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Cytotoxic and Apoptosis-Related Effects of Aqueous Trigonella foenum-graecum Seed Extract on SKOV3- Human Ovarian Cancer Cells

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ABSTRACT

Background: Ovarian cancer remains a major therapeutic challenge because of late diagnosis, recurrence, and treatment resistance. Fenugreek (Trigonella foenum-graecum L.) contains several bioactive constituents with potential anticancer properties. This study investigated the cytotoxic effect of aqueous fenugreek seed extract on SKOV-3 human ovarian cancer cells and evaluated its association with the expression of apoptosis-related genes.

Methods: SKOV-3 cells were treated with aqueous fenugreek seed extract at concentrations of 1, 10, 50, 100, and 200 µg/mL for 24 and 48 h. Cell viability was assessed using the MTT assay, and half-maximal inhibitory concentrations (IC50) were determined by nonlinear regression. To investigate apoptosis-related molecular changes, cells were treated with the experimentally determined IC50 concentration, and the relative mRNA expression levels of BAX, BCL2, and CASP3 were evaluated by quantitative real-time PCR (qRT-PCR) using GAPDH as the reference gene. Relative gene expression was calculated using the 2^−ΔΔCt method.

Results: Aqueous fenugreek extract reduced SKOV-3 cell viability in a concentration- and time-dependent manner. The estimated IC50 values were 38.5 µg/mL at 24 h and 19.7 µg/mL at 48 h, indicating greater cytotoxicity with prolonged exposure. Treatment at the IC50 concentration significantly increased BAX expression to 2.83-fold and CASP3 expression to 3.21-fold, while BCL2 expression decreased to 0.43-fold relative to untreated cells. Consequently, the BAX/BCL2 expression ratio increased approximately 6.6-fold, indicating a shift toward a pro-apoptotic molecular profile.

Conclusion: Aqueous T. foenum-graecum seed extract exerted concentration- and time-dependent cytotoxic effects on SKOV-3 ovarian cancer cells and was associated with coordinated modulation of apoptosis-related genes, characterized by increased BAX and CASP3 expression, decreased BCL2 expression, and an elevated BAX/BCL2 ratio. These findings suggest that modulation of apoptosis-related signaling may contribute to the cytotoxic activity of aqueous fenugreek extract. Further functional and protein-level studies are required to confirm apoptosis and clarify the underlying molecular mechanisms.

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INTRODUCTION

Ovarian cancer represents one of the major malignancies affecting the female reproductive tract and contributes substantially to cancer-related deaths in women. Due to non-specific symptoms, diagnosis at advanced stages, invasiveness and metastasis,

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and the development of drug resistance, this disease remains a significant therapeutic challenge. According to GLOBOCAN 2022 estimates, approximately 324,000 new cases of ovarian cancer and over 206,000 associated deaths occurred worldwide in 2022 (1). In Iran, ovarian cancer is also considered a major malignancy in women, and studies based on national data have demonstrated temporal and geographical variations in the incidence and outcomes of female cancers, including ovarian cancer (2, 3). Despite therapeutic advancements, disease recurrence and chemotherapy resistance remain primary causes of treatment failure; consequently, the identification of natural compounds capable of inhibiting cancer cell growth and inducing cell death has garnered significant interest (4).

Fenugreek (Trigonella foenum-graecum L.), a member of the Fabaceae family, produces seeds that have long been utilized for both medicinal purposes and various applications in the food industry. Phytochemical studies have shown that fenugreek contains a diverse range of bioactive compounds, including steroidal saponins, flavonoids, phenolic compounds, alkaloids, and amino acids. Key compounds include diosgenin, trigonelline, and 4-hydroxyisoleucine. These compounds have attracted attention as potential anti-cancer agents due to their antioxidant and anti-inflammatory activities, as well as their ability to modulate cellular pathways. The type of extraction solvent influences the composition and biological activity of the extract; an aqueous extract can extract a significant portion of fenugreek's polar and water-soluble compounds, making it important to directly investigate its effects in cellular models (5, 6).

Evidence from laboratory studies indicates that fenugreek and certain of its active compounds can inhibit proliferation and induce cell death in various cancer models. For instance, fenugreek extract has been shown to induce cell death in Jurkat lymphoma cells (7). Furthermore, saponin compounds found in medicinal plants including those associated with fenugreek can inhibit cancer cell growth by affecting the cell cycle, oxidative stress, and cell death pathways (5). Studies on ovarian cancer cells have also demonstrated that certain steroidal saponins, such as yamogenin, exhibit anti-proliferative effects by inducing oxidative stress, arresting the cell cycle, and activating apoptosis (8). However, it should be noted that the effects of a pure compound cannot be directly extrapolated to the aqueous fenugreek extract; thus, direct investigation of this extract in ovarian cancer cells is essential.

Programmed cell death (apoptosis) plays an essential role in the removal of damaged or abnormal cells, while disruption of this tightly regulated process is recognized as a key hallmark of cancer. In the mitochondrial apoptotic pathway, the balance between proteins of the BCL-2 family plays a pivotal role in determining cell fate. Bax, a pro-apoptotic protein, facilitates the caspase activation pathway by increasing mitochondrial membrane permeability and promoting the release of cytochrome c, whereas Bcl-2 prevents cell death by maintaining mitochondrial integrity (9). Consequently, an increase in Bax expression and/or a decrease in Bcl-2 expression resulting in an elevated Bax/Bcl-2 ratio may be associated with the activation of the mitochondrial apoptotic pathway. Accordingly, alterations in the expression of these two genes can serve as a suitable indicator for investigating the potential role of a plant extract in inducing apoptosis.

Given the existing evidence regarding the bioactive compounds in fenugreek and their potential to modulate cell death pathways, examining the effect of the plant's aqueous extract on ovarian cancer cells could yield valuable insights into its anti-cancer potential. However, evidence concerning the direct impact of aqueous fenugreek extract on ovarian cancer cell viability and its link to changes in key apoptotic genes remains limited. Therefore, the present study was conducted to investigate the effect of the aqueous extract of Trigonella foenum-graecum on ovarian cancer cell viability using the MTT assay and to evaluate Bax, Bcl-2 and CASP3 gene expression via real-time PCR, thereby assessing the extract's anti-proliferative effects and its potential role in inducing apoptosis in these cells.

MATERIALS AND METHODS

Plant material and preparation of aqueous fenugreek seed extract

Fenugreek seeds were sourced from Tehran, Iran. The botanical identity of the plant material was confirmed by a qualified botanist, and a representative voucher specimen was subsequently archived in the designated herbarium.

The seeds were cleaned to remove foreign materials, washed with distilled water, completely dried, and ground into a fine powder using an electric grinder.

The aqueous fenugreek seed extract was prepared using a maceration method based on previously described procedures. Briefly, 50 g of finely powdered fenugreek seeds was mixed with 500 mL of distilled water (1:10, w/v) in a sterile glass container. The mixture was subjected to continuous agitation on an orbital shaker for 12 h at room temperature (approximately 25 °C) to facilitate the extraction of water-soluble constituents. After completion of the extraction process, insoluble plant residues were separated from the mixture by filtration using Whatman No. 1 filter paper. The obtained aqueous extract was then concentrated under vacuum with a rotary evaporator, while the temperature was maintained below 40 °C throughout the process. The concentrated extract was

subsequently subjected to lyophilization to produce a dry crude aqueous extract. Following freeze-drying, the recovered extract was weighed to calculate the extraction yield, placed in an airtight container, and maintained at 4 °C until further use.

The extraction yield was determined based on the weight of the dried extract, which was then kept in a securely sealed container at 4 °C until required for subsequent experiments.

For cell culture experiments, a stock solution of the aqueous fenugreek extract (10 mg/mL) was freshly prepared by dissolving 100 mg of the dried extract in 10 mL of sterile distilled water. Under aseptic conditions, the stock solution was sterilized by filtration through a 0.22-µm membrane filter. Immediately prior to cell treatment, the filtered stock was diluted in complete culture medium to obtain final working concentrations of 1, 10, 50, 100, and 200 µg/mL.

Working solutions of 1, 10, 50, 100, and 200 µg/mL were freshly prepared from the stock solution using complete culture medium.

SKOV-3 cell culture

SKOV-3 human ovarian adenocarcinoma cells were obtained from the Genetic Resources Center in Tehran, Iran. Cells were propagated in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin and incubated at 37 °C under humidified conditions with 5% CO_2. Fresh culture medium was supplied periodically, and cells were subcultured upon reaching approximately 70–80% confluence. Only actively proliferating cells in the logarithmic growth phase were utilized in the experimental assays.

Evaluation of the cytotoxic effect of aqueous fenugreek extract

Experimental design

The cytotoxic effect of aqueous fenugreek extract on SKOV-3 ovarian cancer cells was initially evaluated to determine its half-maximal inhibitory concentration (IC50). Based on a previous study evaluating aqueous T. foenum-graecum extract in SKOV-3 cells, five concentrations were selected: 1, 10, 50, 100 and 200 µg/mL. Untreated cells receiving only complete culture medium were considered the control group (0 µg/mL) (10). Two exposure periods, 24 and 48 h, were investigated independently. Therefore, the experimental groups for each exposure time consisted of: Control (0 µg/mL), 1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL aqueous fenugreek extract.

MTT cell viability assay

The potential cytotoxic effects of the aqueous fenugreek extract were assessed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). SKOV-3 cells were plated in 96-well plates at a concentration of 5×10^3 cells per well. Cells were initially incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 to ensure adequate attachment. The existing medium was then discarded and substituted with fresh complete culture medium supplemented with aqueous fenugreek extract at final concentrations of 1, 10, 50, 100, or 200 µg/mL. Control cells received complete culture medium without fenugreek extract. Separate plates were prepared for the 24-h and 48-h treatments. At the end of each treatment interval, MTT reagent was introduced into the wells, followed by incubation at 37 °C to promote the formation of purple formazan crystals by viable, metabolically active cells. After incubation, the medium was discarded and the generated formazan was solubilized in dimethyl sulfoxide (DMSO). Optical density (OD) was then measured at approximately 570 nm using a microplate reader. The percentage of viable cells was subsequently determined according to the following formula:

Cell viability (%) = (OD of treated cells / OD of untreated control cells) × 100

The viability of untreated cells was considered 100%. Each concentration was tested in technical replicates, and the experiment was independently repeated at least three times.

Determination of IC50

The percentage of viable SKOV-3 cells was plotted against the logarithm of aqueous fenugreek extract concentration to generate concentration–response curves. The IC50 was defined as the concentration of aqueous fenugreek extract that reduced cell viability to 50% of the untreated control. IC50 values were determined separately for the two exposure periods using nonlinear regression analysis: IC50-24 h and IC50-48 h. The experimentally determined IC50 was subsequently used for the investigation of apoptosis-related gene expression.

Analysis of apoptosis-related gene expression

Treatment of SKOV-3 cells for gene-expression analysis

Following determination of the IC50, a second set of experiments was conducted to investigate whether the cytotoxic activity of aqueous fenugreek extract was associated with modulation of apoptosis-related genes. SKOV-3 cells were seeded into 12-well culture plates at approximately 1×10^5 cells/well, consistent with previously published experiments investigating the effects of fenugreek-derived preparations on SKOV-3 cells. After allowing the cells to attach, the culture medium was replaced with fresh medium. The experimental groups consisted of: Control group: untreated SKOV-3 cells. Treatment group: SKOV-3 cells exposed to aqueous fenugreek extract at the

experimentally determined IC50 concentration. Cells were exposed to the selected treatment before being harvested for RNA extraction.

Total RNA extraction

At the end of the treatment period, the medium was aspirated and the cells were washed with ice-cold phosphate-buffered saline (PBS). Cellular RNA was subsequently extracted with the TRIzol kit (Yekta Tajhiz, Tehran, Iran) following the procedure recommended by the manufacturer. RNA yield and purity were assessed by spectrophotometric determination of absorbance at 260 and 280 nm. Samples that satisfied the required quality and concentration standards were selected for subsequent complementary DNA (cDNA) synthesis.

cDNA synthesis

Equal amounts of total RNA from the different experimental groups were used as templates for cDNA synthesis. Reverse transcription was carried out with a cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's recommended procedure. The generated cDNA was subsequently stored at −20 °C until qRT-PCR analysis.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was employed to quantify the relative mRNA expression of genes involved in apoptosis. The genes selected for expression analysis included:

BAX (BCL2-associated X protein) as a pro-apoptotic marker; BCL2 (B-cell lymphoma 2) as an anti-apoptotic marker; and CASP3 (caspase-3) as a key executioner of apoptosis. Gene-specific primers for BAX, BCL2, CASP3, and the selected housekeeping gene were used for amplification. Each qRT-PCR reaction contained cDNA template, gene-specific forward and reverse primers (Table 1), SYBR Green qPCR Master Mix, and nuclease-free water in a final volume according to the manufacturer's recommendations. The qRT-PCR protocol began with a 10-min denaturation at 95 °C, after which amplification proceeded for 40 cycles, each comprising 15 s at 95 °C and 60 s at 60 °C for denaturation and combined annealing/extension, respectively. Upon completion of the amplification cycles, a melting-curve assessment was performed to verify the specificity of the generated amplicons. Gene expression data were normalized against GAPDH as the endogenous reference, and each sample was analyzed in triplicate.

Relative gene-expression analysis

Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. For each sample:

$\Delta Ct = Ct$ (target gene) – Ct (reference gene)

and:

$\Delta\Delta Ct = \Delta Ct$(treated cells) − ΔCt(control cells).

Relative expression was calculated as:

Relative expression = $2^{-\Delta\Delta Ct}$.

Untreated SKOV-3 cells served as the reference group, with their relative gene expression level set to 1. The expression profiles of BAX, BCL2, and CASP3 were then evaluated in treated cells relative to the control group. Furthermore, the BAX/BCL2 expression ratio was determined to provide an additional measure of the relative contribution of pro-apoptotic and anti-apoptotic signaling. An increase in BAX and CASP3 expression accompanied by a reduction in BCL2 expression and an increased BAX/BCL2 ratio was evaluated in the context of activation of apoptotic signaling.

STATISTICAL ANALYSIS

All experiments were independently repeated at least three times, and quantitative results are reported as mean ± standard deviation (SD). For the MTT data, the effects of aqueous fenugreek extract concentration and exposure duration on SKOV-3 cell viability were evaluated by two-way analysis of variance (two-way ANOVA). Dunnett's multiple-comparisons test was subsequently used to evaluate differences between each extract concentration and its respective untreated control. Dose–response relationships were analyzed by nonlinear regression using a four-parameter logistic model. The IC50 values for the 24- and 48-h treatment intervals were estimated independently, together with their respective 95% confidence intervals. For the qRT-PCR data, differences in ΔCt values between untreated SKOV-3 cells and fenugreek-treated cells were evaluated using a two-tailed Student's t-test after

Table 1. Sequences of primers used for each gene in the qRT-PCR reaction.

Gene	Primer seq	TM (°C)	Size (bp)
BAX	F: CCCGAGAGGTCTTTTTCCGAG R: CCAGCCCATGATGGTTCTGAT	62.1 61.9	155
BCL2	F: GGTGGGGTCATGTGTGTGG R: CGGTTCAGGTACTCAGTCATCC	62.6 61.8	89
CASP3	F: GAAATTGTGGAATTGATGCGTGA R: CTACAACGATCCCCTCTGAAAAA	60.0 60.0	164
GAPDH	F: CTGGGCTACACTGAGCACC R: AAGTGGTCGTTGAGGGCAATG	62.0 62.9	101

confirming data normality. The same statistical test was applied to compare the BAX/BCL2 expression ratios between the control and treatment groups. Differences were regarded as statistically significant when $P < 0.05$. GraphPad Prism, version 10.6.1.892 (GraphPad Software, Boston, MA, USA), was used to perform all statistical analyses.

