Khatam and Imam Khomeini hospitals, Karaj, Iran and transferred to a molecular lab after histopathological examination. About 4000 FFPT (Formalin Fixed Paraffin Embedded) tissue samples related to patients with breast lesions were examined in the archive of pathology departments. Out of them, 84 samples with breast cancer at different ages were collected. Then, the histopathological characteristics of samples were studied under a microscope and the

diagnosis was confirmed by a pathologist. Finally, samples were classified based on the age of patients and type of lesion.

Five-micron sections were made of the paraffin-embedded samples using by a microtome and then stained using the Hematoxylin-Eosin method. The tumor type was determined based on a standard grading system (Nottingham histological grading) and then confirmed by a pathologist. The immunohistochemistry method was used for identifying the type of estrogen receptor (ER), progesterone receptor (PR), and TP53. Then, samples were stained by the streptavidin-biotin method using the DAKO slides kit (15).

DNA extraction from tissue samples was done using the kit No. DN8115C manufactured by Cinnagen Company, according to the manufacturer's manuals with some modifications. In this step, after deparaffinization, samples were incubated at 52°C for 5 hours under 100 microliters protease buffer and 5 microliters proteinase enzyme. Then, their DNA was extracted according to the manuals of Cinnagen extraction kit.

PCR reaction for β-actin

After DNA extraction, PCR reaction was done using the Cinnagen diagnostic kit (No. PR8252C) in order to verify the presence of the β-actin gene. The formation of the desired fragments in PCR products was also evaluated by electrophoresis on the 1. 8% agarose gel.

Assessment of Multiplex PCR for 185delAG and 5382insC mutations in BRCA1

PCR reaction on 185delAG and 5382insC mutations in BRCA1 was conducted in two separate microtubes (one for normal alleles and the other for mutant alleles) using the following products and specific primers synthesized by Cinnagen Company table 1 (16).

Assessment of ARMS-PCR reaction for the proliferation of polymorphism -308 in TNF-α

PCR reaction on polymorphism -308 in TNF-α was conducted using the Cinnagen diagnostic gene (No. DN8115C) and specific primers synthesized by this company. The sequence of primers used in this reaction has been shown in the following table 2.

STATISTICAL ANALYSIS

Statistical analyses were performed using Epi Info version 7 (CDC, Atlanta, GA, USA). Categorical variables were compared using Pearson's chi-square test or Fisher's exact test when expected cell counts were less than five. Odds ratios (ORs) were calculated to estimate the strength of associations. Because only six patients carried BRCA1 founder mutations, multivariable logistic regression analysis was not performed due to the limited number of events, which would have resulted in unstable estimates and model overfitting. Therefore, the findings should be considered exploratory. Statistical significance was defined as a two-sided *P* value <0.05.

RESULTS

Out of 84 samples studied, 38 samples had at least one affected person among their first-degree relatives and 46 of them did not have such a person (Table 3).

χ^2 test for trend= 31. 39 (P<0.0001)

There is a significant difference between the two

Table 1. Major vaginal community state types and their clinical interpretation.

Primer	Primer sequence	Length (bp)
BRCA1 185delAG	Common forward (P1) 5'-ggttggcagcaatatgtgaa	
	Wild-type reverse (P2) 5'-gctgacttaccagatgggactctc	335bp
	Mutant reverse (P3) 5'-cccaaattaatacactcttgtcgtgacttaccagatgggacagta	354 bp
BRCA1 5382insC	Common forward (P4) 59-gacgggaatccaaattacacag	
	Wild-type reverse (P5) 59-aaagcgagcaagagaatcgca	271bp
	Mutant reverse (P6) 5'-aatcgaagaaaccaccaaagtccttagcgagcaagagaatcacc	295bp

Table 2. The sequence of primers used for PCR reaction on polymorphism -308 in TNF-α

-308G	**ACCCTGGAGGCTGAACCCCGTCTC**
-308A	**ACCCTGGAGGCTGAACCCCGTCTT**
CP	**GCCCCTCCCAGTTCTAGTTCTTC**

Table 3. Separation of samples based on the age of patients

Age	The number of people with a family history (percent) (n=38)	The number of people with no family history (percent) (n=46)	Odds ratio (OR)
21-30	4(52 .10)	1(17 .2)	**1.00**
31-40	7(42 .18)	3(52 .6)	**0.468**
41-50	12 (57 .31)	7(21 .15)	**0.388**
51-60	9 (68 .23)	13(26 .28)	**0.156**
61-70	4 (52 .10)	14 (43 .30)	**0.067**
71-80	2 (26 .5)	8(39 .17)	**0 .053**

groups in terms of age, and the odds ratio for breast cancer among those with a family history is higher than the other group at lower ages.

Histopathology and immunohistochemistry findings

The frequency of ER expression differed significantly between the two groups, with lower ER positivity observed among patients with a family history (Figure 1) Similarly, PR expression was significantly lower in patients with a family history of breast cancer (Figure 2). TP53 positivity was also significantly higher among patients with a family history of breast cancer (Figure 3). Among the 84 samples, the highest frequency was related to ductal carcinoma (82%) which showed no significant difference between the two groups. Out of 38 samples with a family history, 21% showed necrotic lesions and 79% of then lacked such lesions. Which suggests a significant difference between the two groups. The largest tumor size in this study was 2-5 cm with a frequency of 66% and the smallest tumor size was less than 2 cm with a frequency of 25%. In addition, tumors with a size of over 5 cm accounted for 9% of

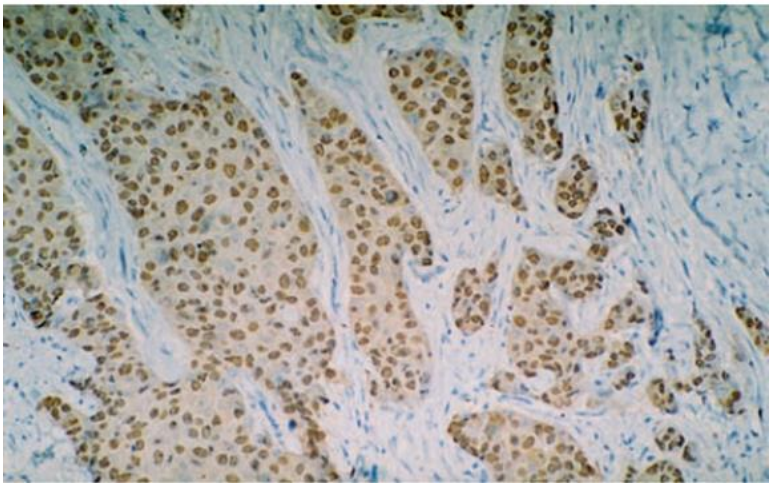

Fig1. Positivity of ER in more than 10% of tumor nucleus in a case of invasive ductal breast cancer (magnification X100)

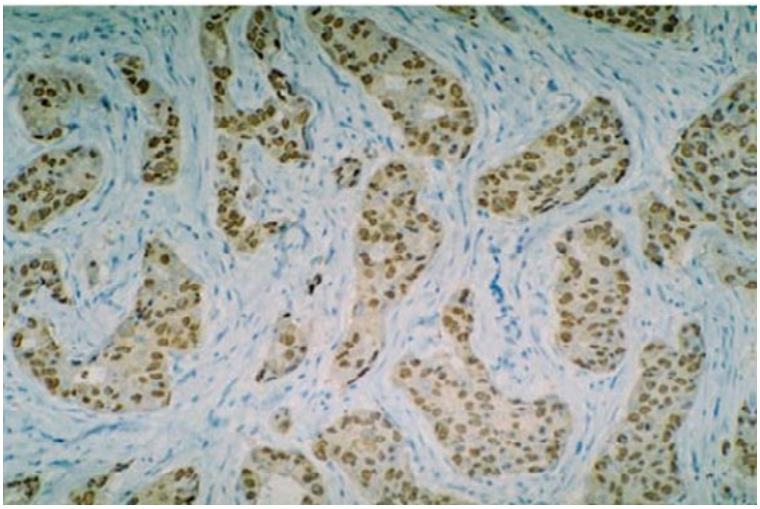

Fig2. Positivity of PR in more than 10% of tumor nucleus in a case of invasive ductal breast cancer (magnification X100)