RESULTS

Effect of aqueous fenugreek extract on SKOV-3 cell viability

The cytotoxic potential of aqueous Trigonella foenum-graecum extract was assessed in SKOV-3 ovarian cancer cells using the MTT assay. Cell viability was evaluated following treatment with different concentrations of the extract for either 24 or 48 h. As shown in Figure 1, treatment with aqueous fenugreek extract resulted in a concentration- and time-dependent reduction in SKOV-3 cell viability. The viability of untreated control cells was considered 100%. After 24 h of treatment, exposure to the lowest concentration of fenugreek extract (1 µg/mL) resulted in only a slight reduction in cell viability to 96.4 ± 3.2%. Increasing the concentration to 10 and 50 µg/mL progressively decreased cell viability to 81.7 ± 4.1% and 42.6 ± 3.8%, respectively. At higher concentrations of 100 and 200 µg/mL, cell viability was further reduced to 27.3 ± 2.9% and 14.8 ± 2.1%, respectively. A greater cytotoxic effect was observed following 48 h of exposure. Cell viability was 92.1 ± 3.5% at 1 µg/mL and decreased to 65.4 ± 3.9% at 10 µg/mL. At 50 µg/mL, only 31.2 ± 3.1% of cells remained viable, while treatment with 100 and 200 µg/mL reduced cell viability to 17.6 ± 2.4% and 8.9 ± 1.7%, respectively.

Determination of IC50 values

To determine the IC50 of aqueous fenugreek extract, concentration–response curves were generated from the MTT viability data and analyzed using nonlinear regression. As illustrated in Figure 2, increasing concentrations of aqueous fenugreek extract progressively reduced SKOV-3 cell viability at both exposure times. The concentration–response curve was shifted toward lower concentrations after 48 h compared with 24 h, indicating an increased cytotoxic effect with prolonged exposure. Nonlinear regression analysis yielded an estimated IC50 of approximately 38.5 µg/mL after 24 h of treatment. In contrast, the estimated IC50 after 48 h was approximately 19.7 µg/mL. Thus, prolonging the exposure time from 24 to 48 h reduced the IC50 by approximately 49%, from 38.5 to 19.7 µg/mL.

Effect of aqueous fenugreek extract on apoptosis-related gene expression

To determine whether the cytotoxic activity of the aqueous fenugreek extract involved alterations in apoptosis-related pathways, the relative mRNA expression of BAX, BCL2, and CASP3 was quantified using qRT-PCR. For this analysis, SKOV-3 cells were exposed to the experimentally established IC50 concentration of the aqueous fenugreek extract, whereas cells receiving no extract treatment served as the control group. Target gene expression was

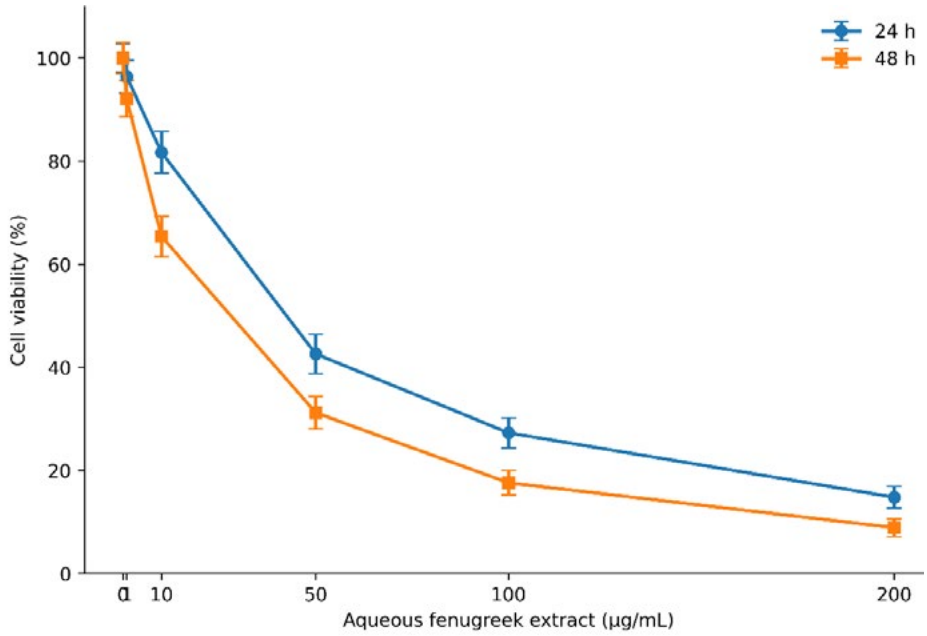


Fig 1. Influence of aqueous Trigonella foenum-graecum extract on the viability of SKOV-3 cells. Cells were exposed to aqueous fenugreek extract at concentrations of 1, 10, 50, 100, and 200 µg/mL for either 24 or 48 h. Cell viability was assessed by the MTT assay and normalized to that of the untreated control group, which was defined as 100% viability. Results are reported as mean ± SD.

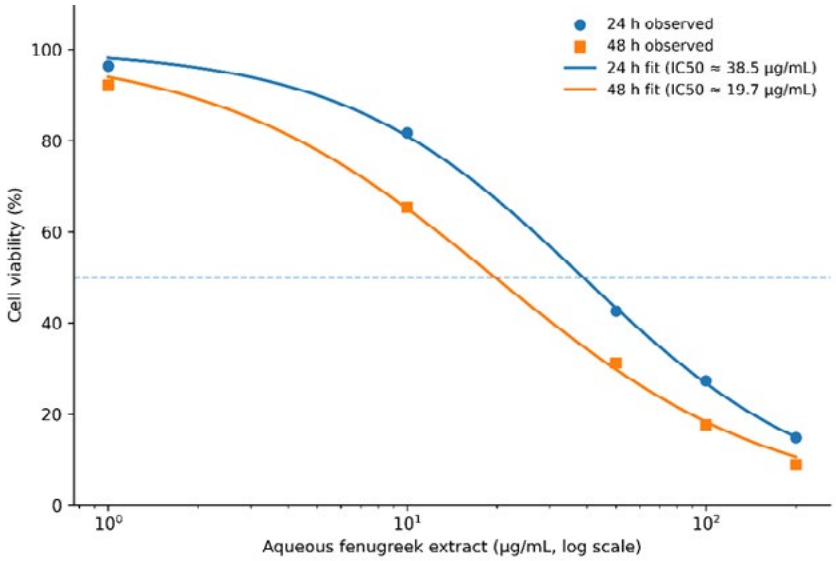


Fig 2. Concentration–response curves and estimated IC50 values of aqueous Trigonella foenum-graecum extract in SKOV-3 cells. SKOV-3 cells were exposed to increasing concentrations of aqueous fenugreek extract for 24 and 48 h. Cell viability determined by MTT assay was fitted using nonlinear regression. The horizontal dashed line represents 50% cell viability. The estimated IC50 values were approximately 38.5 µg/mL for 24 h and 19.7 µg/mL for 48 h.

normalized to the endogenous reference gene, and fold changes in mRNA levels were determined according to the $2^{-\Delta\Delta Ct}$ approach. For comparison, the expression of each target gene in the untreated control group was defined as 1.0.

BAX expression

Exposure of SKOV-3 cells to the aqueous fenugreek extract significantly enhanced the expression of the pro-apoptotic gene BAX. The relative expression level of BAX reached 2.83 ± 0.27-fold in extract-treated cells, compared with 1.00 ± 0.11-fold in the untreated control group. Thus, fenugreek treatment induced an approximately 2.8-fold elevation in BAX expression, representing a statistically significant difference relative to untreated cells ($P < 0.01$).

BCL2 expression

In contrast to BAX, treatment with aqueous fenugreek extract significantly reduced expression of the anti-apoptotic BCL2 gene. Relative BCL2 expression decreased from 1.00 ± 0.09-fold in untreated SKOV-3 cells to 0.43 ± 0.07-fold following treatment with the IC50 concentration of aqueous fenugreek extract. Thus, BCL2 mRNA expression was reduced by approximately 57% compared with untreated cells ($P < 0.01$).

CASP3 expression

The expression of CASP3, a major executioner caspase associated with apoptotic signaling, was also increased following treatment with aqueous fenugreek extract. Relative CASP3 expression increased from 1.00 ± 0.13-fold in control cells to 3.21 ± 0.34-fold in fenugreek-treated cells. This approximately 3.2-fold increase was statistically significant compared with the untreated control ($P < 0.001$).

The relative expression levels of the apoptosis-related genes BAX, BCL2, and CASP3 in SKOV-3 cells following treatment with aqueous fenugreek extract at the IC50 concentration are presented in Figure 3.

Effect of fenugreek extract on the BAX/BCL2 ratio

Since the balance between pro-apoptotic and anti-apoptotic members of the BCL2 family is an important determinant of cellular susceptibility to apoptosis, the BAX/BCL2 expression ratio was also evaluated. Based on relative gene-expression values, the BAX/BCL2 ratio in untreated control cells was approximately: 1.00, whereas treatment with aqueous fenugreek extract increased the ratio to approximately: 6.58. Thus, treatment resulted in an approximately 6.6-fold increase in the BAX/BCL2 ratio compared with untreated SKOV-3 cells. The marked increase in the BAX/BCL2 ratio was primarily associated with simultaneous upregulation of BAX and downregulation of BCL2 expression (figure 4).

DISCUSSION

In this study, the effects of aqueous Trigonella foenum-graecum seed extract on cell viability and apoptosis-associated gene expression were examined in SKOV-3 human ovarian cancer cells. The results revealed a progressive decline in cell viability as both the extract concentration and duration of exposure

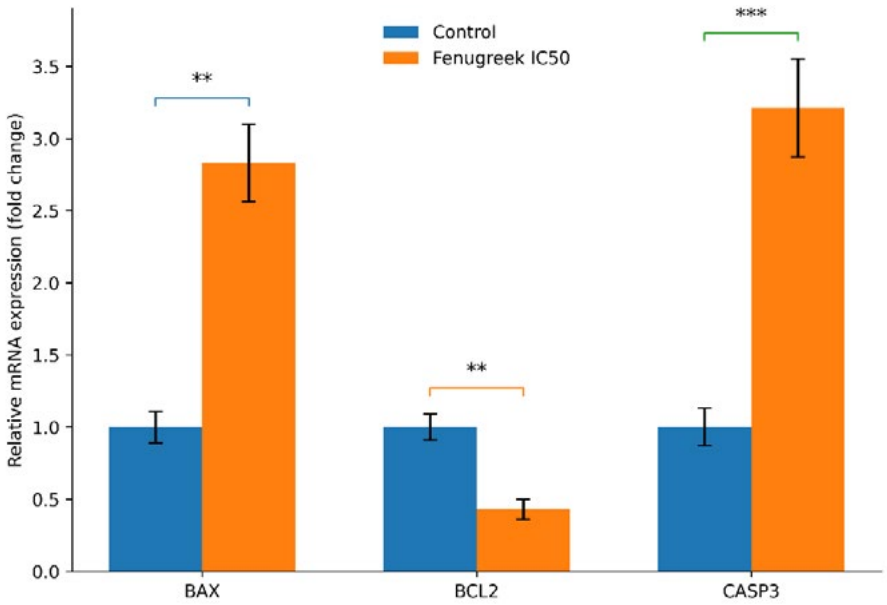


Fig 3. Relative mRNA expression levels of BAX, BCL2, and CASP3 were determined by qRT-PCR and normalized to GAPDH as the reference gene using the 2^-ΔΔCt method.

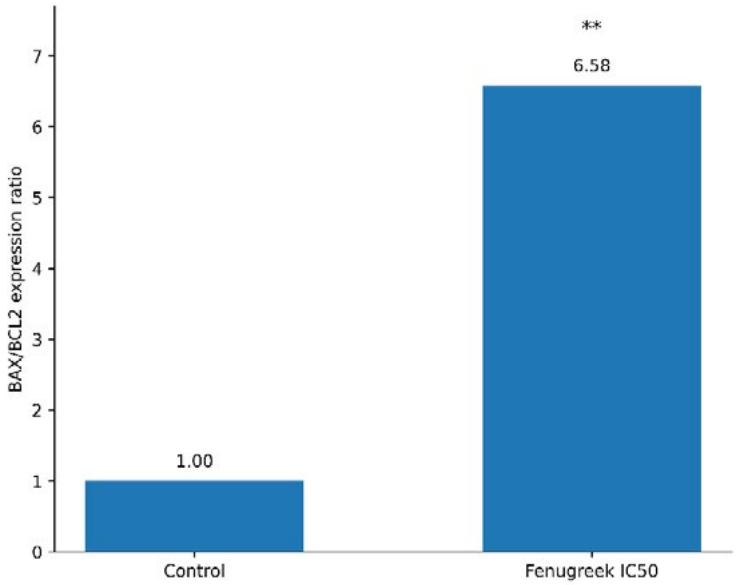


Fig 4. Effect of aqueous Trigonella foenum-graecum extract on the BAX/BCL2 expression ratio in SKOV-3 cells. The ratio of BAX to BCL2 expression was derived from relative mRNA levels quantified by qRT-PCR, with GAPDH serving as the reference gene and relative expression calculated using the 2−ΔΔCt method. Exposure of SKOV-3 cells to the IC50 concentration of aqueous fenugreek extract markedly elevated the BAX/BCL2 expression ratio from 1.00 in the untreated control group to 6.58 in the treated group. This significant increase reflects a shift in apoptosis-related gene expression toward a predominantly pro-apoptotic pattern (**P < 0.01 vs. untreated control).

increased, demonstrating concentration- and time-dependent cytotoxic activity. Moreover, treatment with the experimentally determined IC50 concentration altered the expression pattern of key apoptosis-related genes, as evidenced by increased BAX and CASP3 expression and reduced BCL2 expression. Consistent with these molecular changes, the BAX/BCL2 expression ratio was markedly elevated following treatment. Collectively, these observations indicate that alterations in apoptosis-associated signaling may contribute, at least partially, to the growth-inhibitory effects of aqueous fenugreek extract in SKOV-3 cells.

The MTT assay demonstrated a concentration-dependent decline in cell viability following exposure to the aqueous fenugreek extract. After 24 h of treatment, cell viability decreased from 96.4% at 1 µg/mL to 14.8% at 200 µg/mL, while after 48 h it decreased from 92.1% to 8.9% over the same concentration range. Consistent with these observations, nonlinear regression analysis yielded IC50 values of approximately 38.5 µg/mL

at 24 h and 19.7 µg/mL at 48 h. Thus, extending the exposure time from 24 to 48 h reduced the estimated IC50 by approximately 49%. This finding indicates that prolonged exposure increased the cytotoxic effect of the extract, since a lower concentration was required to produce a 50% reduction in cell viability after 48 h.

The concentration- and exposure time–dependent effects identified in this study are generally in agreement with earlier findings demonstrating the anticancer potential of fenugreek and its biologically active compounds. Of particular relevance, Stefanowicz-Hajduk et al. investigated standardized fenugreek seed extracts, fractions, and secondary metabolites in SKOV-3 ovarian cancer cells and demonstrated cytotoxic activity accompanied by apoptosis-associated cellular responses (11). The agreement between those findings and the present results is important because both studies involve the SKOV-3 cell line, although differences in extract composition and experimental conditions prevent direct numerical comparison of potency. Shokrzadeh et al. also investigated an aqueous preparation of T. foenum-graecum in SKOV-3 cells and demonstrated that these cells are responsive to aqueous fenugreek extract (10). Therefore, the present observation that aqueous fenugreek extract suppresses SKOV-3 viability is supported by previous direct evidence in the same ovarian cancer model.

Additional evidence supporting the susceptibility of SKOV-3 cells to fenugreek-associated compounds was provided by Stefanowicz-Hajduk et al. (2022), who investigated yamogenin, a steroidal sapogenin occurring in fenugreek and other plant species. Yamogenin reduced SKOV-3 viability and induced cellular changes associated with oxidative stress, cell-cycle disturbance, and apoptosis. Although an isolated phytochemical cannot be considered equivalent to a crude aqueous fenugreek extract, these observations provide a biologically plausible link between constituents naturally associated with fenugreek and the cytotoxic response of ovarian cancer cells (8). Fenugreek seeds contain several classes of potentially bioactive compounds, including steroidal saponins and sapogenins, flavonoids, alkaloids such as trigonelline, and other phytochemicals. Therefore, the cytotoxic effect of the aqueous extract observed in the present study may reflect the combined actions of multiple constituents rather than the activity of a single compound.

An important finding of the present study was that the reduction in cell viability was accompanied by marked alterations in apoptosis-related gene expression. Treatment of SKOV-3 cells with the IC50 concentration of aqueous fenugreek extract increased BAX expression approximately 2.83-fold, whereas BCL2 expression decreased to approximately 0.43-fold relative to untreated cells. BAX and BCL2 are functionally opposing regulators of mitochondrial apoptosis. BAX promotes mitochondrial outer membrane permeabilization under pro-apoptotic conditions, whereas BCL2 promotes cell survival by suppressing mitochondrial apoptotic signaling. Therefore, the simultaneous increase in BAX and decrease in BCL2 observed in the present study suggests a shift in the molecular balance toward a pro-apoptotic state.

The direction of these molecular changes is consistent with previous studies investigating fenugreek in other cancer models. Khalil et al. (2015) demonstrated that fenugreek treatment induced apoptosis in HepG2 hepatocellular carcinoma cells and was associated with alterations in apoptosis-related factors, including increased Bax and caspase-3. Similarly, Alrumaihi et al. (2021) reported that methanolic fenugreek seed extract induced apoptosis-associated alterations in breast cancer cells, including mitochondrial depolarization, increased reactive oxygen species generation, and modulation of the Bax/Bcl-2 balance. Although these studies were conducted in different cancer cell lines, their findings support the hypothesis that regulation of mitochondrial apoptosis-related factors may represent an important component of the anticancer activity of fenugreek (12, 13).

The concurrent upregulation of BAX and downregulation of BCL2 markedly shifted the BAX/BCL2 expression ratio, increasing it from approximately 1.0 in untreated SKOV-3 cells to 6.58 after fenugreek treatment. This observation may be particularly relevant because the balance between pro- and anti-apoptotic BCL2-family members can provide more mechanistic information than changes in either gene alone. An increase in the BAX/BCL2 ratio indicates a shift toward pro-apoptotic signaling and may increase cellular susceptibility to mitochondrial apoptotic stimuli. Similar alterations in the Bax/Bcl-2 balance have been reported following treatment of cancer cells with fenugreek preparations (13). Therefore, the marked increase in the BAX/BCL2 ratio observed in the present study provides a plausible molecular link between fenugreek treatment and the reduction in SKOV-3 cell viability.

In parallel with the changes in BAX and BCL2, CASP3 expression increased approximately 3.21-fold following treatment with aqueous fenugreek extract. Caspase-3 is a major executioner component of apoptotic signaling and participates in the cleavage of multiple cellular substrates during the execution phase of apoptosis. Previous investigations have similarly suggested involvement of caspase-associated pathways in the anticancer effects of fenugreek. Khoja et al. (2011) reported that fenugreek induced apoptosis in MCF-7 human breast cancer cells and altered the expression of several apoptosis-associated

genes, including Bax and members of the caspase pathway (14). Khalil et al. (2015) also reported increased caspase-3 in fenugreek-treated HepG2 cells (12). Moreover, the direct investigation of fenugreek-derived preparations in SKOV-3 cells by Stefanowicz-Hajduk et al. (2021) included analysis of caspase-3/7 activity, further supporting the potential involvement of executioner caspases in the response of ovarian cancer cells to fenugreek-derived compounds (11).

Taken together, the coordinated increase in BAX expression, reduction in BCL2 expression, elevation of the BAX/BCL2 ratio, and increase in CASP3 expression are compatible with the involvement of apoptosis-related signaling, potentially involving the mitochondrial pathway. A plausible interpretation is that treatment with the aqueous fenugreek extract alters the balance between pro- and anti-apoptotic BCL2-family members, thereby creating cellular conditions favorable for mitochondrial apoptotic signaling and downstream engagement of the caspase machinery. This interpretation is supported by previous studies in which fenugreek preparations or fenugreek-associated compounds produced mitochondrial alterations, oxidative stress, modulation of Bax/Bcl-2 signaling, and caspase-associated responses in cancer cells (8, 12, 13).

However, the present findings should not be interpreted as definitive evidence of functional activation of the mitochondrial apoptotic pathway. In particular, increased CASP3 mRNA expression does not necessarily indicate activation of caspase-3 protein, since qRT-PCR measures transcriptional abundance rather than proteolytic cleavage or enzymatic activity. Similarly, altered BAX and BCL2 mRNA expression does not directly demonstrate mitochondrial membrane permeabilization. Therefore, the current results are more appropriately interpreted as demonstrating a shift in apoptosis-related gene expression that is consistent with, but does not independently prove, activation of apoptosis. Functional assays such as Annexin V/propidium iodide flow cytometry, caspase-3/7 activity measurement, detection of cleaved caspase-3 and cleaved PARP, mitochondrial membrane potential analysis, or assessment of cytochrome-c release would provide stronger evidence for the proposed mechanism.

Nevertheless, the agreement between the MTT and qRT-PCR findings strengthens the biological interpretation of the present results. MTT analysis demonstrated a progressive decrease in metabolic viability with increasing extract concentration and exposure duration, while qRT-PCR demonstrated a coordinated increase in BAX and CASP3 together with suppression of BCL2. A reduction in MTT signal alone cannot distinguish between apoptosis, inhibition of proliferation, cell-cycle arrest, metabolic suppression, or other forms of cell death. However, when the viability results are considered together with the observed changes in apoptosis-associated genes, an apoptotic contribution to the cytotoxic effect becomes biologically plausible. This interpretation is also consistent with previous fenugreek studies in which reduced cancer-cell viability was accompanied by molecular or functional evidence of apoptosis (8, 11-14).

The greater cytotoxicity observed after 48 h compared with 24 h is also noteworthy. The reduction in the estimated IC50 from approximately 38.5 µg/mL at 24 h to 19.7 µg/mL at 48 h suggests that prolonged exposure increased the apparent cytotoxic potency of the extract. This response may reflect progressive accumulation of cellular stress, sustained suppression of proliferation, or gradual engagement of cell-death pathways. However, numerical differences between IC50 values obtained in the present study and those reported in previous investigations should not necessarily be interpreted as conflicting findings. Extraction solvent, extraction procedure, geographical origin and chemical composition of the plant material, concentration of individual phytochemicals, cancer cell type, initial cell density, duration of treatment, and analytical approach used for dose–response modeling can all substantially influence calculated IC50 values. In particular, aqueous, methanolic, lipid, standardized, and purified preparations of fenugreek are chemically different and should not be expected to demonstrate identical cytotoxic potency.

Overall, the present findings indicate that aqueous T. foenum-graecum seed extract exerts a concentration- and time-dependent inhibitory effect on SKOV-3 ovarian cancer cells and that this response is accompanied by coordinated modulation of apoptosis-related genes. The simultaneous increase in BAX and CASP3, decrease in BCL2, and pronounced elevation of the BAX/BCL2 ratio indicate a shift toward a pro-apoptotic molecular profile. Further studies incorporating functional apoptosis assays, protein-level analysis, additional ovarian cancer cell lines, non-malignant control cells, and phytochemical characterization of the aqueous extract are required to establish the precise molecular mechanisms and selectivity of the observed effects.

CONCLUSION

The results demonstrate that aqueous Trigonella foenum-graecum seed extract exerts cytotoxic effects on SKOV-3 human ovarian cancer cells, with the reduction in cell viability becoming more pronounced as the extract concentration and treatment duration increase. Fenugreek treatment was further accompanied by upregulation of BAX and CASP3, downregulation of BCL2, and a pronounced elevation in the BAX/BCL2 expression ratio, collectively reflecting a shift toward a pro-apoptotic gene expression pattern. These molecular alterations suggest that regulation of apoptosis-associated pathways may be involved in the growth-inhibitory activity of the extract against ovarian

cancer cells. Nevertheless, confirmation of this proposed mechanism requires further investigation incorporating functional assays of apoptosis, assessment of apoptosis-related proteins, additional ovarian cancer cell models, and non-malignant cells. Such studies would also help determine the selectivity and potential anticancer significance of aqueous fenugreek extract.

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Ethics Approval and Consent to Participate

No ethics approval or consent statement is provided in the article. Since the study involved laboratory experiments using a human ovarian cancer cell line (SKOV-3), please confirm whether ethical approval was required and obtained, or whether an exemption applies.

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Regenerative Biotechnology of the Auditory System: Recent Advances in Cell Therapy, Tissue Engineering, Gene Therapy, and Genome Editing Toward Personalized Medicine

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ABSTRACT

Sensorineural hearing loss ranks among the most common auditory disorders, arising mainly from injury to the cochlear sensory hair cells and their associated afferent neurons. Because the mammalian cochlea possesses only a minimal capacity for self-repair, achieving a definitive cure remains a persistent clinical obstacle. Recent advances in biotechnology, particularly in the fields of stem cell biology, tissue engineering, gene therapy, and genome editing, have opened new avenues for auditory system regeneration. This article surveys how hair cells differentiate at the molecular level, assesses the regenerative capabilities of different stem cell populations for repairing the inner ear, and reviews applications of newer tools such as RNA interference, CRISPR/Cas9-mediated editing, viral and non-viral gene-delivery systems, inner-ear organoid models, and bioengineered scaffolds. Preclinical studies indicate that integrating stem cell–based therapies with advanced biotechnological approaches can enhance the structural and functional regeneration of the inner ear. Despite these promising findings, further research is required to improve the efficacy, safety, and clinical translation of these technologies before they can be widely implemented in therapeutic practice.

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INTRODUCTION

Cell-based therapy has emerged as a promising strategy for treating numerous diseases, and developing novel therapeutic interventions for hearing loss is of particular importance. The application of stem cells for the treatment of deafness and hearing impairment has become one of the most active areas of biomedical research (1, 2). The differentiation of embryonic and adult stem cells into auditory hair cells, followed by their transplantation into the organ of Corti, represents a critical objective of regenerative medicine for hearing restoration (3). These approaches rely on the intrinsic capacity of stem cells to engraft, proliferate, differentiate, and survive within the target tissue, serving as replacement or adjunctive therapies for damaged cells. Most congenital and acquired hearing loss results from destruction of cochlear hair cells and/or auditory neurons (4). In mammals, this damage is essentially irreversible, since hair cells cannot be replaced through cell division or differentiation of other inner ear epithelial cells (5); yet no definitive cure exists, leaving only partial rehabilitative compensation available. In recent years, stem cell–based therapy has been proposed to replace damaged or lost hair cells in mammals. Initial studies showed that embryonic, adult inner ear, and neural stem cells can be induced to differentiate into hair cells, strengthening the prospect of treating hearing loss through regenerative medicine (4). Cell therapy and tissue engineering have therefore attracted considerable attention as approaches to

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restore both structural and functional integrity of the auditory system, though challenges remain in cell-source selection, control of differentiation, efficient delivery/engraftment, and achieving durable outcomes. Therefore, this review aims to critically examine current evidence on cell therapy and tissue engineering in auditory system regeneration, evaluate the potential and limitations of these approaches, and discuss future perspectives for developing effective therapies for hearing loss and deafness.

Anatomy and Underlying Developmental Mechanisms of the Inner Ear

The inner ear is organized into two distinct functional units: a hearing-related component and a balance-related component. The latter, which incorporates the semicircular canals, governs the maintenance of equilibrium (Figure 1).

The auditory portion of the inner ear centers on the cochlea, a spiral structure built from four main compartments. The scala vestibuli lies next to the semicircular canals and holds perilymph, divided from the cochlear duct by Reissner's membrane. Parallel to it, the scala tympani sits adjacent to the tympanic membrane, likewise perilymph-filled, and is partitioned from the cochlear duct by the basilar membrane. At the cochlear apex, these two chambers merge at a point known as the helicotrema. Between them runs the scala media (cochlear duct), an endolymph-containing space enclosed by Reissner's, the reticular lamina, and the basilar membrane; it houses the organ of Corti, the true auditory sensory structure (Figure 1). Mechanical displacement of the endolymph caused by incoming sound waves bends the stereocilia atop the hair cells, gating open ion channels permeable to K^+ and Ca^{2+}; the resulting membrane depolarization drives glutamate release at the presynaptic terminal, generating the electrical impulse relayed to the brain. **Modiolus:** the cone-shaped bony core at the center of the cochlea. **Cochlear cupula (apex):** the tip of the cochlea, where the bony canal completes ~2.5 turns around the modiolus (6, 7).

Each stage of inner ear development is regulated by the coordinated activation of defined gene sets. The precise expression of the *Lfng*, *Six1*, *Eya1*, *Ngn1*, *Sox2*, and *Delta1* genes is essential for the development of the otic cup. *Ngn1* and *Delta1* play pivotal functions in the differentiation of the sensory–neural domain. Specifically, when the Notch ligand Delta1 is expressed, it suppresses a subset of prosensory epithelial cells from committing to a neuroblast fate. In contrast, the expression of *Ngn1* (neurogenin 1), which encodes a basic helix–loop–helix (bHLH) transcription factor, in adjacent sensory epithelial cells promotes their differentiation into supporting cells, auditory hair cells, and several other specialized cell types (8).

Genetic and Molecular Pathways Governing Hair Cell Fate Specification

Atoh1 (also referred to as Math1) stands as the earliest and most critical gene linked to the emergence of hair cells. This bHLH transcription factor is expressed, during embryonic life, exclusively within cells that have been directed towards becoming hair cells (9).

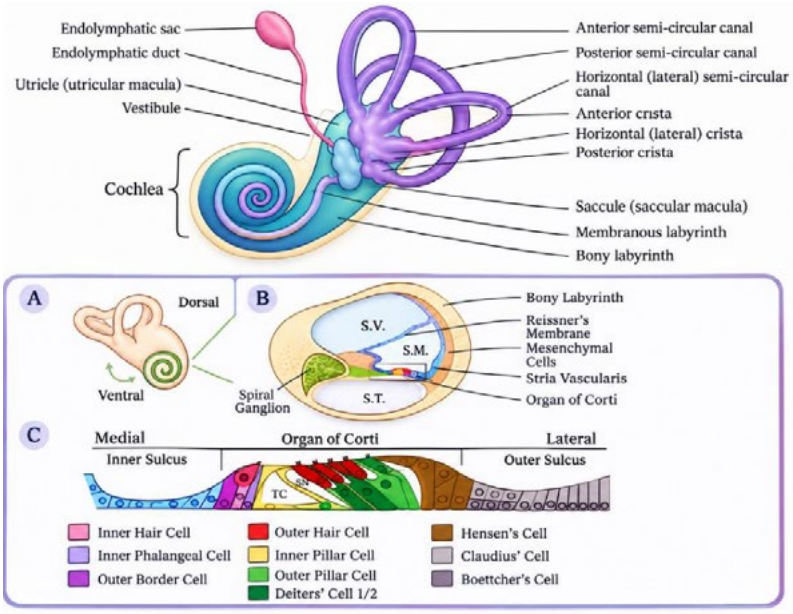


Fig 1. Structural organization of the inner ear, cochlea, and organ of Corti. (A) General layout of the inner ear and the position of the cochlea. (B) Cross-sectional view depicting three fluid-filled compartments — the scala vestibuli, scala media, and scala tympani — separated by Reissner›s, basilar, and tectorial membranes, with the organ of Corti resting atop the basilar membrane and receiving innervation from spiral ganglion neurons. (C) Magnified depiction of the organ of Corti, delineating inner and outer hair cells alongside pillar cells, Deiters› cells, and Hensen›s cells.