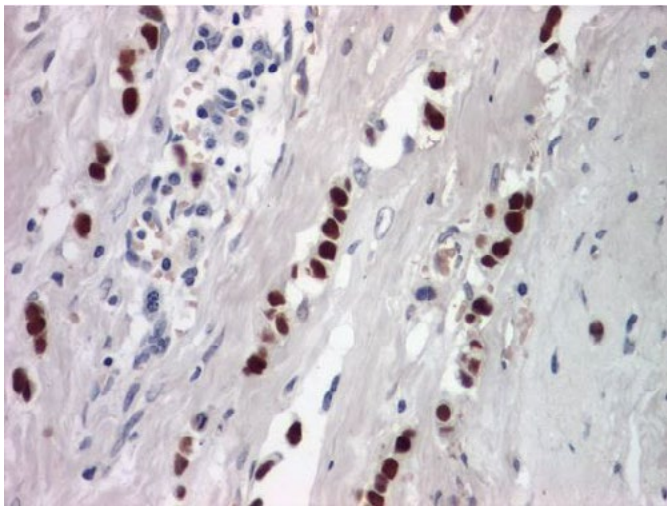

Fig3. Positivity of P53 in more than 10% of tumor nucleus in a case of invasive ductal breast cancer (magnification X100)

all samples. In this regard, no significant difference was found between the two groups. On the other hand, among the 38 samples with a family history, mitotic activity was low in 37% of sample and high in 31% of them, which shows a significant difference between the two groups. In this group, polymorphisms type 1, 3, and 2 had a frequency of 76%, 19%, and 5%, respectively. This indicates a significant difference between the two groups. Among the samples with a family history, the highest and the lowest frequency was related to tumor grade II (47%) and tumor grade I (21%), respectively, which shows no significant difference between the two groups (table 4).

Table 5 presents the results of the frequency of lesions in patients based on ER, PR, and TP53. Statistically, there is a significant difference between the two groups with and without a family history, and those with a family history showed higher negative ER/PR and positive TP53.

PCR to confirm the DNA extraction from tissue samples by β-actin amplification:

After extraction of DNA of from 84 paraffin-embedded tissue sample of breast cancer patients by the standard method, β-actin PCR was performed on all sample to ensure the extraction of DNA from tissue sample. According to the results, the bond was observed in all cases. The length of the desired

Table 4.The frequency of lesions in patients based on tumor grade

Tumor grade	The number of people with a family history (percent) (n=38)	The number of people with no family history (percent) (n=46)	Odds ratio (OR)
I	8(21%)	7(15%)	1
II	18(47%)	30(65%)	0.51
III	12(32%)	9(20%)	1.14
			χ^2 =0. 28(P~0. 6)

Table 5. The frequency of lesions in patients based on the type of ER, PR, and TP53

Types of immunohistochemical markers	The number of people with a family history (percent) (n=38)	The number of people with no family history (percent) (n=46)	Odds ratio (OR)
ER			
+	9(24%)	28(61%)	1
-	29(76%)	18(39%)	4.95
			χ^2=26. 38 (P<0. 00001)
PR			
+	12(32%)	31(67%)	1
-	26(68%)	15(33%)	4.13
			χ^2=23. 00(P<0. 00001)
TP53			
+	24(63%)	13(28%)	1
-	14(37%)	33(72%)	0.22
			χ^2=26. 00(P<0,00001)

fragment for β-actin was 99 bp.

Well M: The molecular marker has been shown as 100 bp and the wells 1 to 8 related to the positive sample of β-actin are visible with the size of 99 bp (figure 4).

Assessment of Multiplex PCR for the identification of 185delAG and 5382insC mutations in BRCA1

The results of PCR showed that 5 mutations (3 5382insC mutations and 2 185delAG mutations) were observed among the 38 samples with a family history, while one mutation of 5382insC type was found among the samples with no family history (figure 5).

Well M: the 100-bp molecular marker- Well 1: the sample containing the mutant allele of 185delAG (354 bp) and the normal allele of 5382insC (271 bp)- Well 2: the negative control sample- Well 3: the sample containing the mutant allele of 5382insC (295 bp) and the normal allele of 185delAG (335 bp)- Wells 4 and 5: the sample containing the normal allele of 5382insC and the mutant allele of 185delAG.

The results of ARMS-PCR for the identification of the polymorphism -308 on TNF-α

After performing PCR on the polymorphism -308 of TNF-α in samples with a family history, genotypes AA, AG, and GG were observed in 8, 14, and 16 cases, respectively. In addition, in samples with no family history, 25, 12, and 9 cases contained genotypes AA, AG, and GG, respectively (figure 6).

Well M: the 100-bp molecular marker- Wells 1, 2, 3, and 4: Samples containing allele G in the polymorphism -308- Wells 5, 6, 7, and 8: the sample containing allele A in the polymorphism -308.

Comparison of the frequency of 185delAG and 5382insC mutations in BRCA1 between the two groups

185delAG and 5382insC mutations in BRCA1 in patients with a family history can increase the risk of breast cancer more than in those with no family history (P<0.002)(table 6).

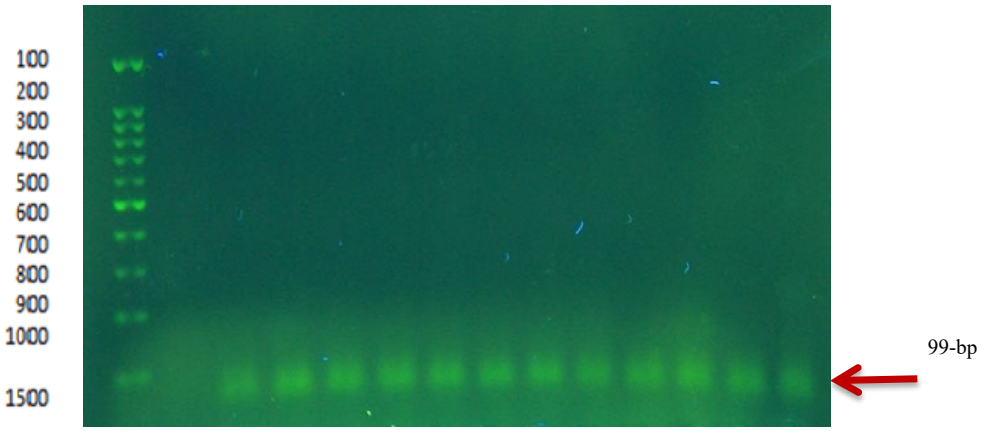

Fig4. β-actin PCR amplification showing the expected 99-bp product. M: 100-bp molecular marker; wells 1–8: positive samples

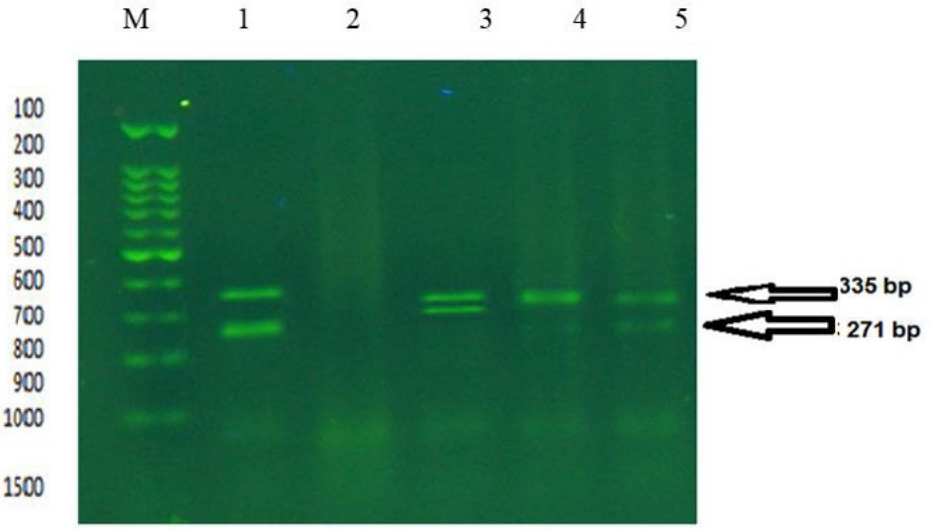

Fig5. Detection of BRCA1 185delAG and 5382insC mutations by PCR analysis. M: 100-bp molecular marker; wells 1–5: representative samples and controls