Multiple regulators acting in parallel govern both how much Atoh1 is expressed and where. As depicted in Figure 2, Sox2 present across the entire sensory domain is required to switch on Atoh1; yet prolonged Sox2 expression works against Atoh1-mediated hair cell differentiation, whereas lowering Sox2 levels leads to a greater number of hair cells forming (8, 10). A separate regulatory layer comes from the Id (Inhibitors of Differentiation) proteins Id1, Id2, and Id3 which are abundant in the cochlear duct during early development but decline sharply once hair cell differentiation begins, consistent with a role as Atoh1 antagonists: forcing Id3 expression blocks hair cell formation, pointing to the Id family as candidate targets for future therapeutic intervention (11). The most extensively studied pathway regulating *Atoh1* is Notch signaling, in which membrane-bound receptors and ligands restrict signal transduction to directly contacting cells, allowing multipotent progenitors to diverge into distinct lineages (12). expressed in epithelial cells, while its ligands Delta-like 1 (Dll1) and Jagged2 are restricted to hair-cell-committed cells (13, 14), and the inhibitory bHLH factors Hes and Hey1 are expressed exclusively in supporting cells (15, 16). Genetic deletion of individual Notch pathway components increases hair cell number, with the magnitude depending on the specific gene disrupted (17).

Key Molecular Markers for Understanding the Differentiation of Inner Ear Stem Cells into Auditory Hair Cells

The path by which inner ear stem cells become hair cells unfolds across three sequential phases prosensory specification, lineage commitment toward hair cells, and final maturation. Prior to E12, the future sensory epithelium is broadly marked by Notch1, Jagged1, and Sox2 expression (17, 18). Once E12 is reached, climbing levels of the cell-cycle inhibitors $p27^{Kip1}$, $p19^{Ink4d}$, and Rb push prosensory cells out of the cell cycle, such that by E14 every $Sox2^{+}$ cell has also become $p27^{Kip1+}$. After this point of mitotic arrest, a fraction of these cells switches on *Atoh1* the pivotal determinant of hair cell identity with the Notch ligands *Jagged2* and *Delta1* coming online shortly afterward; these ligands, in turn, trigger Notch signaling in neighboring cells and induce *Hes1*/*Hes5*, steering those neighbors toward becoming supporting cells instead (19). By E16–E17, *Notch1*, *Jagged1*, p27, and *Hes5* have become confined to the supporting-cell population, with Sox2 levels now considerably higher in supporting cells than in hair cells; come P0, hair cells no longer retain any Sox2, whereas supporting cells continue to express it. Over the interval from birth through P7, the hair-cell-associated markers *Atoh1*, *Jagged2*, and *Delta1* gradually fade out (accompanied by a parallel decline in *Notch1*/*Hes5* within supporting cells), while Sox2, p27, and *Jagged1* remain detectable in supporting cells right through to full maturation (18).

Converting inner ear stem cells into functional auditory hair cells unfolds through a multistep sequence, one that hinges on the tightly coordinated interplay of numerous transcription factors and signaling cascades (Figure 3). It is this same regulatory circuitry that underpins present-day cell-based and tissue-engineering approaches aimed at hair cell regeneration in sensorineural hearing loss, and reproducing these developmental steps both in vitro and in a living organism continues to stand as a

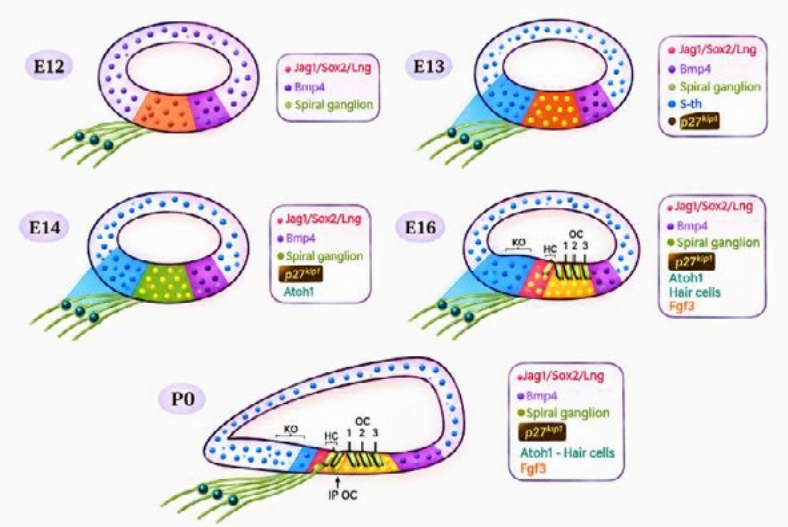


Fig2. Role of Sox2 in regulating Atoh1 expression during inner ear development. At E12, the prosensory domain expresses Jag1, Sox2, and Lng; BMP4 then refine the sensory domain boundaries while p27kip1 drives cell cycle exit. Atoh1 expression begins at E14, initiating hair cell differentiation. Fgfr3 subsequently marks pillar and Deiters› cells, and by birth (P0) the organ of Corti reaches its mature architecture one row of inner and three rows of outer hair cells with supporting cells.

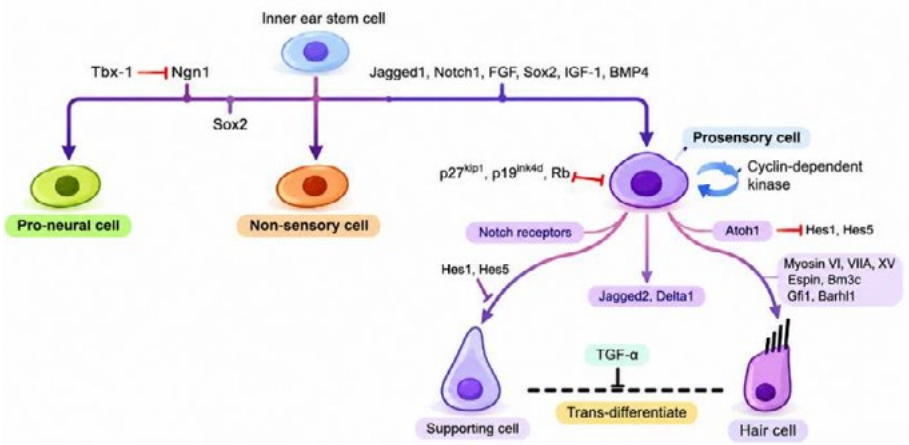


Fig3. Signaling pathways governing inner ear stem cell fate decisions. Stem cells of the inner ear give rise to three lineages — pro-neural, non-sensory, and prosensory — with commitment to the prosensory lineage driven by Notch/Jagged signaling together with Sox2, Atoh1, FGF/BMP4/IGF-1, and the cell-cycle regulators p27Kip1 and p19Ink4d. From this prosensory pool, both hair cells and supporting cells subsequently emerge: Notch signaling, acting through Hes1/Hes5, keeps the two populations in balance, while Atoh1 pushes cells toward the hair cell fate and switches on hair-cell-specific genes such as Myosin VI/VIIA/XV, Espin, Brn3c, Gfi1, and Barhl1.

core objective for the field (4, 18).

Prospects for the Application of Stem Cells in the Treatment of Deafness

Work in recent years has made it clear that success in cell-engineering and regenerative-medicine therapeutics hinges largely on how well the molecular circuitry controlling self-renewal, differentiation, and plasticity in stem cells can be regulated. It follows that pinpointing and manipulating these regulatory pathways holds real potential for boosting the effectiveness of stem-cell-based approaches to regenerating specialized tissue (20). Broadly, stem cells are undifferentiated cells capable of self-renewal; they are grouped by origin into embryonic and adult types, and by their differentiation range into toti-, pluri-, multi-, or unipotent categories. Applications span much of regenerative medicine, extending from neural tissue to bone, cartilage, and the cornea. Embryonic stem cells (ESCs) in particular originate from the blastocyst's inner cell mass, growing as tightly packed, Sox2-positive colonies that divide indefinitely and retain the ability to give rise to all three germ layers (2, 3, 21), and have been investigated for infertility, neurological, cardiovascular, and metabolic disease (22). Worldwide, congenital deafness impacts hundreds of millions of individuals, most commonly stemming from injury to the cochlea's hair cells and the neurons connected to them (23); Since these specialized mechanosensory cells are responsible for translating sound into neural impulses and possess only a narrow window for spontaneous repair, the resulting damage tends to be permanent (24). No cure currently exists, leaving hearing aids and cochlear implants as the sole clinical options — devices that come with drawbacks, not least their reliance on external hardware, which has spurred interest in regenerative alternatives instead. Foundational work mapping the genes driving auditory system development and regeneration across rodents, birds, and amphibians (25-28), together with the extensive use of rodent models for their genetic and physiological similarity to humans (29), established a key distinction: unlike fish, amphibians, and birds, mammalian hair cells cannot regenerate once lost, since they cannot be replaced through cell division or endogenous regeneration. This fundamental difference has made cell-based therapy, alongside other stem cell sources such as inner ear epithelial and cochlear supporting cells, one of the leading strategies for inner ear repair (4, 30, 31). The first study testing this approach stem cell transplantation into ototoxin-deafened mice found accelerated hearing recovery (27), prompting numerous follow-up studies (28, 32, 33). Building on this, later research established several complementary findings: ESC-derived progenitor cells were capable of turning into hair-cell-like cells and taking up residence within injured inner ear epithelium (34), while ESC-derived neural progenitors, once transplanted into the cochlear nerve trunk, extended outgrowths oriented specifically toward the organ of Corti (35). Elsewhere, neural stem cells placed in co-culture with hair cells were driven towards an auditory identity (36), and inner ear stem cells proved able to give rise to sensory neurons that were functionally active (37). Human embryonic auditory stem cells, for their part, could be expanded in culture and directed to differentiate into both neurons and hair cells, yielding useful models for studying ototoxicity and for drug-screening applications (38). Differentiation conditions were further shown to influence the mechanosensitivity of hair-cell-like clusters derived from ESCs and induced pluripotent stem cells (iPSCs) (25), while mouse ESCs cultured in stromal-conditioned medium (39), or engineered to overexpress *Atoh1* in co-culture with spiral ganglion neurons, likewise acquired

mechanosensory, hair-cell-like properties (26). Non-sensory supporting cells show similar promise: in co-culture systems, cells located closest to sensory hair cells display the greatest capacity for hair-cell regeneration (28, 32). Among the stem cell types investigated, adult mesenchymal stem cells (MSCs) have emerged as one of the most promising candidates in preclinical research; the following sections review the principal cell sources and their applications in auditory system regeneration.

Application of Adult (Mesenchymal) Stem Cells

Mesenchymal stem/stromal cells (MSCs) are mesoderm-derived progenitors characterized by fibroblast-like morphology, plastic adherence, self-renewal, and differentiation into osteogenic, adipogenic, and chondrogenic lineages; their immunomodulatory and regenerative properties have made them one of the most widely used cell types in regenerative medicine (40). Three principal cell sources have been examined as candidates for inner ear regeneration, namely those derived from bone marrow (BM-MSCs), adipose tissue (AD-MSCs), and umbilical cord tissue (UC-MSCs), and the evidence supporting each differs considerably. Of the three, BM-MSCs carry the most substantial experimental backing when it comes to restoring auditory function and driving regeneration of both hair cells and spiral ganglion neurons a strength attributable to their ability to migrate toward sites of injury, release neurotrophic factors, and reshape cochlear architecture. AD-MSCs are more accessible and proliferate more readily, making them attractive for clinical translation, but evidence for their direct differentiation into auditory cell types remains limited. UC-MSCs combine high proliferative potential with low immunogenicity and anti-inflammatory activity, though preclinical evidence in inner ear regeneration is still sparse. Overall, BM-MSCs currently represent the most promising candidate for auditory cell therapy (41). Numerous studies have investigated different strategies to enhance the survival, regenerative potential, and differentiation capacity of mesenchymal stem/stromal cells (MSCs) under both in vivo and in vitro conditions. In a study conducted by Lin et al. in 2012, adipose-derived mesenchymal stem cells isolated from the subcutaneous adipose tissue of mice (of mesodermal origin) were co-cultured with spiral ganglion neurons isolated from the organ of Corti of the mouse cochlea (of ectodermal origin) to induce the differentiation of MSCs into auditory hair cells under in vitro conditions. The authors concluded that spiral ganglion neurons secrete factors that are required for the differentiation of mesenchymal stem cells into auditory hair cell-like cells. RT-PCR served to profile the expression of the relevant molecular markers, whereas immunocytochemistry was employed to examine cellular morphology and structural characteristics (42). By contrast, when human BM-MSC-derived neural progenitor-like cells were co-cultured with chick utricular stromal cells under retinoic acid, EGF, and Sonic Hedgehog signaling, neuronal markers increased but hair-cell markers (Myosin VI and VIIa) remained absent indicating that these these culture conditions were insufficient to induce the complete differentiation of mesenchymal stem cells into functional auditory hair cells (43). Numerous studies have employed UC-MSCs for differentiation into various cell types under in vitro culture conditions. For example, in a research conducted by Yoo et al. in 2010, human UC-MSCs were co-cultured with hair follicle-derived cells to induce their differentiation into dermal papilla cells, which possess the potential to promote hair formation, as a potential therapeutic approach for alopecia (44), but no study to date has reported their differentiation into auditory hair cells.

Application of Embryonic Stem Cells (ESC)

Evidence for ESC-based hair cell generation comes from two complementary approaches. Co-culturing inner ear-derived auditory stem cells with Math1/nGFP mouse ESCs directed sequential differentiation first into Nestin^{+} progenitor cells, then into hair cell-like cells confirmed by Myosin VIIa, Espin, *Math1*, and *Atoh1* expression alongside loss of the supporting-cell marker pan-cytokeratin (34). Building on this, a comparative co-culture study found that intact ESC-derived embryoid bodies generated significantly more neuron-like cells when paired with auditory hair cells than with auditory neurons, indicating that hair cells provide a more effective inductive microenvironment than neurons for directing stem cell differentiation (36).

Strategies for Reprogramming Stem Cells Through Genetic Manipulation and Modulation of Key Gene Expression

Among the applied fields within biotechnology, stem cell research occupies a particularly prominent place, given the fresh possibilities it offers for cell-based therapy and the replacement of damaged tissue. A turning point came in 1998, when the first human embryonic stem cells were successfully isolated; combined with their striking versatility in generating diverse cell and tissue types spanning neurons, hepatocytes, and insulin-secreting cells this discovery ushered in a new phase for tissue engineering and drew biological science and clinical practice closer together. Even so, considerable obstacles continue to stand between stem cells' therapeutic promise and their actual clinical use. Our grasp of the molecular processes steering stem cell differentiation has advanced substantially thanks to progress across developmental biology, tissue and scaffold engineering, genetic engineering,

and nanoscale cell manipulation (3, 45). Gene transfer or modulation of gene expression, achieved by delivering gene expression cassettes into target cells, can influence not only the behavior of the transplanted cells and their interactions with neighboring host cells, but also their responsiveness to environmental changes such as hypoxia, hormonal fluctuations, and pharmacological exposure. A variety of delivery methods have been developed for this purpose: viral vectors for stable, long-term gene expression, and non-viral approaches DNA plasmids together with physical and chemical transfection techniques including liposomes, microinjection, microporation, nucleofection, electroporation, and magnetofection. Compared with viral approaches, non-viral delivery systems tend to trigger a weaker immune response and carry a lower chance of insertional mutagenesis, although this comes at the cost of reduced transfection efficiency and expression that is only short-lived. On the viral side, four vector types retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAVs) dominate current practice for gene delivery, whether the setting is in vivo or in vitro. Retroviral and lentiviral vectors achieve stable gene transfer through genomic integration, but this remains technically challenging in mesenchymal and hematopoietic stem cells because of their relatively low transduction efficiency; moreover, the random integration of viral genomes into the host genome can disrupt endogenous or pluripotency-associated genes, raising the risk of insertional mutagenesis and tumorigenesis. Lentiviral vectors are most commonly used for genetically modifying hematopoietic stem cells for example, to deliver the β-globin gene in mouse models of β-thalassemia (46-48) and lentiviral HSP70 overexpression has similarly been used to improve MSC survival under ischemic stress (49), yet the limited tissue penetration of these vectors in vivo makes them poorly suited to transfecting inner ear sensory epithelium. Adenoviral and Sendai virus vectors, by contrast, achieve high, non-integrating transduction efficiency across diverse inner ear cell types and are considered comparatively safe despite a risk of immune activation at high doses, making them the preferred vehicle for auditory gene delivery (50). A great deal of research has focused on how stem cells transform into the specialized cell types that make up different tissues and organs. As one illustration, Talens-Visconti and colleagues (2006) set out to compare transduction efficiency between bone marrow- and adipose-derived mesenchymal stem cells, delivering the transcription factors C/EBPα and C/EBPβ both known to control expression of numerous hepatocyte-specific genes — via adenoviral vectors, with the goal of pushing these cells toward a hepatocyte-like identity (51). As noted earlier, *Atoh1* ranks as the earliest and most consequential gene tied to hair cell formation: this bHLH transcription factor is expressed, during embryonic development, only within cells already committed to becoming hair cells, and a substantial body of work has pinpointed its upregulation as the pivotal molecular trigger for hair cell differentiation. Oshima and colleagues explored this by examining how stem cells were distributed across the auditory and vestibular regions of the inner ear in Math1/nGFP transgenic mice. What they found was evidence of separate stem cell populations, each with its own regenerative capacity, within the inner ear a distinction they argued carries real implications for designing stem cell-based therapeutic strategies aimed at auditory system regeneration (52). A large number of studies have also examined the potential of adenoviral vectors to promote stem cell differentiation under in vivo conditions. Kawamoto et al. used adenoviral vectors carrying the *LacZ* reporter gene to deliver the *Math1* gene into the endolymphatic compartment of the cochlea in adult guinea pigs and to monitor transgene expression. Four days after viral inoculation, the animals were sacrificed, and cochlear tissues were analyzed to evaluate the expression of hair cell differentiation markers (53). Similarly, Izumikawa et al. investigated the effect of adenovirus-mediated *Atoh1* overexpression on the differentiation of auditory hair cells in deaf guinea pigs under in vivo conditions. Their findings confirmed the essential role of *Atoh1* in hair cell differentiation and further demonstrated the high efficiency of adenoviral vectors in mediating gene transfer to cochlear cells (54). Accordingly, Chen et al. employed the simultaneous expression of Pax2 and Math1 in supporting cells isolated from the cochleae of neomycin-treated mice to generate hair cells. Their results confirmed the high efficiency of adenoviral vectors for delivering therapeutic genes into supporting cells and further demonstrated their potential for promoting hair cell regeneration (55). In these studies, only cellular differentiation was evaluated using techniques such as the analysis of marker gene expression, and no investigations were conducted to assess the electrophysiological responsiveness of the differentiated hair cells to external stimuli. In addition to the aforementioned studies, numerous investigations have examined the differentiation of MSCs, ESCs, and iPSCs into auditory hair cells under *in vitro* conditions. Owing to their long lifespan, high homing capacity, and ability to evade immune rejection, mesenchymal stem cells are considered promising candidates for regenerative therapies and cellular differentiation.

Non-viral delivery has also been tested directly against this viral evidence. Plasmid-mediated delivery of *Math1*/GFP into MSC-derived progenitors successfully induced Myosin VIIA expression, confirming feasibility, but transfection efficiency reached only ~2% illustrating the efficiency trade-off

of non-viral methods relative to adenoviral approaches (33). Because of their capacity to give rise to all three embryonic germ layers, ESCs and iPSCs likewise stand out as potent starting material for differentiation studies. Work carried out in 2006-2007 revealed that embryonic stem cells could be pushed towards becoming neural progenitors and, further along, auditory sensory neurons. A later study, published in 2010, went a step further by devising a multistep protocol that steered embryonic stem cells down the ectodermal path and ultimately towards auditory cell generation; this work examined how Wnt and TGF-β signaling shaped iPSC differentiation using iPSCs reprogrammed from mouse fibroblasts alongside embryonic stem cells sourced from Math1/nGFP transgenic mouse blastocysts across a range of different culture substrates (25). Then, in 2012, Ouji and colleagues took a different route, using a doxycycline (DOX)-controlled Tet-on inducible system to drive embryonic stem cells towards a hair-cell-like fate; although the approach worked, differentiation efficiency remained low (39). A parallel strategy exploits RNA interference miRNAs, siRNAs, and shRNAs which post-transcriptionally silence target genes by forming double-stranded complexes with their mRNA, either blocking translation or triggering degradation, offering a specific (if imperfect, given seed-region-driven off-target effects) tool for probing gene function without permanent genomic modification (56, 57). This approach has been used to direct MSC differentiation toward bone, cartilage, liver, and neural lineages (57-59). For example, Weimin Qiu (2014) demonstrated that transfection of human mesenchymal stem cells with a miR-141-3p mimic using the lipid-based transfection reagent Lipofectamine RNAiMAX significantly increased miR-141-3p expression. This upregulation directly targeted the *CDC25A* gene, resulting in G1 cell cycle arrest, reduced alkaline phosphatase activity, and inhibition of the osteogenic differentiation of these cells. Taken together, these results underscore just how central a role microRNAs play in governing both the proliferation and differentiation of stem cells, while also pointing to their promise as tools within cellular engineering and regenerative medicine (60). The same interference tools have directly tested the Notch–*Atoh1* axis introduced earlier. Genetic deletion of Notch pathway components increases hair cell number to a degree that depends on the specific gene disrupted: *Hes1*$^{+}$□$^{-}$;*Hes5*$^{-}$□$^{-}$ and *Hes1*$^{-}$□$^{-}$;*Hes5*$^{+}$□$^{-}$ mutant mice show a marked rise in both hair cell number and *Math1* expression (19, 61, 62). Furthermore, siRNA-mediated silencing of Math1 in ESCs reduced Brn3c expression and hair cell number, and reporter assays confirmed that Math1 directly activates Brn3c transcription (63). Turning to the Notch pathway itself, Jung and colleagues (2013) examined its contribution to hair cell regeneration by knocking down Hes5 with a targeted siRNA in mice whose utricles had been damaged using an aminoglycoside. What emerged was that suppressing Hes5 pushed Atoh1 expression upwards, and this in turn drove supporting cells to convert into newly formed hair cells. Taken together, this work made the case that using RNA interference (RNAi) to selectively knock down downstream elements of the Notch pathway offers a viable route towards encouraging hair cell regeneration within the inner ear (64).

Emerging Technologies for Auditory System Regeneration

One of the most significant recent advances in auditory tissue engineering and cell-based therapy has been the development of three-dimensional inner ear organoids derived from ESCs and iPSCs. These three-dimensional culture systems are capable of recapitulating many of the developmental and functional characteristics of the sensory epithelium of the inner ear and can generate auditory hair cells and auditory neurons with morphological and molecular properties closely resembling those of native tissue. In addition to providing an appropriate model for investigating inner ear development and hereditary hearing disorders, these organoids have emerged as promising platforms for evaluating gene therapy, cell-based therapy, and drug screening strategies in regenerative medicine. Nevertheless, enhancing the functional maturation of differentiated cells, establishing the spatial organization characteristic of the human cochlea, and improving synaptic connectivity with auditory neurons remain among the major challenges facing this technology (65, 66). A systematic review published in 2025 evaluated both preclinical and clinical studies investigating cell-based therapies for sensorineural hearing loss. The review demonstrated that mesenchymal stem cells, embryonic stem cells, and inner ear-derived stem cells were able to produce partial improvements in hearing thresholds in animal models through the secretion of neurotrophic factors, modulation of inflammatory responses, protection of spiral ganglion neurons, and stimulation of auditory hair cell regeneration. Despite these encouraging findings, the authors emphasized that heterogeneity in cell delivery methods, differences in cell sources, limited survival of transplanted cells, and the lack of large-scale clinical trials remain the principal barriers to the clinical translation of these therapeutic approaches (67). In recent years, CRISPR/Cas-based genome editing has emerged as one of the most promising biotechnological tools for the treatment of hereditary hearing loss and the enhancement of inner ear regenerative strategies. By introducing precise and targeted genomic modifications, this technology enables the correction of pathogenic mutations, inactivation

of dominant disease-causing alleles, and reactivation of molecular pathways involved in auditory hair cell differentiation. Preclinical studies have demonstrated that CRISPR/Cas9, base editing, and prime editing platforms can successfully correct mutations in genes such as *TMC1*, *MYO6*, *KCNQ4*, and *Stereocilin* in animal models, resulting in measurable improvements in auditory function. Furthermore, the integration of CRISPR technology with iPSCs has enabled the generation of genetically corrected auditory hair cells, which may serve as valuable resources for cell-based therapy, disease modeling, and drug discovery. Despite these remarkable advances, several important challenges remain, including the targeted delivery of genome-editing systems to inner ear cells, reduction of off-target effects, improvement of editing efficiency in postnatal cells, and the establishment of long-term safety. Nevertheless, the convergence of CRISPR-based genome editing with tissue engineering, biological nanocarriers, and advanced gene delivery systems has created a promising outlook for the development of personalized regenerative therapies for patients with sensorineural hearing loss (68-71). Recent advances in base editing technology have opened new avenues for the treatment of hereditary hearing loss. In a study published in Nature Biomedical Engineering in 2024, an adenine base editor (ABE) was employed to correct a pathogenic mutation in the *OTOF* gene in a mouse model of congenital deafness. The genome-editing system was delivered to the inner ear via adeno-associated virus (AAV) vectors through the round window membrane, enabling precise correction of the genetic mutation without inducing DNA double-strand breaks. As a result, Otoferlin protein expression was restored in approximately 88% of inner hair cells, synaptic transmission was significantly improved, and hearing function was maintained at near-normal levels for more than 18 months, with no significant off-target effects observed. These findings suggest that next-generation base-editing technologies may provide a safer and more efficient alternative to conventional CRISPR/Cas9 genome editing for the treatment of hereditary inner ear disorders (72). One of the most promising emerging technologies in inner ear tissue engineering is the application of three-dimensional (3D) bioprinting and bioactive scaffolds to create a microenvironment that closely mimics the extracellular matrix (ECM) and thereby directs stem cell behavior. This strategy relies on bioinks made from hydrogels and biomaterials either natural or synthetic, including gelatin methacryloyl (GelMA), alginate, collagen, fibrin, hyaluronic acid, and polyethylene glycol (PEG) to keep cells viable while encouraging stem cells to proliferate, migrate, and take on the identity of auditory hair cells and spiral ganglion neurons. Beyond this, such scaffolds can be designed to load and gradually release growth factors among them brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), fibroblast growth factor-2 (FGF2), and insulin-like growth factor-1 (IGF-1) together with regulatory molecules from pathways like Atoh1 and Notch, all of which work to boost tissue regeneration right at the injury site. Evidence from recent work shows that pairing bioactive scaffolds with either MSCs or iPSCs does more than just help transplanted cells survive; it also improves how they organize spatially and encourages them to form structures resembling sensory epithelium. That said, some obstacles persist: accurately rebuilding the complex architecture of the organ of Corti, replicating the precise arrangement of inner and outer hair cells, and forming working synaptic links with auditory neurons remain among the toughest barriers to bringing this technology into clinical use. Still, despite these hurdles, recent progress in smart bioinks, multimaterial bioprinting, and stimuli-responsive bioactive scaffolds has opened up encouraging prospects for regenerative treatment of sensorineural hearing loss (72, 73). In addition, recent evidence has showed that long non-coding RNAs (lncRNAs) role as key regulators of gene expression networks by interacting with microRNAs (miRNAs) and messenger RNAs (mRNAs) to modulate diverse cellular signaling pathways (74, 75). Accordingly, targeting these regulatory molecules has appeared as a encouraging approach in genetic engineering and regenerative medicine, particularly for directing stem cell differentiation and promoting the regeneration of damaged tissues.

Personalized Medicine in Auditory Regeneration

Beyond these molecular and biomaterial-based strategies, the increasing accessibility of genetic diagnosis has positioned personalized medicine as an increasingly central paradigm in the field of auditory regeneration. Hearing loss is genetically and phenotypically highly heterogeneous, with pathogenic variants identified in well over 100 genes underlying non-syndromic forms alone; this diversity means that no single therapeutic strategy is likely to benefit all patients equally. Advances in next-generation sequencing (NGS) have transformed diagnostic capability, enabling comprehensive gene-panel testing that identifies a likely causative variant in a substantial proportion of patients, informs accurate genetic counseling, and directly shapes clinical management and prognosis on a patient-by-patient basis (76). Beyond diagnosis alone, NGS-based approaches have been shown to meaningfully improve precision medicine workflows in hearing loss by refining variant classification and guiding family-specific counseling and surveillance strategies (77). This genotype-first framework has begun to translate directly into genotype-specific

therapy. A clear example is *DB-OTO*, a dual adeno-associated virus gene therapy designed specifically for patients with biallelic pathogenic variants in *OTOF* (encoding otoferlin); in a first-in-human trial, a single intracochlear infusion restored clinically meaningful hearing in the majority of treated children, with several achieving normal hearing thresholds (78). Because this therapy is only applicable to patients with a confirmed *OTOF* genotype, it exemplifies how precise molecular diagnosis and targeted gene replacement together constitute personalized medicine in its most concrete clinical form within the auditory field. These developments converge naturally with the cell- and gene-based strategies discussed throughout this review. Patient-derived induced pluripotent stem cells, in particular, offer a personalized platform: since they carry a patient's own genetic background, they can in principle be used to model an individual's specific form of hearing loss in vitro and to evaluate candidate gene- or cell-based therapies before clinical application, allowing treatment selection to be tailored to genotype rather than applied uniformly. As genetic diagnosis becomes faster and more accessible, integrating this personalized framework with the regenerative approaches reviewed here represents a logical and increasingly attainable next step toward individualized treatment of hearing loss and deafness.

FUTURE PERSPECTIVES

Rapid advances in biotechnology and regenerative medicine have created a encouraging outlook for the development of effective therapies for hearing disorders. In the coming years, the convergence of cell therapy, tissue engineering, genome editing, and nanotechnology is expected to overcome many of the current limitations associated with inner ear regeneration. The development of next-generation bioactive scaffolds and smart hydrogels capable of mimicking the native microenvironment of the organ of Corti, together with controlled-release systems for growth factors, may enhance the survival of transplanted cells, promote their lineage-specific differentiation, and improve their functional integration with host tissue. Furthermore, the use of nanocarriers, exosomes, and hybrid delivery vectors for the transport of genes, regulatory RNAs, and genome-editing systems has opened new avenues for the precise treatment of genetic disorders of the inner ear. At the same time, advances in CRISPR/Cas, Base Editing, and Prime Editing technologies are expected to enable the precise correction of disease-causing mutations with minimal off-target effects. Integrating these technologies with iPSCs and three-dimensional inner ear organoids will make it possible to generate patient-specific hair cells and auditory neurons, thereby paving the way toward personalized therapeutic strategies. In addition, the continued development of three-dimensional culture systems, inner ear organoids, and 3D bioprinting technologies will provide more accurate models for investigating inner ear development, drug screening, and evaluating the safety and efficacy of novel therapeutic approaches. Another emerging trend is the development of targeted delivery systems for the inner ear. The application of guided delivery through the round window membrane, biodegradable microneedles, magnetic nanoparticles, and image-guided delivery systems may enable more precise administration of therapeutic cells and molecules while minimizing damage to the delicate cochlear structures. These advances, together with improvements in the design of biological delivery vectors, are expected to increase the feasibility of minimally invasive, single-stage therapeutic interventions for many patients.