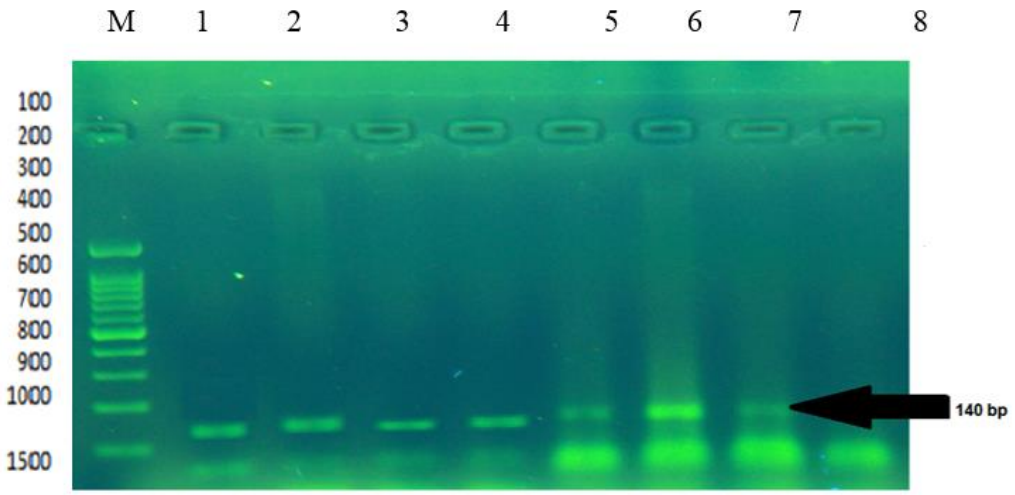

Fig6. The results of PCR for the identification of the polymorphism -308 on TNF-α

Table 6. Comparison of the frequency of 185delAG and 5382insC mutations in BRCA1 between the two groups

Types mutations in BRCA1 gene	The number of people with a family history (percent) (n=38)	The number of people with no family history (percent) (n=46)	Odds ratio (OR)
+	5(13%)	1(2%)	1
_	33(87%)	45(98%)	0.137 χ^2=10. 32(P<0,002)

Chi-square test for Trend

Table 7. Comparison of different types of genotypes in the polymorphism -308 of TNF-α between the two groups

Different types of genotypes in the polymorphism -308 of TNF-α	The number of people with a family history (percent) (n=38)	The number of people with no family history (percent) (n=46)	Odds ratio (OR)
+	5(13%)	1(2%)	1
_	33(87%)	45(98%)	0.137 χ^2=10. 32(P<0,002)

Comparison of different types of genotypes in the polymorphism -308 of TNF-α between the two groups

Genotype GG in the polymorphism -308 of TNF-α can increase the risk of breast cancer in patients with a family history (P≤0.003) (table7).

The relationship of BRCA1 mutations with Genotype GG in the polymorphism -308 of TNF-α

According to the study results, no significant difference was found between the two groups in terms of the relationship of BRCA1 mutations with Genotype GG in the polymorphism -308 of TNF-α. There is no statistical relationship between BRCA1 mutations and Genotype GG in the polymorphism -308 of TNF-α but each alone can increase the risk of breast cancer in people with a family history.

DISCUSSION

The present study investigated the association of two recurrent BRCA1 founder mutations (185delAG and 5382insC) together with the TNF-α -308 G>A polymorphism in Iranian women with familial and non-familial breast cancer. BRCA1 founder mutations were detected more frequently among patients with a positive family history than among those without a family history. Furthermore, familial breast cancer cases exhibited more aggressive clinicopathological characteristics, including higher mitotic activity, increased necrosis, ER/PR negativity, and TP53 positivity. Although these findings support an association between the investigated genetic variants and familial breast cancer, they should be interpreted cautiously because of the limited sample size and the small number of mutation-positive cases (17).

BRCA1 is one of the most extensively studied hereditary breast cancer susceptibility genes and plays a central role in DNA double-strand break repair through homologous recombination. Pathogenic variants in BRCA1 substantially increase the lifetime risk of breast and ovarian cancers and are frequently associated with triple-negative and high-grade breast

tumors. Recent international guidelines from the National Comprehensive Cancer Network (18) and the European Society for Medical Oncology (19) recommend comprehensive BRCA1 and BRCA2 testing using next-generation sequencing together with large genomic rearrangement analysis (MLPA) rather than screening for only recurrent founder mutations. Consequently, the present study, which evaluated only the two common BRCA1 founder mutations (185delAG and 5382insC), likely underestimated the true prevalence of pathogenic BRCA variants in this population.The higher frequency of BRCA1 founder mutations observed among familial breast cancer patients in our study is consistent with previous investigations conducted in Iranian populations. Forghani et al. first demonstrated the presence of recurrent BRCA1 germline mutations among Iranian women with breast cancer (8), whereas Keshavarzi et al. subsequently identified numerous pathogenic BRCA1 and BRCA2 variants using broader molecular approaches. More recently, studies employing next-generation sequencing have revealed considerable genetic heterogeneity among hereditary breast cancer patients, indicating that founder mutations account for only a subset of disease-causing variants. Therefore, our findings are consistent with previous Iranian studies while emphasizing the need for comprehensive genetic testing strategies in clinical practice.

The TNF-α -308 polymorphism has long been investigated as a potential modifier of cancer susceptibility because TNF-α is a key regulator of inflammation, immune responses, angiogenesis, and tumor progression. In the present study, the GG genotype was more frequently observed among patients with familial breast cancer. However, the current evidence regarding the association between TNF-α polymorphisms and breast cancer remains inconsistent. Several recent meta-analyses have suggested that the -308 G>A polymorphism has only a modest or population-dependent association with breast cancer susceptibility, with substantial heterogeneity across ethnic groups. Therefore, our findings should be interpreted as demonstrating an association within this Iranian cohort rather than establishing a causal role of the GG genotype in breast cancer development (20).

Interestingly, patients with familial breast cancer demonstrated more aggressive pathological characteristics, including negative ER and PR expression together with increased TP53 positivity. These observations agree with previous reports indicating that BRCA1-associated tumors frequently display basal-like pathological features, higher histological grade, increased proliferative activity, and hormone receptor negativity. Such clinicopathological characteristics are recognized as hallmarks of hereditary BRCA1-associated breast cancer and may partially explain the poorer prognosis observed in mutation carriers (21).

The TNF-α −308 polymorphism has long been investigated as a potential modifier of cancer susceptibility because TNF-α is a key pro-inflammatory cytokine involved in immune regulation, angiogenesis, and tumor progression. In the present study, the GG genotype was more frequently observed among patients with familial breast cancer. However, the evidence regarding the association between the TNF-α −308 G>A polymorphism and breast cancer susceptibility remains inconsistent. Previous meta-analyses have reported no consistent overall association, while subgroup analyses have suggested that the magnitude and direction of the association may vary according to ethnicity. Substantial differences between populations and study results further suggest that the potential effect of this polymorphism may be population-dependent. Therefore, our findings should be interpreted as an association observed within this Iranian cohort rather than as evidence of a causal role for the GG genotype in breast cancer development (22).