Even with these notable gains, moving these technologies out of the lab and into everyday clinical practice is still contingent on clearing a set of significant hurdles among them, standardizing how therapeutic cells are manufactured, guaranteeing safety over the long run, keeping immune reactions in check, sharpening delivery precision, cutting down off-target effects during genome editing, and tracking functional outcomes well beyond the initial treatment period. That said, mounting preclinical evidence, together with the launch of the earliest clinical trials testing gene therapy and genome editing for auditory disorders, points to a new chapter opening up for inner ear regenerative medicine. Looking ahead to the next ten years, bringing together biotechnology, tissue engineering, biomaterials science, nanotechnology, and precision medicine is likely to pave the way for personalized treatments capable not just of restoring hearing, but of addressing the molecular roots of disease itself and achieving lasting repair of the inner ear's structure and function alike.

Overall, the convergence of emerging biotechnological approaches including cell therapy, tissue engineering, nanotechnology, genome editing, and precision medicine has the potential to transform the treatment paradigm for hearing disorders from supportive interventions to regenerative and disease-modifying therapies, thereby opening a new horizon for the long-term treatment of hearing loss and deafness in the near future.

Conflict of Interest

The authors declared no conflict of interest.

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Author Contributions

Marzieh Alaei: Investigation, methodology, data collection, and writing original draft. Sogol Javaherian-Nia: Investigation, data analysis, and review and editing. Roya Chabi: Conceptualization, supervision, project administration, and writing review and editing. All authors read and approved the final manuscript.

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Not applicable.

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Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Genetic Profiles That Increase Susceptibility to Multiple Sclerosis after Epstein–Barr Virus Infection: Focus on HLA, EBV-Specific Immune Response, and Polygenic Immune Risk

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ABSTRACT

The Epstein–Barr virus (EBV) is recognized as the primary infectious agent linked to multiple sclerosis (MS), although the presence of EBV infection alone does not suffice to explain the progression of the illness, given that most patients infected with EBV do not manifest MS. This review examines host genetic profiles that may increase MS susceptibility among EBV-exposed individuals, with emphasis on HLA-mediated antigen presentation, EBV-specific immune responses, and non-HLA immune pathways. The strongest genetic risk signal is HLA-DRB115:01, often within the HLA-DR15 haplotype, which may promote presentation of EBV-derived antigens such as EBNA1 and facilitate CD4+ T-cell activation, molecular mimicry, and CNS-directed autoimmunity. In contrast, HLA-A02:01 appears protective, potentially through more effective CD8+ T-cell-mediated control of EBV-infected B cells; therefore, absence of HLA-A02:01 may represent an important risk-modifying feature. Emerging evidence also implicates HLA-E01:01, particularly in individuals with a history of infectious mononucleosis, suggesting a role for altered NK-cell and CD8+ T-cell regulation in EBV-associated MS risk. Beyond HLA, non-HLA loci such as CD86, CD40, IL2RA, and IL7R may amplify EBV-driven immune activation by modulating antigen-presenting cell function, B/T-cell costimulation, cytokine signaling, and immune tolerance. High anti-EBNA1 antibody titers, especially in HLA-DRB115:01-positive individuals, may serve as an immunological marker of dysregulated host–EBV interaction. Overall, the most plausible high-risk profile combines EBV positivity, infectious mononucleosis or elevated anti-EBNA1 responses, HLA-DRB115:01 positivity, absence of HLA-A02:01, possible HLA-E01:01 carriage, and increased non-HLA immune risk burden. These profiles remain research-based risk-enrichment models rather than clinical prediction tools. Longitudinal investigations focusing on functional genomics, single-cell methodologies, HLA peptidomics, and EBV vaccination are required to understand the reasons behind the selective development of MS in certain EBV-infected individuals and to shape preventive strategies.

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INTRODUCTION

Multiple sclerosis (MS) is a persistent immune-mediated disorder that results in demyelination within the central nervous system (CNS), marked

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by inflammatory lesions, neuroaxonal damage, and progressive neurological impairment (1). While the specific etiology is not fully elucidated, MS is generally seen as a complex ailment stemming from the convergence of genetic vulnerability, environmental influences, and immunological irregularities (2). Among environmental influences, the Epstein Barr virus (EBV) has surfaced as the most reliably linked infectious agent in the etiology of multiple sclerosis. EBV is a widespread gamma-herpesvirus that induces lifelong latency in memory B cells, forming a scientifically credible connection between viral persistence, B-cell dysfunction, and central nervous system autoimmunity (3, 4).

The association between EBV and MS has been strengthened substantially by longitudinal epidemiological evidence. In a substantial group of over 10 million young adults within the U.S. military, seroconversion for EBV occurred prior to the onset of MS, with the likelihood of developing MS escalating nearly 32 times following EBV infection, while infections from other viruses did not exhibit a similar correlation (3). This finding shifted the EBV–MS relationship from a long-standing epidemiological observation toward a more causal framework. However, EBV infection alone cannot explain MS occurrence, because more than 90% of adults worldwide are EBV-seropositive, whereas only a small minority develops MS (5). This discrepancy suggests that host factors, particularly genetic susceptibility, determine whether EBV infection remains clinically silent or contributes to autoimmune demyelination (6).

Genetic susceptibility to MS is strongly shaped by the major histocompatibility complex (MHC), especially the human leukocyte antigen (HLA) region (7). The most robust genetic risk factor identified to date is the HLA-DRB115:01 allele, commonly part of the HLA-DR15 haplotype (8). In contrast, HLA-A02:01 has been repeatedly associated with protection against MS (9). These HLA associations are biologically relevant in the context of EBV because HLA molecules determine the repertoire of viral and self-peptides presented to T cells. Therefore, specific HLA profiles may influence the quality, magnitude, and specificity of EBV-directed immune responses, potentially promoting molecular mimicry, bystander activation, or survival of autoreactive lymphocytes (10).

Recent mechanistic studies have begun to connect EBV infection directly with MS-associated HLA risk. CD4+ T cells restricted by HLA-DRB115:01 have been shown to recognize EBV-derived antigens, supporting the concept that HLA-DR15 may shape pathogenic anti-EBV immunity in genetically susceptible individuals (11). In addition, experimental and immunopeptidomic work suggests that EBV infection may alter antigen presentation in a way that interacts with HLA-DR15 and promotes MS-relevant immune responses. These data provide a mechanistic bridge between two of the strongest MS risk factors: EBV infection and HLA-DRB115:01 carriage (10).

Beyond classical HLA-DRB115:01 risk, emerging evidence indicates that additional HLA and non-HLA variants may modify EBV-associated MS susceptibility. A recent population-based study reported that carriers of HLA-E01:01 with a history of infectious mononucleosis (IM), a clinical manifestation of primary EBV infection, had an increased risk of MS independently of other MS-associated HLA alleles. This finding suggests that genetic variation affecting non-classical antigen presentation and antiviral immune control may help explain why symptomatic EBV infection confers higher MS risk in some individuals (12). Similarly, studies in pediatric-onset MS have implicated interactions between childhood EBV seropositivity, HLA-DRB1*15, and variants in genes involved in antigen-presenting cell function, including CD86 (13).

The overarching genetic framework of MS is polygenic. Extensive genome-wide association studies have discovered over 200 non-MHC susceptibility variations, a significant number of which are associated with immunological pathways related to T cells, B cells, monocytes, microglia, cytokine signaling, and antigen presentation. These variants do not appear to be EBV-specific. Still, they may create an immune background that amplifies EBV-driven inflammation, impairs viral control, or lowers the threshold for loss of self-tolerance. Thus, EBV-associated MS risk is likely not determined by a single allele, but by a composite genetic profile involving classical HLA risk alleles, protective HLA variants, EBV-specific immune response genes, and a broader polygenic autoimmune burden (11, 14, 15).

Despite rapid progress, the genetic determinants that distinguish EBV-infected individuals who develop MS from those who remain healthy remain incompletely defined. Understanding these determinants is clinically important because it may enable risk stratification after EBV infection, clarify mechanisms of virus-triggered CNS autoimmunity, and inform preventive strategies such as EBV vaccination, antiviral approaches, or early immune monitoring in genetically susceptible populations. This review aims to summarize current evidence on genetic profiles that increase the risk of MS among EBV-infected individuals, with emphasis on HLA-DRB115:01, absence of protective HLA-A02:01, EBV-specific humoral and cellular immunity, emerging HLA-E associations, non-HLA immune loci, and polygenic risk models.

EBV as a prerequisite or major trigger for MS

Overview: EBV as the leading infectious candidate in MS

EBV has emerged as the most significant infectious

agent associated with the development of MS (16). EBV is a widespread human gamma-herpesvirus that infects over 90% of people globally and maintains lifelong latency, mostly among memory B cells (17). This unique biology provides a plausible framework for its involvement in MS, a disease in which B cells, antigen presentation, ectopic lymphoid inflammation, and antibody-mediated immune responses have central pathogenic roles (18). Nonetheless, the widespread occurrence of EBV infection among the general population and the relatively low rate of MS suggest that EBV is probably not enough by itself. EBV is increasingly seen as an essential precursor or significant catalyst that operates with host genetic predisposition and several environmental risk factors (19).

Prospective epidemiological evidence linking EBV to MS

The most persuasive epidemiological data comes from extensive prospective studies indicating that EBV infection occurs before the start of MS (20). In a significant longitudinal investigation including over 10 million young individuals in the U.S. military, the occurrence of EBV seroconversion was associated with a notable rise in the risk of multiple sclerosis. Among individuals who later developed MS, nearly all were EBV-seropositive before disease onset, and the risk of MS increased approximately 32-fold after EBV infection. Importantly, this association was not observed with other common viruses, including cytomegalovirus, supporting a relatively specific link between EBV and MS rather than a nonspecific consequence of viral exposure or immune activation. The serum neurofilament light chain, an indicator of neuroaxonal damage, rose only after EBV seroconversion, hence reinforcing the chronological order in which EBV infection occurs prior to observable CNS harm (3).

EBV seronegativity and the timing of MS risk

Earlier prospective serological studies also support the concept that EBV infection is strongly associated with future MS risk. Individuals who remain EBV-seronegative appear to have an extremely low risk of developing MS, whereas risk rises after EBV seroconversion. In addition, elevated antibody responses to EBV nuclear antigen 1, particularly anti-EBNA1 IgG, have repeatedly been associated with increased MS risk years before clinical onset. EBNA1 is expressed during EBV latency, and heightened anti-EBNA1 responses may reflect altered host control of latent infection, enhanced EBV-specific immune activation, or a propensity toward cross-reactive immune responses (2, 4). These observations suggest that not only EBV infection itself, but also the quality and magnitude of the host immune response to EBV, may be relevant to MS susceptibility. (20-22).

IM as a clinical marker of high-risk EBV infection

IM, the symptomatic manifestation of primary EBV infection, further strengthens the EBV–MS association. A history of IM has consistently been associated with increased MS risk, suggesting that the intensity, timing, or immunological context of primary EBV infection may influence later autoimmune susceptibility (23).

Delayed primary EBV infection during adolescence or early adulthood often produces stronger systemic immune activation than asymptomatic childhood infection. This may help explain why IM is a particularly relevant clinical marker in MS risk studies. From the perspective of this review, IM is especially important because it provides a bridge between EBV exposure and host genetic susceptibility. Genetic variation may influence whether primary EBV infection is efficiently controlled, remains clinically silent, or develops into a symptomatic and immunologically intense infection that increases the likelihood of later autoimmune events (24, 25).

EBV latency in B cells and MS-relevant immune dysregulation

Several biological mechanisms may explain how EBV contributes to MS. First, EBV establishes latency in B cells, a cell population now recognized as central to MS immunopathology. B cells may function as antigen-presenting cells, generate pro-inflammatory cytokines, experience clonal proliferation, and facilitate T-cell activation. The therapeutic effectiveness of B-cell-depleting treatments in MS reinforces the significance of B-cell biology in disease processes (19, 26).

EBV-infected B cells may therefore contribute to MS by promoting abnormal antigen presentation, sustaining autoreactive lymphocyte populations, or creating a chronic reservoir of immune activation. Because EBV persists within the memory B-cell compartment, it has the potential to influence immune function over long periods, consistent with the long interval that can occur between primary EBV infection and clinical MS onset. This persistent viral latency may be particularly relevant in individuals whose genetic background favors inefficient EBV immune control or exaggerated EBV-directed immune responses (26, 27).

EBNA1-specific immunity and molecular mimicry

A second major mechanism by which EBV may promote MS is molecular mimicry between viral and CNS antigens. EBNA1 has received particular attention because it is expressed during EBV latency and because elevated anti-EBNA1 immune responses have been associated with increased MS risk before disease onset (19, 28).

A key mechanistic study showed that clonally

expanded B cells in MS can recognize both EBV EBNA1 and the CNS protein glial cell adhesion molecule, GlialCAM. The study provided evidence that anti-EBNA1 immune responses can cross-react with GlialCAM, and that EBNA1 immunization worsened disease in an experimental model (28). These findings offer a direct mechanistic link between EBV-specific immunity and CNS-directed autoimmunity. Nevertheless, molecular mimicry is unlikely to explain all EBV-associated MS cases. It is more likely one component of a broader pathogenic framework involving B-cell dysregulation, antigen presentation, chronic immune activation, and genetically determined loss of immune tolerance.

Interaction between EBV and host genetic background

A third and particularly relevant mechanism for this review is the interaction between EBV and the host genetic background. This is especially important for HLA alleles, which determine how viral and self-peptides are presented to T cells. The strong association between HLA-DRB1*15:01 and MS suggests that certain individuals may present EBV-derived peptides in a way that promotes autoreactive CD4+ T-cell responses or facilitates cross-reactivity with CNS antigens (10). Conversely, protective alleles such as HLA-A*02:01 may support more effective antiviral immune surveillance or reduce the presentation of pathogenic peptide repertoires (9).

Thus, EBV may act as a trigger whose pathological consequences depend strongly on genetically determined antigen presentation. This concept helps explain why EBV infection is common, while MS is rare. In most EBV-infected individuals, antiviral immunity resolves primary infection and maintains long-term viral control without CNS autoimmunity (29). In genetically susceptible individuals, however, EBV-directed immune responses may be shaped in a way that promotes persistent B-cell activation, autoreactive T-cell responses, or antibody cross-reactivity with CNS antigens (29, 30).

Figure 1 summarizes this model, showing how EBV infection may initiate MS-relevant immune pathways, while host genetic susceptibility determines whether these responses remain controlled or progress toward CNS autoimmunity.

HLA class II genetic susceptibility

*HLA-DRB1*15:01 as the major genetic risk factor for MS*

The most robust and consistently observed genetic risk factor in MS is found in the MHC class II region, namely the HLA-DRB1*15:01 allele, often inherited as part of the HLA-DR15 haplotype. This allele has reliably been identified as the primary genetic risk

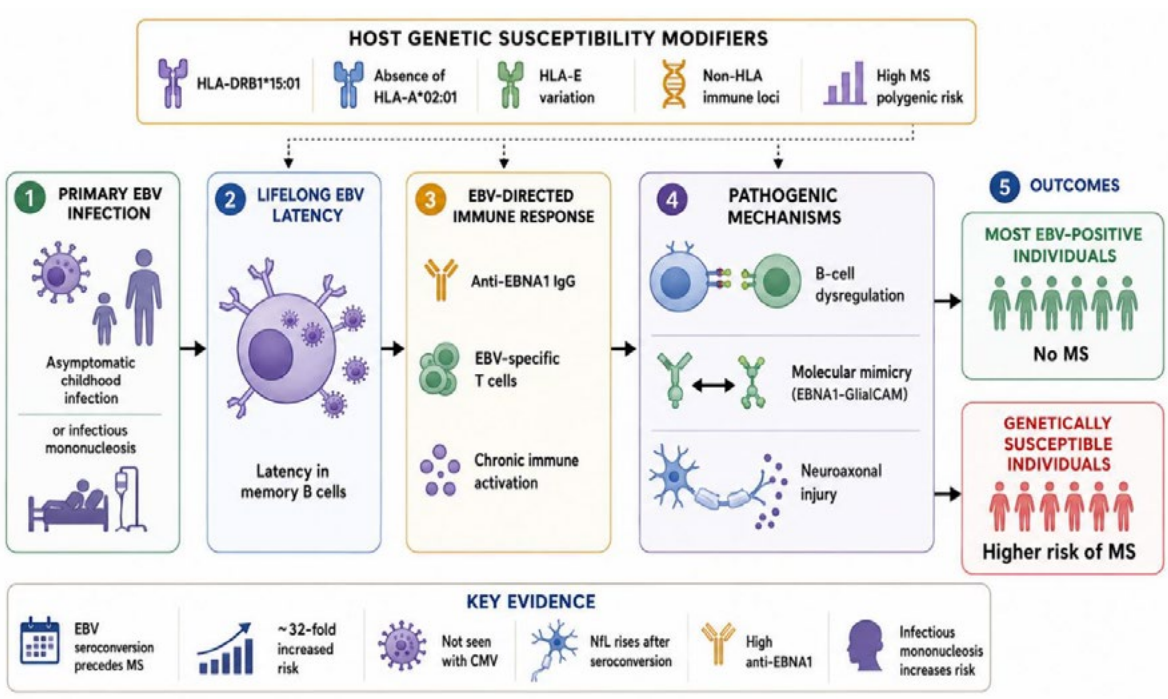


Fig 1. The conceptual framework of EBV as a nearly essential but inadequate catalyst for MS. EBV infection occurs before the development of MS and induces lifelong latency in memory B cells. In people with genetic predispositions, immune responses aimed against EBV may lead to B-cell dysregulation, irregularities in antigen presentation, molecular mimicry between EBNA1 and CNS antigens like GlialCAM, and persistent immunological activation. Given that EBV infection is prevalent and MS is seldom, genetic predisposition in the host is necessary to explain why only a fraction of those infected with EBV progress to MS.

determinant for MS in several groups, particularly among those of European descent. Its pivotal function in MS vulnerability underscores the significance of CD4+ T-cell-mediated immunity and antigen presentation in the development of the illness (31, 32).

The relevance of HLA-DRB115:01 becomes particularly important in the context of EBV, because HLA class II molecules determine which extracellular, endosomal, or B-cell-derived antigens are presented to CD4+ T cells (33). Since EBV establishes lifelong latency in B cells, and B cells are professional antigen-presenting cells, the combination of EBV persistence and HLA-DRB115:01-mediated antigen presentation provides a plausible biological bridge between the strongest environmental and genetic risk factors for MS. Recent reviews and mechanistic studies increasingly support the view that HLA-DR15 may shape EBV-specific CD4+ T-cell immunity in a way that contributes to MS-relevant immune dysregulation (34).

HLA-DR15 and antigen presentation of EBV-derived peptides

The HLA-DR15 haplotype comprises HLA-DRB1*15:01 and associated class II molecules that are involved in the presentation of peptide antigens to CD4+ T lymphocytes. In the context of EBV infection, this is especially significant since EBV-infected B cells are capable of processing and presenting viral antigens via MHC class II pathways. If these peptides produced from EBV are displayed in an immunologically favorable environment, they might facilitate the proliferation of EBV-specific CD4+ T cells, sustained immune activation, or cross-reactive reactions targeting CNS antigens (10, 35).

A recent study directly linked HLA-DRB115:01 to EBV-specific CD4+ T-cell recognition. Drosu and colleagues showed that CD4+ T cells restricted by **HLA-DRB115:01** can recognize EBV glycoprotein-derived antigens capable of intracellular antigen presentation (10). This finding is important because it provides a mechanistic connection between HLA-DRB1*15:01 and EBV-directed adaptive immunity, suggesting that the MS-associated HLA class II background can shape the repertoire of EBV-specific CD4+ T-cell responses (10).

This mechanism may be particularly relevant in EBV-infected B cells. Unlike many acute viral infections, EBV persists in the memory B-cell compartment, allowing repeated or chronic opportunities for viral antigen presentation (36). In individuals carrying HLA-DRB1*15:01, EBV-infected B cells may therefore act not only as viral reservoirs but also as antigen-presenting cells capable of sustaining CD4+ T-cell activation. This provides a biologically plausible route through which EBV latency, B-cell biology, and HLA class II genetic risk converge in MS (35).

EBNA1 as a key antigenic link between EBV and CNS autoimmunity

EBV nuclear antigen 1, or EBNA1, has become one of the most important EBV antigens in MS research. EBNA1 is expressed during latent EBV infection and is required for maintenance of the viral episome in infected cells. Elevated immune responses against EBNA1, especially anti-EBNA1 IgG, have repeatedly been associated with increased MS risk before disease onset. This makes EBNA1 not only a marker of EBV-directed immune activity but also a potential mechanistic contributor to disease development (37, 38).

In the context of HLA class II susceptibility, EBNA1 is important for two reasons. First, EBNA1-derived peptides may be presented to CD4+ T cells by MS-associated HLA class II molecules, including HLA-DRB115:01. Second, certain EBNA1 epitopes may share structural or sequence similarity with CNS antigens, creating the possibility of molecular mimicry. Therefore, an individual with HLA-DRB115:01 who mounts a strong EBNA1-directed immune response may have a higher probability of developing cross-reactive immune responses relevant to CNS autoimmunity (39).

This concept is supported by studies showing that anti-EBNA1 antibody titers and HLA-DR15 are both associated with MS risk. De Jager and colleagues reported that anti-EBNA1 antibody titers were associated with MS risk independently of the DR15 allele, suggesting that EBV-directed humoral immunity and HLA-DRB1*15:01 represent partially independent but potentially convergent risk dimensions (40).

Molecular mimicry involving EBNA1 and CNS antigens

A prominent mechanistic rationale for EBV-related MS is molecular mimicry, when immune responses aimed at EBV antigens inadvertently target structurally similar CNS proteins. The most often examined instance pertains to EBNA1 and GlialCAM, a central nervous system protein produced by glial cells. Lanz and associates revealed that clonally proliferated B lymphocytes from multiple sclerosis patients could attach to both EBV EBNA1 and GlialCAM, indicating a strong molecular resemblance between a dormant EBV antigen and a central nervous system target (28).

This finding is highly relevant to HLA-DRB115:01-mediated susceptibility. HLA-DRB115:01 may facilitate the activation or maintenance of CD4+ T-cell responses that support B-cell maturation, antibody production, and epitope spreading after EBV infection. In this model, EBNA1-specific immune responses may begin as antiviral responses but later extend toward CNS antigens such as GlialCAM. Recent evidence also suggests that antibody responses against EBNA1

and CNS-mimic antigens, including GlialCAM, may be elevated in individuals with MS carrying HLA-DRB1*15:01, supporting an additive interaction between genetic risk and EBV-related autoimmunity (41).

However, molecular mimicry should not be viewed as the only mechanism linking EBV and HLA-DRB1*15:01 to MS. It is more likely that molecular mimicry operates together with other processes, including B-cell dysregulation, altered antigen presentation, chronic EBV-specific T-cell stimulation, impaired viral control, and bystander inflammatory activation. This broader interpretation is important because MS is heterogeneous, and different patients may develop the disease through partially overlapping but non-identical EBV-related pathways (42).

EBV-induced immunopeptidome changes in the HLA-DR15 context

Recent mechanistic studies have broadened the understanding of EBV–HLA interaction beyond mere peptide imitation. Wang and associates indicated that EBV infection has the capacity to alter both the transcriptome and immunopeptidome of B cells affected by the MS-related HLA-DR15 haplotype. Their research indicates that EBV infection might modify the array of peptides shown by HLA-DR15 molecules, including both viral and self-originated antigens (35).

This is significantly vital for comprehending genetic predisposition in persons positive for EBV. If EBV infection alters antigen processing and presentation in HLA-DR15-positive B cells, then HLA-DRB1*15:01 could not just present specific EBV peptides; it might establish a more extensive antigen-presentation milieu that promotes the activation of autoreactive CD4+ T cells. This concept offers a more cohesive elucidation of how EBV infection and the inheritance of HLA-DR15 may collaboratively enhance immunological processes linked with MS (36, 43).

In practical terms, this means that the risk profile is unlikely to be explained by EBV seropositivity alone. Rather, MS risk may be enriched in individuals in whom EBV infection occurs within an HLA class II background that promotes persistent or pathogenic antigen presentation (44). This provides a direct rationale for considering EBV-positive / HLA-DRB1*15:01-positive individuals as a biologically meaningful high-risk subgroup.

*HLA-DRB1*15:01 as a possible co-receptor for EBV entry*

Another proposed mechanism linking HLA-DRB115:01 to EBV involves viral entry into B cells. A study reported that HLA-DRB115:01 may act as a co-receptor for EBV infection in a B-cell line, suggesting a direct molecular connection between the major MS genetic risk allele and EBV biology (45).

Although this finding requires further validation across primary human cells and diverse populations, it is conceptually important. If HLA-DRB115:01 facilitates EBV infection or alters the efficiency of viral entry, it could contribute to a higher burden of EBV-infected B cells or a qualitatively different pattern of EBV persistence. This would add another layer to the EBV–HLA interaction: HLA-DRB115:01 may influence not only antigen presentation after infection, but potentially the biology of EBV infection itself (46).

Integrative model: HLA-DR15 as a genetic amplifier of EBV-associated MS risk

Taken together, current evidence supports a model in which HLA-DRB1*15:01/HLA-DR15 acts as a genetic amplifier of EBV-associated MS risk. In this model, EBV provides the environmental trigger and persistent viral reservoir, whereas HLA-DRB1*15:01 shapes the antigenic and cellular immune context in which EBV is recognized. This may occur through several overlapping mechanisms: enhanced presentation of EBV-derived peptides, activation of EBV-specific CD4+ T cells, support of EBNA1-directed humoral immunity, facilitation of molecular mimicry with CNS antigens, and EBV-induced remodeling of the HLA-DR15 immunopeptidome. This framework helps explain why EBV infection alone is not sufficient to cause MS. Most EBV-infected individuals maintain lifelong viral latency without developing CNS autoimmunity. In contrast, individuals carrying HLA-DRB115:01 may be more likely to generate persistent, dysregulated, or cross-reactive EBV-directed immune responses. Therefore, the **EBV-positive / HLA-DRB115:01-positive** profile represents one of the most biologically plausible and best-supported genetic risk profiles for MS development.

This HLA class II–centered mechanism is illustrated in Figure 2, which summarizes how HLA-DRB1*15:01 may shape EBV antigen presentation and promote downstream EBNA1-directed immunity and CNS cross-reactivity.

While HLA-DRB115:01 represents the dominant HLA class II risk allele for MS, disease susceptibility is further modified by other HLA variants, including protective class I alleles such as HLA-A02:01.

HLA class I protection and risk modification

HLA class I alleles as modifiers of MS susceptibility

While the most significant genetic correlation in MS is found in the HLA class II area, namely HLA-DRB1*15:01, HLA class I alleles also play a role in vulnerability to the disease. HLA-A*02:01 is often identified as a protective allele. This protective connection has been reported across numerous independent investigations and does not seem to be

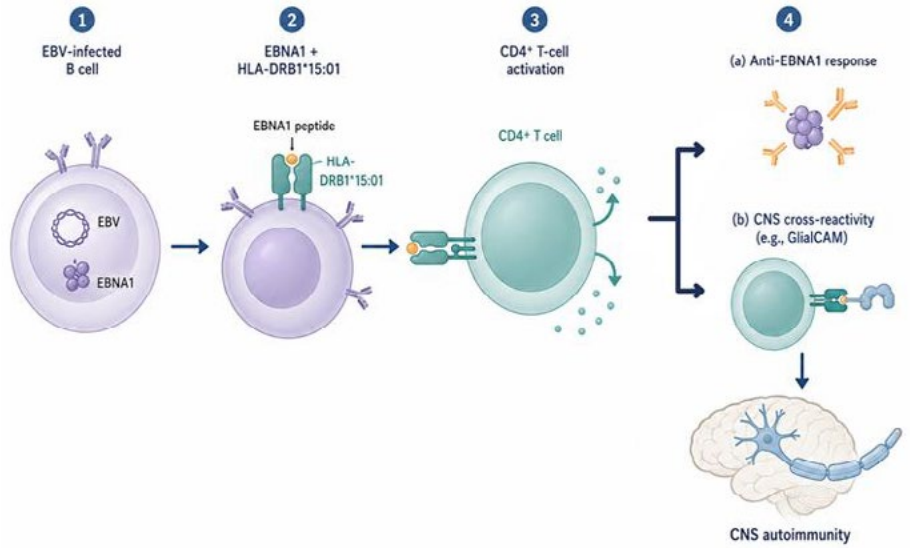


Fig 2. HLA-DRB1*15:01-mediated presentation of EBV antigens and CNS cross-reactivity. EBV-infected B cells may present EBNA1-derived peptides through HLA-DRB1*15:01, leading to activation of EBV-specific CD4+ T cells. In genetically susceptible individuals, this response may support anti-EBNA1 immunity and cross-reactive recognition of CNS antigens such as GlialCAM, thereby contributing to CNS autoimmunity and increased MS risk.

purely due to linkage disequilibrium with class II alleles, indicating that HLA-A*02:01 may have an independent immunological impact on MS risk (7, 47).

*HLA-A*02:01 as a protective allele in MS*

The protective effect of HLA-A02:01 has been confirmed in population-based and genetic association studies. Bergamaschi and colleagues reported a strong protective effect of **HLA-A02**, independent of HLA-DRB1*15 and other HLA-DRB1 alleles, supporting the idea that class I-mediated immune mechanisms modify MS susceptibility independently of classical class II risk (47). Similarly, Link and colleagues emphasized the importance of both HLA class I and class II effects in MS and confirmed the protective contribution of HLA-A*02 in Scandinavian cohorts (48). These findings indicate that MS risk is shaped not only by alleles that enhance susceptibility, but also by alleles that may actively reduce disease risk.

Biological rationale: CD8+ T-cell surveillance and EBV control

The biological relevance of HLA-A02:01 in EBV-associated MS risk is plausible because HLA class I molecules present intracellular peptides to CD8+ cytotoxic T cells, which are central to antiviral immune surveillance. Since EBV establishes latent infection in B cells, effective CD8+ T-cell recognition of EBV-derived peptides may be important for controlling the burden and activity of EBV-infected B cells. In this context, HLA-A02:01 may protect against MS by supporting efficient antiviral immune control, thereby reducing persistent EBV-driven B-cell activation and downstream autoimmune risk (49, 50).

*HLA-A*02:01 and EBV viral load in MS*

Evidence directly linking HLA-A02 to EBV control has been reported in MS cohorts. Agostini and colleagues found that MS patients expressing **HLA-A02** had significantly lower EBV viral load compared with HLA-A02-negative patients. This observation suggests that HLA-A02 may contribute to more effective immune control of EBV-infected cells, providing a mechanistic explanation for its protective association in MS (50). Although viral load is only one measure of EBV immune control, these data support a model in which HLA-A*02:01 reduces MS risk by limiting EBV persistence, reactivation, or antigenic stimulation.

*Absence of HLA-A*02:01 as part of a high-risk genetic profile*

The absence of HLA-A*02:01 can therefore be considered a risk-modifying feature rather than a primary risk allele. In individuals who already carry strong susceptibility factors such as HLA-DRB1*15:01, lack of HLA-A02:01 may remove a protective layer of antiviral immune surveillance. This produces a biologically plausible high-risk configuration: **HLA-DRB115:01-positive / HLA-A*02:01-negative**. In such individuals, HLA class II-mediated presentation may favor CD4+ T-cell activation against EBV or self-antigens, while the absence of protective class I-mediated CD8+ control may allow more persistent EBV-related immune stimulation (7, 48, 50).

*HLA-A*02:01, EBV-specific CD8+ T cells, and immune phenotype*

Recent studies further support the concept that MS-associated and MS-protective HLA class I alleles may

shape EBV-specific CD8+ T-cell responses differently. Work comparing EBV epitope-specific CD8+ T-cell responses in the context of HLA-A2 and higher-risk HLA class I backgrounds suggests that HLA-A2-restricted responses may recognize a broader or more effective EBV epitope repertoire, whereas some MS-associated class I contexts may be linked to more restricted or dysfunctional EBV-specific CD8+ T-cell responses (51). Although these data are still emerging, they are highly relevant to the present review because they connect HLA class I genetics, EBV immune surveillance, and MS risk modification.