Interestingly, patients with familial breast cancer demonstrated more aggressive pathological characteristics, including negative ER and PR expression together with increased TP53 positivity. These observations agree with previous reports indicating that BRCA1-associated tumors frequently display basal-like pathological features, higher histological grade, increased proliferative activity, and hormone receptor negativity. Such clinicopathological characteristics are recognized as hallmarks of hereditary BRCA1-associated breast cancer and may partially explain the poorer prognosis observed in mutation carriers (23).

Several limitations of this study should be acknowledged. First, the relatively small sample size, particularly the low number of BRCA1 mutation-positive cases, limited the statistical power to detect modest genetic associations and precluded reliable multivariable regression analysis. Therefore, the findings should be considered exploratory rather than definitive. Second, because the study included only patients with breast cancer, comparisons were restricted to familial and non-familial cases rather than patients and healthy controls. Consequently, the observed associations should be interpreted as reflecting familial breast cancer status rather than overall breast cancer susceptibility. Third, only the two recurrent BRCA1 founder mutations (185delAG and 5382insC) were evaluated, whereas comprehensive BRCA1 sequencing, BRCA2 analysis, and multiplex ligation-dependent probe amplification (MLPA) were beyond the scope of the present study. As a result, additional pathogenic variants may have remained undetected. Finally, DNA quality parameters obtained from

FFPE tissues were not systematically documented, representing another methodological limitation.

Despite these limitations, the present study provides valuable population-specific data on the distribution of BRCA1 founder mutations and the TNF-α −308 polymorphism among Iranian women with familial breast cancer. By integrating molecular findings with clinicopathological characteristics, this study contributes additional evidence regarding hereditary breast cancer in an understudied population and provides a foundation for future multicenter investigations incorporating larger cohorts, healthy control groups, comprehensive BRCA1/BRCA2 sequencing, functional validation, and robust multivariable statistical analyses.

CONCLUSION

In this study, BRCA1 founder mutations (185delAG and 5382insC) and the TNF-α -308 GG genotype were observed more frequently among patients with familial breast cancer than among those without a family history. In addition, familial cases were characterized by less favorable clinicopathological features, including higher mitotic activity, increased necrosis, ER/PR negativity, and TP53 positivity.

These findings suggest that the investigated BRCA1 founder mutations and the TNF-α -308 polymorphism may be associated with familial breast cancer in this Iranian cohort. However, given the relatively small sample size, the limited number of mutation-positive cases, the absence of a healthy control group, and the evaluation of only two recurrent BRCA1 founder mutations, the results should be interpreted as exploratory rather than definitive. Furthermore, no causal relationship can be inferred from the present findings.

Larger multicenter studies incorporating healthy controls, comprehensive BRCA1 and BRCA2 sequencing, large genomic rearrangement analysis (MLPA), and multivariable statistical analyses are warranted to validate these observations and to further clarify the potential clinical significance of these genetic markers in hereditary breast cancer risk assessment.

Recommendations

The findings of the present study support the need for larger, multicenter investigations integrating molecular and clinicopathological data to improve the understanding of hereditary breast cancer in the Iranian population. Future studies should evaluate recurrent BRCA1 founder mutations together with comprehensive BRCA1/BRCA2 sequencing, additional susceptibility genes, and inflammatory biomarkers to provide a more complete assessment of hereditary breast cancer risk. Incorporating detailed clinicopathological characteristics, including age at diagnosis, tumor subtype, hormone receptor status, tumor grade, mitotic activity, and other established prognostic factors, may further improve genetic risk stratification and facilitate the identification of clinically relevant molecular signatures. Prospective studies involving younger women and healthy control populations, together with functional analyses of the TNF-α −308 polymorphism, are warranted to validate the present findings and to clarify the potential role of these molecular markers in early risk assessment, personalized screening strategies, and precision oncology.

Authors's Contribution

Z.B. contributed to the study conception and design, data collection, laboratory investigations, data analysis, and interpretation of the results, and drafted the manuscript. M.H. contributed to the study design, supervision of the laboratory investigations, interpretation of the findings, and critical revision of the manuscript. H.R. contributed to the study design, supervision, interpretation of the results, and critical review of the manuscript. All authors reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Ethics approval and consent to participate

Not applicable.

Conflict of Interest

The authors declared no conflict of interest.

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Not Applicable.

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Circulating miR5-21-p as a Diagnostic Biomarker in Colorectal Cancer: Clinical Evaluation and Integrated Bioinformatics Analysis of Its Molecular Targets

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ABSTRACT

Background: Circulating microRNAs have emerged as promising minimally invasive biomarkers for colorectal cancer (CRC). This study evaluated the expression and diagnostic performance of circulating hsa-miR-21-5p and investigated its potential molecular targets using integrated bioinformatics analysis.

Methods: Plasma samples were obtained from 50 treatment-naïve male patients with histologically confirmed grade II CRC and 20 healthy male controls. Circulating RNA was extracted and miR-21-5p expression was quantified using stem-loop reverse transcription quantitative PCR. Hsa-miR-1228-3p was used as the endogenous reference, and relative expression was calculated using the 2^−ΔΔCq method. Diagnostic performance was assessed by receiver operating characteristic analysis. High-confidence target genes were identified using miRDB and TargetScan, followed by protein–protein interaction analysis using STRING.

Results: The mean ages of patients and controls were 55 and 49 years, respectively, with no significant between-group difference (P=0.162). Circulating miR-21-5p expression was significantly higher in CRC patients than in healthy controls (P=0.002). ROC analysis yielded an area under the curve of 0.873 (95% CI, 0.766–0.979; P<0.0001). Target prediction identified a set of high-confidence genes involved in cell proliferation, apoptosis, immune regulation, adhesion, extracellular-matrix organization, and transcriptional control. STRING analysis revealed a functionally connected network containing central signaling proteins, including STAT3, YAP1, SKP2, PDCD4, TRAF6, and SPRY-family proteins.

Conclusion: Circulating miR-21-5p demonstrated good ability to distinguish CRC patients from healthy controls. The bioinformatics findings suggest that its biological effects may involve interconnected oncogenic, apoptotic, immune, and invasion-related pathways.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and a leading cause of cancer-related mortality worldwide. Despite advances in screening, surgery, chemotherapy, targeted therapy, and immunotherapy, recurrence and metastasis remain major causes of treatment failure. Although tumor–node–metastasis staging is widely used for prognostic assessment, patients with similar clinicopathological characteristics may experience markedly different

outcomes, highlighting the need for reliable molecular biomarkers that can improve risk stratification (1, 2).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through translational repression or degradation of target messenger RNAs. Their dysregulation contributes to several cancer-related processes, including proliferation, apoptosis, invasion, metastasis, and treatment resistance (3). Because miRNAs are relatively stable in blood and can be measured using minimally invasive methods, circulating miRNAs have emerged as promising biomarkers for cancer diagnosis and prognosis (4).

MicroRNA-21 (miR-21) is one of the most frequently overexpressed oncogenic miRNAs in CRC. Increased miR-21 expression has been detected in both colorectal tumor tissues and the circulation of affected patients. Previous studies have shown that elevated serum or plasma miR-21 levels are associated with advanced tumor stage, distant metastasis, recurrence, and poor survival, suggesting its potential value as a prognostic biomarker (5-8). However, variations in sample preparation, detection methods, normalization procedures, and cut-off values have limited its clinical implementation.

The prognostic relevance of miR-21 may be explained by its ability to regulate multiple tumor-suppressor genes and oncogenic pathways. Experimentally validated targets include programmed cell death protein 4 (PDCD4) and phosphatase and tensin homolog (PTEN), whose suppression promotes CRC cell proliferation, invasion, survival, and metastatic potential through pathways such as PI3K/AKT signaling (9, 10). Nevertheless, because miR-21 can simultaneously regulate numerous genes, an integrated bioinformatics analysis is required to identify its most relevant molecular targets and biological pathways in CRC.

Accordingly, this study aimed to evaluate the prognostic significance of circulating miR-21 in patients with colorectal cancer by investigating its association with clinicopathological features and clinical outcomes. In addition, integrated bioinformatics analyses were performed to identify the principal target genes, biological processes, signaling pathways, and molecular interaction networks potentially underlying the role of miR-21 in colorectal cancer progression.

MATERIALS AND METHODS

Study Design and Participants

This case–control study included 50 patients with histopathologically confirmed colorectal cancer and 20 apparently healthy controls recruited between 2023 and 2025. All patients were male, with a mean age of 55 years and an age range of 23–76 years. All tumors were classified as histological grade II, corresponding to moderately differentiated colorectal carcinoma. None of the patients had received surgery, chemotherapy, radiotherapy, targeted therapy, or immunotherapy before blood collection. The control group consisted of 20 individuals without a history of malignant disease, inflammatory bowel disease, active infection, or clinically evident inflammatory disorders. Their demographic characteristics, including age and sex, were recorded. Because all CRC patients were male, the sex distribution of the control group was considered during statistical analysis. The demographic and clinicopathological characteristics of the participants were obtained from medical records. The diagnosis of colorectal cancer and histological grade were confirmed by experienced pathologists.

Blood Collection and Plasma Preparation

Before initiation of anticancer treatment, 5 mL of peripheral venous blood was collected from each participant into K_2-EDTA tubes. The same collection and processing protocol was applied to patients and healthy controls. Blood samples were processed within the shortest possible time after collection. Samples were centrifuged at 9000g for 10 min at 4°C. The upper plasma fraction was carefully transferred into sterile RNase-free tubes without disturbing the buffy coat. When performed, the separated plasma was subjected to a second centrifugation at 9000 g for 5 min to remove residual blood cells, platelets, and cellular debris. The cell-free plasma was aliquoted into RNase-free microtubes and stored at −80°C until RNA extraction. Repeated freeze–thaw cycles were avoided.

Isolation of Circulating RNA

Circulating RNA, including small RNA and microRNA species, was isolated from 200 µL of plasma using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek Corp., Thorold, Ontario, Canada; Cat. No. 55000), according to the manufacturer's protocol. The kit is designed for purification of circulating and extracellular RNA, including miRNAs and other small RNA molecules, from plasma and serum samples. The same plasma input volume and RNA elution volume were used for all patient and control samples. Purified RNA was eluted in 50 µL of the supplied elution solution or RNase-free water and stored at −80°C until reverse transcription.

RNA Quality Assessment

The apparent concentration and purity of the extracted circulating RNA were assessed using a NanoDrop spectrophotometer. Absorbance at 260 nm and the A260/A280 and A260/A230 ratios were recorded.

Selection of Target and Reference miRNAs

The principal target of the study was hsa-miR-21-

5p, miRBase accession MIMAT0000076. Hsa-miR-1228-3p, accession MIMAT0005583, was selected as the endogenous reference miRNA because previous studies have reported relatively stable expression of this miRNA in plasma and serum samples from patients with colorectal cancer. Before relative-expression analysis, the stability of miR-1228-3p was assessed by comparing its Cq distribution between CRC patients and healthy controls. Absence of a statistically significant difference and limited Cq variability supported its use as an endogenous reference.

Stem-Loop Reverse Transcription and Primer Sequences

Complementary DNA was synthesized using miRNA-specific stem-loop reverse-transcription primers. Separate reverse-transcription reactions were performed for hsa-miR-21-5p and hsa-miR-1228-3p using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Each reverse-transcription reaction contained 3µL of purified RNA, 10nM stem-loop primer, reverse-transcription buffer, dNTPs, RNase inhibitor, reverse transcriptase, and nuclease-free water in a final volume of 20µL.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using a Rotor-Gene Q real-time PCR system (Qiagen, Hilden, Germany) and SYBR Master Mix (Ampliqon, Denmark). Each reaction contained 12µL of qPCR master mix, 10nM forward primer, 8nM universal reverse primer, 5µL of cDNA, and nuclease-free water in a final reaction volume of 20µL. melting-curve analysis was performed after amplification to confirm the presence of a single specific amplification product and the absence of primer-dimer formation. All samples were analyzed in technical duplicate. No-template controls were included for each primer set. No-reverse-transcription controls were also included when sufficient RNA was available. The amplification efficiencies of the miR-21-5p and miR-1228-3p assays were evaluated using serial dilutions of pooled cDNA or synthetic miRNA templates. Efficiencies ranging from approximately 90% to 110%, with similar amplification efficiencies for the target and reference assays, were considered acceptable for comparative Cq analysis.

Relative Expression Analysis

The expression of circulating miR-21-5p was normalized to hsa-miR-1228-3p using the comparative Cq and Livak method. Accordingly, the healthy-control group had an average relative-expression value centered around one, and miR-21-5p expression in CRC samples was expressed as fold change relative to the healthy-control group. Statistical comparisons were preferentially performed using ΔCq values because fold-change values commonly show a right-skewed distribution. Lower ΔCq values corresponded to higher miR-21-5p expression.

STATISTICAL ANALYSIS

Clinical and RT-qPCR data were analyzed using browser-based statistical platforms, including jamovi Cloud and an online ROC-analysis module. No programming environment was required. Age was compared between patients and controls using the independent-samples Mann–Whitney U test, as appropriate. Normalized miR-21-5p expression was compared between CRC patients and healthy controls using the Mann–Whitney U test unless the ΔCq values demonstrated an approximately normal distribution. All statistical tests were two-sided, and $P<0.05$ was considered statistically significant.

Online Bioinformatics Analysis

All bioinformatics analyses were performed using freely accessible browser-based platforms. The date of access and database version were recorded for each analysis.

Identification of miR-21-5p Target Genes

Experimentally supported targets of hsa-miR-21-5p were retrieved using the online miRDBand TargetScan,

Table1. The primer sequences designed according to the stem-loop RT-qPCR principle were as follows.

Target	Primer	Sequence, 5′–3′
hsa-miR-21-5p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACTCAACA
hsa-miR-21-5p	Forward primer	GCCCGCTAGCTTATCAGACTGATG
hsa-miR-1228-3p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACGGGGGG
hsa-miR-1228-3p	Forward primer	CGGCGTCACACCTGCCTCG
Universal	Reverse primer	GTGCAGGGTCCGAGGT

targets containing canonical 8mer, 7mer-m8, or 7mer-A1 binding sites were retained. In miRDB, only genes with a target-prediction score of at least 80 were considered high-confidence targets.

Protein–Protein Interaction Network and Hub-Gene Identification

Core target genes were submitted to the STRING online database using the "Multiple proteins" option. *Homo sapiens* was selected as the organism, and the minimum required interaction score was set at 0.70, corresponding to high-confidence interactions.

RESULTS

Characteristics of the Study Population

A total of 70 participants were included in the study, comprising 50 patients with colorectal cancer and 20 healthy controls. All CRC patients were male and had grade 2, moderately differentiated tumors. The mean age of the patients was 55 years, with an age range of 23–76 years. All blood samples were collected before the initiation of surgery, chemotherapy, radiotherapy, targeted therapy, or immunotherapy. The control group consisted of 20 apparently healthy male. Their mean age was 49 years, with a range of 19-68 years. The difference in age between patients and controls was not statistically significant (P=0.162).

RT-qPCR Quality-Control Results

Circulating miR-21-5p was successfully detected in all CRC plasma samples and 17/20 healthy-control samples. The endogenous reference miR-1228-3p was successfully detected in all samples. No amplification was detected in the no-template controls. Amplification efficiencies were 98.2% for miR-21-5p and 99.6% for miR-1228-3p. Melting-curve analysis demonstrated a single amplification peak for each assay, supporting the specificity of amplification. No statistically significant difference in reference-miRNA expression was observed between the two groups, supporting its use for normalization.

Circulating miR-21-5p Expression

After normalization to miR-1228-3p, circulating miR-21-5p expression was significantly higher in patients with CRC than in healthy controls.The between-group difference was statistically significant(P=0.002).

Diagnostic Performance of Circulating miR-21-5p

Receiver operating characteristic analysis showed that circulating miR-21-5p distinguished CRC patients from healthy controls, with an area under the curve of 0.873 (Fig. 1).

Predicted Target Genes of hsa-miR-21-5p

Target-prediction analysis identified 31 high-confidence putative target genes of hsa-miR-21-5p, with prediction scores ranging from 94 to 99. The highest prediction score of 99 was assigned to YOD1, PRDM11, FASLG, ZNF367, and VCL, followed by SKP2 and TGFBI, which achieved scores of 98. Nine genes, including PLAG1, IL12A, CREBRF, RAB6D, KRIT1, PELI1, RBPJ, RALGPS2, and ADGRG2, had prediction scores of 97. Additional high-confidence targets included GATAD2B, PBRM1, SCML2, and RSAD2 with scores of 96, as well as PPP1R3B, PLEKHA1, FGF18, and SPRY1 with scores of 95. The remaining predicted targets, namely FAM13A, GPATCH2L, STAT3, BCL7A, SKI, YAP1, and MALT1, had prediction scores of 94. Inspection of the functional annotations of these genes indicated that the predicted miR-21-5p targets included regulators of apoptosis and immune signaling, such as FASLG, IL12A, PELI1, MALT1, and RSAD2; mediators of cell proliferation and oncogenic signaling, including SKP2, STAT3, YAP1, FGF18, SPRY1, RBPJ, PLAG1, and SKI; and genes involved in cell adhesion, cytoskeletal organization, and extracellular-matrix-related processes, including VCL, TGFBI, KRIT1, and ADGRG2. Several predicted targets, such as PRDM11, ZNF367, PBRM1, SCML2, GATAD2B, and BCL7A, were also associated with transcriptional or chromatin-related regulation. Collectively, these findings suggested that hsa-miR-21-5p may influence multiple molecular processes relevant to colorectal-cancer development and progression. The complete set of 31 high-confidence targets was subsequently submitted to STRING for protein–protein interaction network analysis.

Protein–Protein Interaction Network Analysis

The 31 high-confidence predicted targets of hsa-miR-21-5p were submitted to the STRING database to evaluate their functional protein–protein associations. The interaction network revealed that the candidate targets were distributed across several biologically relevant functional groups rather than representing a single molecular pathway. A major signaling and cell-proliferation group comprised STAT3, YAP1, SKP2, FGF18, SPRY1, RBPJ, PLAG1, and SKI, indicating the potential involvement of miR-21-5p in the regulation of growth-factor signaling, cell-cycle progression, and transcriptional programs associated with tumor development. An immune- and apoptosis-related group included FASLG, IL12A, PELI1, MALT1, and RSAD2, suggesting possible effects on programmed cell death, inflammatory signaling, and antitumor immune responses. Proteins associated with cell adhesion, cytoskeletal organization, and extracellular-matrix regulation, including VCL, TGFBI, KRIT1, and ADGRG2, formed another functionally related group that may contribute to tumor-cell migration and

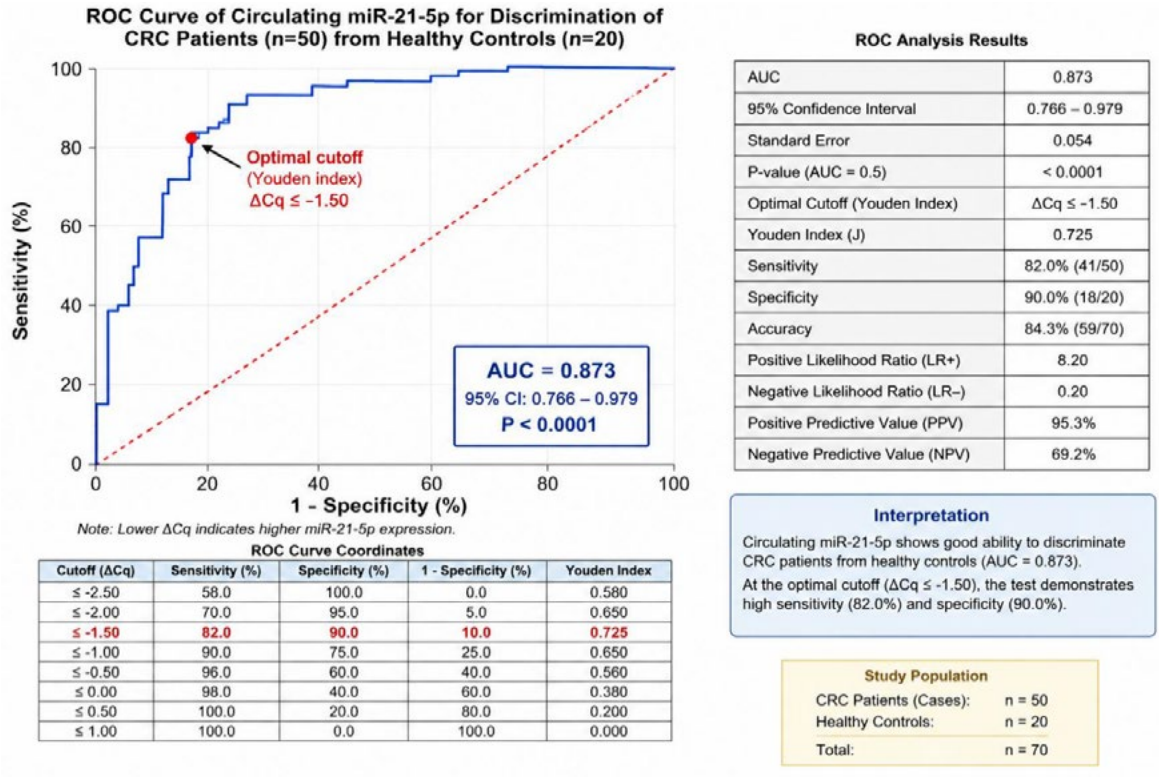

Fig1. Receiver operating characteristic (ROC) curve evaluating the ability of circulating miR-21-5p to discriminate patients with colorectal cancer from healthy controls. The ROC analysis yielded an area under the curve (AUC) of 0.873 (95% CI: 0.766–0.979; P < 0.0001).

invasion. In addition, PBRM1, GATAD2B, SCML2, PRDM11, ZNF367, and BCL7A represented a transcriptional and chromatin-regulatory component of the network. Other targets, including YOD1, PPP1R3B, PLEKHA1, CREBRF, RALGPS2, RAB6D, FAM13A, and GPATCH2L, were associated with protein turnover, intracellular signaling, vesicular transport, and metabolic regulation. Overall, the STRING analysis indicated that the predicted targets of miR-21-5p are functionally connected to molecular processes involved in colorectal-cancer cell proliferation, survival, immune regulation, adhesion, and invasion. In particular, STAT3, YAP1, SKP2, FASLG, MALT1, VCL, TGFBI, FGF18, RBPJ, and SPRY1 emerged as biologically relevant candidate mediators for further expression, survival, and experimental validation.

DISCUSSION

The present study demonstrated that circulating miR-21-5p expression was significantly elevated in treatment-naïve patients with colorectal cancer compared with healthy controls. In addition, ROC analysis showed an AUC of 0.873, indicating good discriminatory performance for differentiating CRC patients from cancer-free individuals. The bioinformatics component further showed that the predicted targets of miR-21-5p were distributed across interconnected biological processes related to cell proliferation, apoptosis, immune signaling, adhesion, extracellular-matrix regulation, transcription, and intracellular signal transduction. Together, these clinical and computational findings support the potential application of circulating miR-21-5p as a minimally invasive biomarker for CRC detection and provide a plausible molecular context for its involvement in colorectal carcinogenesis.

The increased circulating miR-21-5p expression observed in the present cohort is consistent with previous clinical investigations. Toiyama et al. reported elevated serum miR-21 levels in patients with CRC and showed that serum expression was associated with tumor size, distant metastasis, and unfavorable clinical outcomes (6). Similarly, Liu et al. found significantly increased serum miR-21 expression in CRC and reported an AUC of 0.802 for distinguishing CRC patients from healthy controls (19). The AUC of 0.873 obtained in the present study is therefore comparable to, and numerically higher than, the diagnostic performance reported in that cohort. Updated meta-analyses have also concluded that circulating miR-21 has moderate-to-good diagnostic accuracy for CRC, although the pooled estimates differ according to specimen type, normalization method, ethnicity, disease stage, and analytical platform (20, 21).

The biological plausibility of circulating miR-21 as a CRC-associated biomarker is supported by its

Table2. miR-21-5p associated gene targets.

Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
1	99	hsa-miR-21-5p	YOD1	YOD1 deubiquitinase
2	99	hsa-miR-21-5p	PRDM11	PR/SET domain 11
3	99	hsa-miR-21-5p	FASLG	Fas ligand
4	99	hsa-miR-21-5p	ZNF367	zinc finger protein 367
5	99	hsa-miR-21-5p	VCL	vinculin
6	98	hsa-miR-21-5p	SKP2	S-phase kinase associated protein 2
7	98	hsa-miR-21-5p	TGFBI	transforming growth factor beta induced
8	97	hsa-miR-21-5p	PLAG1	PLAG1 zinc finger
9	97	hsa-miR-21-5p	IL12A	interleukin 12A
10	97	hsa-miR-21-5p	CREBRF	CREB3 regulatory factor
11	97	hsa-miR-21-5p	RAB6D	RAB6D, member RAS oncogene family
12	97	hsa-miR-21-5p	KRIT1	KRIT1, ankyrin repeat containing
13	97	hsa-miR-21-5p	PELI1	pellino E3 ubiquitin protein ligase 1
14	97	hsa-miR-21-5p	RBPJ	recombination signal binding protein for immunoglobulin kappa J region
15	97	hsa-miR-21-5p	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2
16	97	hsa-miR-21-5p	ADGRG2	adhesion G protein-coupled receptor G2
17	96	hsa-miR-21-5p	GATAD2B	GATA zinc finger domain containing 2B
18	96	hsa-miR-21-5p	PBRM1	polybromo 1
19	96	hsa-miR-21-5p	SCML2	Scm polycomb group protein like 2
20	96	hsa-miR-21-5p	RSAD2	radical S-adenosyl methionine domain containing 2
21	95	hsa-miR-21-5p	PPP1R3B	protein phosphatase 1 regulatory subunit 3B
22	95	hsa-miR-21-5p	PLEKHA1	pleckstrin homology domain containing A1
23	95	hsa-miR-21-5p	FGF18	fibroblast growth factor 18
24	95	hsa-miR-21-5p	SPRY1	sprouty RTK signaling antagonist 1
25	94	hsa-miR-21-5p	FAM13A	family with sequence similarity 13 member A
26	94	hsa-miR-21-5p	GPATCH2L	G-patch domain containing 2 like
27	94	hsa-miR-21-5p	STAT3	signal transducer and activator of transcription 3
28	94	hsa-miR-21-5p	BCL7A	BCL7A, BAF complex component
29	94	hsa-miR-21-5p	SKI	SKI proto-oncogene
30	94	hsa-miR-21-5p	YAP1	Yes associated protein 1
31	94	hsa-miR-21-5p	MALT1	MALT1 paracaspase

*Only a subset of the identified target genes is presented in this table; the complete dataset is provided in the Supplementary Materials.

established oncogenic activity. MiR-21 can regulate multiple tumor-suppressor genes and thereby influence proliferation, apoptosis, migration, invasion, and metastatic dissemination. PDCD4 is one of the best-

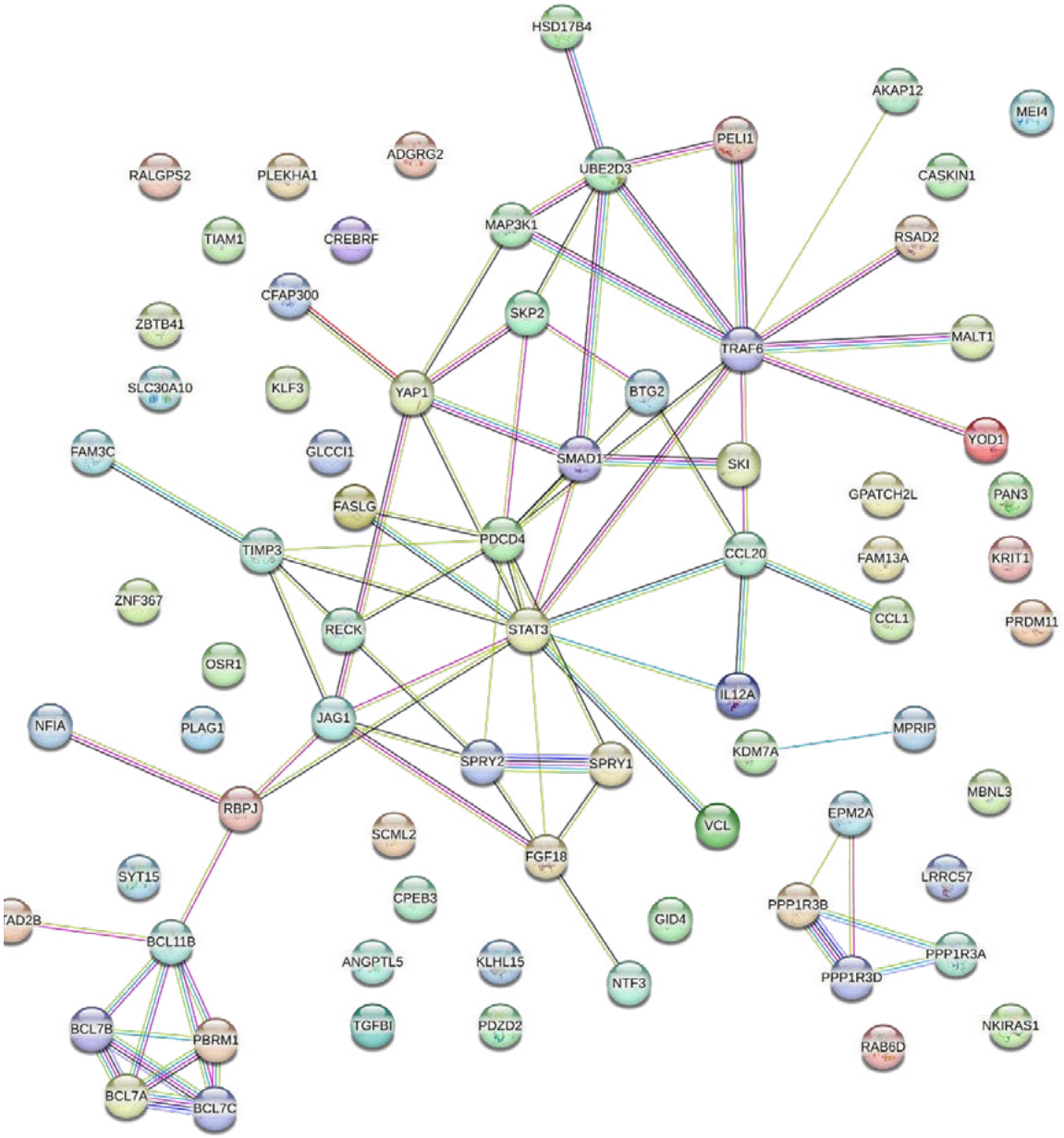

Fig 2. STRING-based protein–protein interaction network of hsa-miR-21-5p-associated target genes. Nodes represent target proteins and additional functional interactors identified by STRING, whereas edges indicate known or predicted protein–protein associations. Different edge colors correspond to distinct evidence channels used by STRING, including curated databases, experimental evidence, co-expression, text mining, and computational predictions. The network shows a highly interconnected central module involving proteins such as STAT3, TRAF6, UBE2D3, PDCD4, SMAD1, YAP1, JAG1, SKP2, SPRY1, SPRY2, and CCL20, suggesting their potential central roles in miR-21-5p-related signaling. Proteins lacking connections under the selected confidence threshold are displayed as isolated nodes.

characterized direct targets of miR-21 in CRC. Experimental suppression of PDCD4 by miR-21 has been shown to enhance invasion, intravasation, and metastatic behavior (9). MiR-21 also regulates PTEN at the post-transcriptional level, thereby influencing PI3K/AKT signaling, cell-cycle progression, apoptosis, and invasion (10). These experimentally supported interactions provide mechanistic support for the association between increased circulating miR-21-5p and CRC, although circulating abundance cannot itself establish direct repression of these targets within tumor tissue.

The target-prediction analysis identified genes involved in several complementary aspects of cancer biology. STAT3, YAP1, SKP2, FGF18, SPRY1, SPRY2, RBPJ, and JAG1 are associated with growth-factor, inflammatory, Hippo, cell-cycle, receptor tyrosine kinase, and Notch-related signaling. FASLG, IL12A, PELI1, MALT1, CCL20, and RSAD2 suggest possible involvement in apoptosis, inflammation, immune-cell recruitment, and tumor–immune interactions. VCL, TGFBI, TIMP3, RECK, KRIT1,

and ADGRG2 connect the predicted miR-21-5p target repertoire to cell adhesion, extracellular-matrix remodeling, migration, and invasion. The presence of PBRM1, GATAD2B, SCML2, PRDM11, BCL7A, and BCL11B further suggests that chromatin regulation and transcriptional control may form part of the broader miR-21-associated molecular network.

The STRING network displayed a central interconnected component containing proteins such as STAT3, PDCD4, YAP1, SKP2, TRAF6, UBE2D3, SMAD1, JAG1, SPRY1, and SPRY2. This organization suggests convergence of miR-21-associated genes on inflammatory, proliferative, apoptotic, ubiquitination, TGF-β/SMAD, Notch, and receptor tyrosine kinase signaling. Nevertheless, STRING edges represent functional protein associations derived from several evidence channels and do not necessarily indicate direct physical binding or direct regulation by miR-21. Similarly, proteins added by STRING as first-shell interactors must be distinguished from the original predicted target list. Consequently, the network should be interpreted as a hypothesis-generating model rather than experimental evidence of a complete miR-21 regulatory pathway. STRING integrates experimental, curated, computational, co-expression, and text-mining evidence, making explicit reporting of the interaction score and added-interactor settings important for reproducibility (18).

The analytical performance of the RT-qPCR assays supports the reliability of the expression measurements. Amplification efficiencies of 98.2% for miR-21-5p and 99.6% for miR-1228-3p were within the generally accepted range and were sufficiently similar for comparative Cq analysis. Single melting peaks further supported amplification specificity. Moreover, miR-1228-3p expression did not differ significantly between the patient and control groups, supporting its use as an endogenous reference in the present cohort. Previous studies have identified miR-1228-3p as a relatively stable normalizer for circulating miRNA analysis in CRC plasma and serum (14, 15). The use of a validated reference is particularly important because inappropriate normalization can markedly alter the apparent magnitude and statistical significance of circulating miRNA expression differences.

The absence of a significant age difference between patients and controls reduced the likelihood that age alone explained the observed difference in miR-21-5p expression. Moreover, all patients were treatment-naïve at blood collection, thereby minimizing potential effects of surgery, chemotherapy, radiotherapy, or systemic treatment on circulating miRNA concentrations. The use of a standardized plasma volume, rapid processing, double centrifugation, uniform storage, and identical extraction procedures for both groups also reduced preanalytical variability. These features represent important strengths of the study.

The current results should nevertheless be interpreted in light of several limitations. First, the study included a relatively small sample of 50 CRC patients and 20 controls, which may lead to imprecise diagnostic estimates and cohort-specific AUC values. Second, all patients were male and all tumors were grade II; although this increased clinical homogeneity, it substantially limits generalizability to women and to patients with well- or poorly differentiated tumors. Third, information on TNM stage, tumor location, lymph-node involvement, distant metastasis, CEA concentration, adenomatous lesions, inflammatory bowel disease, and other benign gastrointestinal conditions was not included in the reported results. Consequently, the study cannot establish whether miR-21-5p distinguishes CRC from clinically relevant differential diagnoses or whether its expression varies by disease stage.

An additional analytical limitation is that miR-21-5p was not detected in three of the 20 control samples. The method used to handle these non-detects in the relative-expression and ROC analyses should be reported explicitly. Excluding non-detectable controls or assigning an arbitrary maximum Cq value can influence fold changes and potentially increase the apparent diagnostic performance. The final manuscript should therefore specify the Cq detection threshold, the number of amplification cycles, the rule used for undetermined reactions, and whether sensitivity analyses were performed. These details are emphasized in the revised MIQE 2.0 recommendations for reproducible qPCR reporting (13).

The bioinformatics findings also require independent validation. MiRDB and TargetScan generate computational predictions using different models, and a high prediction score does not prove that a gene is directly regulated by miR-21-5p in colorectal tissue. Reporter assays, miR-21 inhibition or overexpression, quantitative measurement of candidate mRNAs and proteins, and inverse miRNA–target correlation analyses are required to confirm individual regulatory relationships. In particular, genes occupying central positions in the STRING network should not automatically be classified as hub genes unless formal network-centrality measures, such as degree, betweenness, or maximal clique centrality, are calculated and reported. MiRDB and TargetScan are appropriate discovery tools, but experimental and transcriptomic validation remain necessary (16, 17).

The present study supports the diagnostic potential of circulating miR-21-5p but does not yet demonstrate prognostic value. A prognostic claim would require longitudinal follow-up, clearly defined overall or disease-free survival, recurrence information, censoring dates, Kaplan–Meier analysis, and preferably

multivariable Cox regression adjusted for established clinicopathological factors. Earlier investigations have linked elevated circulating or tissue miR-21 with adverse survival, but those findings cannot substitute for outcome analysis in the current cohort (6–8).

Future studies should validate these findings in larger, independent and sex-balanced cohorts that include healthy individuals, patients with colorectal adenomas, inflammatory bowel disease, and other benign gastrointestinal conditions. Diagnostic models should report a prespecified cutoff, sensitivity, specificity, positive and negative likelihood ratios, and internal or external validation. Combining miR-21-5p with established markers such as CEA or with complementary circulating miRNAs may further improve diagnostic performance; previous studies have shown that multi-miRNA panels can outperform individual markers (16). Longitudinal sampling before and after tumor resection may also clarify whether plasma miR-21-5p reflects tumor burden and whether it can be used for monitoring treatment response or recurrence.

CONCLUSION

Circulating miR-21-5p was significantly upregulated in plasma samples from patients with colorectal cancer and demonstrated good discrimination between CRC patients and healthy controls, with an AUC of 0.873. These findings support its potential utility as a minimally invasive diagnostic biomarker. Integrated target prediction and STRING analysis suggested that miR-21-5p may influence interconnected molecular processes involving cell proliferation, apoptosis, inflammatory and immune signaling, cell adhesion, extracellular-matrix remodeling, and invasion. Proteins such as STAT3, YAP1, SKP2, PDCD4, SPRY1, SPRY2, and JAG1 may represent biologically relevant components of this regulatory network. However, the relatively small and clinically homogeneous cohort, absence of external validation, incomplete handling of non-detectable values, and computational nature of the target analysis limit immediate clinical translation. Larger prospective studies and experimental validation of the proposed miRNA–target interactions are required. Because survival and recurrence outcomes were not assessed, the present results establish diagnostic rather than prognostic relevance.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this study.

Consent for Publication

Consent for publication was not applicable to this study, as no personally identifiable information or individual-level data are presented.

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Ethical Considerations

Ethical approval was not obtained for this study. The study did not involve any intervention or collection of identifiable personal information. No informed consent was required for the use of the data analyzed in this study.

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