HLA class I protection in the broader genetic–environmental model

The protective role of HLA-A02:01 should be interpreted within a broader gene–environment framework. Environmental factors such as EBV infection, smoking, vitamin D deficiency, and adolescent obesity may interact with HLA-mediated immune pathways to influence MS risk. For example, prior work has suggested that smoking-related MS risk may be more pronounced in individuals lacking protective **HLA-A02:01** or carrying HLA-DRB1*15:01, indicating that HLA class I protection may modify susceptibility across multiple environmental contexts, not only EBV infection (52). This reinforces the concept that absence of HLA-A*02:01 is best viewed as a risk amplifier within a multilayered susceptibility profile.

Integrative interpretation

Taken together, current evidence supports the view that HLA-A*02:01 is an important protective HLA class I allele in MS, likely acting through mechanisms related to CD8+ T-cell-mediated immune surveillance and control of EBV-infected B cells. Conversely, absence of HLA-A02:01 may increase vulnerability to EBV-associated immune dysregulation, particularly when combined with **HLA-DRB115:01**, high anti-EBNA1 responses, or other immune risk variants. Therefore, the EBV-positive / HLA-DRB115:01-positive / HLA-A02:01-negative profile represents a biologically coherent and clinically relevant high-risk configuration for MS susceptibility (7, 50, 51).

In addition to traditional HLA-A and HLA-DR alleles, recent findings indicate that non-classical HLA molecules, especially HLA-E, might also alter the risk of EBV-related MS by affecting NK-cell and CD8+ T-cell reactions to EBV-infected B cells.

HLA-E and EBV immune control

HLA-E as an emerging modifier of EBV-associated MS risk

Beyond classical HLA class I and II molecules, recent evidence suggests that the non-classical HLA class I molecule HLA-E may modify the association between EBV infection and MS. HLA-E has a specialized immunological role because it interacts with receptors expressed on natural killer (NK) cells and CD8+ T cells, particularly the inhibitory receptor NKG2A/CD94, thereby influencing antiviral immune surveillance and immune regulation. This is relevant to EBV-associated MS because EBV persists in B cells, and effective long-term control of EBV-infected cells depends not only on conventional HLA-restricted T-cell responses but also on NK-cell and CD8+ T-cell mechanisms (30, 49).

HLA-E allelic variation and infectious mononucleosis

The potential relevance of HLA-E to MS emerged from studies showing that HLA-E allelic variation can influence the clinical outcome of primary EBV infection. IM, the symptomatic manifestation of primary EBV infection, is associated with strong immune activation and has consistently been linked to increased MS risk (12). A mechanistic study by Vietzen and colleagues showed that HLA-E-restricted immune responses are important for the control of EBV-associated diseases, including IM. The findings indicated that the prominently expressed HLA-E*01:03/01:03 genotype conferred protection against IM, in part by stimulating EBV BZLF1-specific HLA-E-restricted CD8+ T-cell responses that may restrict viral spread (30).

*HLA-E*01:01, infectious mononucleosis, and MS susceptibility*

A 2025 population cohort study directly connected HLA-E variation with MS susceptibility in the context of IM. Nova and colleagues reported that carriers of HLA-E*01:01 who had been diagnosed with infectious mononucleosis were at considerably increased risk of MS, independent of other MS-associated HLA alleles. Importantly, the association between HLA-E01:01 and MS was observed primarily among individuals with a history of IM, suggesting a gene–environment interaction rather than a general HLA-E effect on MS susceptibility (12). This finding positions HLA-E01:01 as an emerging EBV-context-dependent risk modifier.

Dose-dependent risk and independence from classical HLA effects

The reported HLA-E01:01–IM interaction appears to show a dose-dependent pattern. In summaries of the 2025 cohort study, MS risk among individuals with a history of IM was higher in heterozygous HLA-E01:01 carriers and even higher in HLA-E01:01 homozygotes, compared with individuals carrying two HLA-E01:03 alleles (12, 53). The study also reported that this effect persisted after accounting for other MS-related HLA variants, including HLA-DRB1*15:01 and HLA-A*02:01 (12). This is important because it

suggests that HLA-E may add a distinct layer of risk beyond the classical HLA class II risk and class I protection framework.

Possible mechanism: weaker EBV immune control

The most plausible interpretation is that HLA-E*01:01 could be related to less efficient immune control of EBV, particularly during or after symptomatic primary infection. In contrast, HLA-E01:03 has been associated with stronger HLA-E-restricted CD8+ T-cell responses against EBV antigens, including BZLF1, and reduced susceptibility to IM (30). If HLA-E01:01 supports weaker NK-cell or CD8+ T-cell-mediated control of EBV-infected B cells, this could result in greater viral persistence, more intense immune activation, or prolonged antigenic stimulation after IM. These processes could plausibly increase the likelihood of EBV-driven B-cell dysregulation and CNS-directed autoimmunity in genetically susceptible individuals (12, 30).

NK-cell and CD8+ T-cell pathways in EBV-associated autoimmunity

HLA-E is particularly interesting because it links EBV biology to both innate-like and adaptive antiviral pathways. HLA-E-presented peptides can regulate NK-cell activity through inhibitory NKG2A/CD94 signaling, while HLA-E-restricted CD8+ T cells can directly recognize EBV-derived peptides and contribute to viral control. In EBV infection, this balance may determine whether infected B cells are efficiently contained or whether viral persistence promotes chronic immune stimulation. Since MS involves both B-cell dysregulation and CD8+ T-cell accumulation in CNS lesions, HLA-E-mediated antiviral control provides a biologically plausible bridge between symptomatic EBV infection and later CNS autoimmunity (30, 54).

Interpretation within the EBV–genetics model of MS

Within the broader model of EBV-associated MS, HLA-E should be viewed as an emerging risk modifier, not yet as established as HLA-DRB1*15:01 or HLA-A*02:01. The current evidence suggests that HLA-E01:01 may increase MS risk specifically in individuals who experienced infectious mononucleosis, possibly by weakening EBV immune control or altering NK/CD8+ T-cell regulation. Therefore, a clinically meaningful high-risk configuration may include **EBV-associated IM + HLA-E01:01**, especially when combined with HLA-DRB1*15:01, absence of HLA-A*02:01, high anti-EBNA1 responses, or elevated MS polygenic risk. However, replication in additional ancestries and functional studies using primary human immune cells are needed before HLA-E typing can be incorporated into formal MS risk prediction (12, 53).

Table 1 summarizes HLA-mediated profiles that may modify MS risk among EBV-exposed individuals.

EBV-specific humoral immunity and host genetics

EBV antibody profiles as markers of MS-relevant immune response

EBV-specific antibody responses provide an important link between viral exposure and host immune susceptibility in MS. Because EBV seropositivity is highly prevalent in adults, simple EBV positivity has limited value for risk stratification. In contrast, quantitative and antigen-specific antibody patterns, particularly elevated antibodies against EBNA1, appear more informative. High anti-EBNA1 IgG titers have repeatedly been associated with increased MS risk years before clinical onset, suggesting that MS susceptibility is related not only to EBV infection itself, but also to an exaggerated or dysregulated immune response to latent EBV antigens (40, 55).

EBNA1 is especially relevant because it is expressed during EBV latency and is required for maintenance of the viral episome in infected B cells. Persistently elevated anti-EBNA1 IgG may reflect chronic antigenic stimulation, impaired control of EBV-infected B cells, or a tendency toward cross-reactive immune responses (56). This is consistent with mechanistic studies showing that EBNA1-directed antibodies can cross-react with CNS-associated antigens such as GlialCAM, providing a potential bridge between EBV-specific humoral immunity and CNS autoimmunity (28, 41).

Antibodies against EBV viral capsid antigen (anti-VCA IgG) are useful markers of prior EBV infection and are commonly used to define EBV serostatus in epidemiological studies. However, because anti-VCA IgG is widespread among EBV-exposed individuals, it appears less specific than anti-EBNA1 for MS risk prediction. Therefore, anti-VCA is best interpreted as a marker of EBV exposure history, whereas anti-EBNA1 may better reflect MS-relevant immune dysregulation related to latent EBV infection (21, 56).

Anti-EBNA1 titers, HLA-DRB1*15:01, and MS risk

The correlation between anti-EBNA1 reactions and MS is especially significant when considering host genetics. HLA-DRB1*15:01, the most significant genetic risk allele for multiple sclerosis, may affect CD4+ T-cell reactions to peptides produced from EBV and assist in T-cell support for EBNA1-specific B cells (57). De Jager and colleagues found that anti-EBNA1 antibody levels correlated with the incidence of multiple sclerosis, irrespective of HLA-DRB1*15:01, suggesting that EBV-targeted humoral immunity and HLA class II genetic predisposition are separate but intersecting factors in MS vulnerability (40).

The combination of HLA-DRB1*15:01 carriage and high anti-EBNA1 IgG titers may therefore

Table 1. HLA class I and class II profiles modifying EBV-associated MS risk.

HLA profile	Direction of effect on MS risk	EBV-related implication	Proposed mechanism	Strength of evidence
HLA-DRB1*15:01 positive / HLA-DR15 positive	Increased risk	May enhance pathogenic EBV antigen presentation	Presentation of EBV-derived peptides, including EBNA1-related antigens, to CD4+ T cells; possible promotion of EBV-specific and cross-reactive CNS-directed immune responses	Strong
HLA-A*02:01 positive	Protective	May improve antiviral immune surveillance	More effective CD8+ T-cell-mediated control of EBV-infected B cells; lower EBV viral load reported in HLA-A*02-positive MS patients	Strong to moderate
HLA-A*02:01 negative	Risk-modifying; loss of protection	May reduce protective EBV immune control	Absence of a protective class I pathway may permit greater EBV persistence or chronic EBV-driven immune stimulation	Moderate
HLA-DRB115:01 positive / HLA-A02:01 negative	Higher-risk combined profile	Combines class II risk with loss of class I protection	Potentially enhanced CD4+ T-cell activation against EBV/self-antigens together with reduced CD8+ antiviral control	Strong biological plausibility; moderate direct EBV-specific evidence
HLA-E*01:01 carrier + infectious mononucleosis history	Increased risk	May identify individuals with weaker EBV control after symptomatic primary infection	Altered HLA-E-mediated NK-cell and CD8+ T-cell regulation; possible reduced control of EBV-infected B cells after IM	Emerging 2025 cohort evidence
HLA-E*01:03/01:03	Potentially protective against IM; possible indirect MS relevance	May support stronger control of primary EBV infection	Induction of EBV BZLF1-specific HLA-E-restricted CD8+ T-cell responses that limit viral dissemination	Moderate for EBV/IM; indirect for MS
HLA-DRB115:01 positive + HLA-A02:01 negative + HLA-E*01:01 carrier + IM history	Proposed high-risk EBV-associated profile	Integrates EBV exposure, symptomatic infection, class II risk, loss of class I protection, and non-classical HLA risk	Persistent EBV antigenic stimulation, altered B-cell antigen presentation, weaker antiviral control, and increased likelihood of CNS cross-reactivity	Conceptual; requires validation

define a biologically meaningful high-risk profile among EBV-infected individuals. In this model, HLA-DRB1*15:01 may promote presentation of EBV-derived peptides to CD4+ T cells, while persistent anti-EBNA1 responses may amplify B-cell activation, antibody production, and potential CNS cross-reactivity. Although the statistical interaction varies across cohorts, the biological rationale for considering this combined profile is strong (58).

Recent studies have also suggested that anti-EBNA1 responses may be refined beyond total IgG titers. Antibodies directed against specific EBNA1 regions, including autoreactivity-associated domains, may better capture MS-relevant humoral responses than total anti-EBNA1 levels alone. This supports a shift from simple EBV serostatus toward more detailed EBV antibody profiling combined with HLA genotype (41, 59).

Integrative interpretation

Overall, EBV-specific humoral immunity represents a measurable interface between EBV exposure and genetically shaped immune risk. High anti-EBNA1 IgG, particularly in HLA-DRB1*15:01-positive individuals, is one of the most consistent EBV-related immune profiles associated with MS susceptibility. Anti-VCA IgG remains useful for documenting EBV exposure, but it is less specific for MS risk than anti-EBNA1. Thus, the profile EBV-seropositive / high anti-EBNA1 IgG / HLA-DRB1*15:01-positive, especially when combined with absence of protective HLA-A*02:01 or history of infectious mononucleosis, represents a plausible high-risk immunogenetic pattern (40, 58).

However, elevated EBV antibody titers should not be interpreted as diagnostic markers of MS. Many individuals with high anti-EBNA1 titers never

develop MS, indicating that humoral EBV responses act as risk markers within a broader framework that includes HLA background, non-HLA immune loci, environmental exposures, and possibly early biomarkers of neuroaxonal injury. In this context, EBV antibody profiling is best viewed as a readout of host virus interaction rather than a stand-alone predictor of disease (59).

Non-HLA loci and antigen-presenting cell pathways

Non-HLA genetic architecture of MS and immune-cell pathways

Although HLA alleles account for the strongest genetic effects in MS, the broader genetic architecture of the disease is highly polygenic. The large 2019 International MS Genetics Consortium study analyzed 47,429 MS cases and 68,374 controls and identified 200 autosomal non-MHC susceptibility variants, in addition to MHC and chromosome X signals. Many of these non-HLA variants map to immune-cell pathways involving peripheral immune cells and microglia, supporting the concept that MS susceptibility is shaped by multiple genes involved in immune activation, antigen presentation, cytokine signaling, and lymphocyte regulation (14, 60, 61).

This non-HLA genetic background is particularly relevant to EBV-associated MS because EBV persists in B cells, which can function as antigen-presenting cells. EBV-infected B cells may present viral or self-antigens, supply costimulatory signals to T cells, and sustain chronic immune activation. Therefore, genetic variants affecting B-cell activation, antigen-presenting cell function, and B/T-cell costimulation may influence whether EBV infection remains controlled or contributes to autoreactive immune responses (14).

CD86 and B/T-cell costimulation in EBV-associated MS risk

Among non-HLA loci, CD86 is especially relevant because it encodes a costimulatory molecule expressed on antigen-presenting cells, including B cells and dendritic cells. CD86 interacts with CD28 and CTLA-4 on T cells, thereby influencing T-cell activation, tolerance, and immune regulation (62). In the setting of EBV infection, altered CD86-mediated costimulation could amplify T-cell responses to EBV-infected B cells and promote a more persistent or dysregulated adaptive immune response (14).

A notably significant article for this analysis is the 2024 publication about gene-environment interactions in pediatric-onset MS. Ziaei and associates presented findings indicating an interaction between childhood EBV infection and both HLA-DRB1*15 and the GG genotype of the CD86 risk variation rs2255214. The researchers determined that these results underscore a significant function of antigen-presenting cells in the likelihood of pediatric-onset MS linked with EBV. This positions CD86 as one of the most evident non-HLA instances presently documented that connects EBV exposure, APC biology, and vulnerability to MS (63).

CD40 and EBV-driven B-cell activation

CD40 is another biologically relevant non-HLA locus because it plays a central role in T-cell-dependent B-cell activation, germinal center responses, immunoglobulin class switching, and antigen presentation (64). In MS, B cells are not only antibody-producing cells but also antigen-presenting cells capable of shaping T-cell responses. Because EBV establishes latency in B cells, CD40-mediated signaling may influence the survival, activation, and antigen-presenting function of EBV-infected B cells (4).

EBV biology further strengthens the relevance of CD40-related pathways. EBV latent membrane protein 1, or LMP1, can mimic aspects of CD40 signaling and promote B-cell activation, survival, and upregulation of antigen-presentation machinery. Recent EBV–MS reviews note that EBV LMP1 expression can enhance expression of MHC class I, MHC class II, and costimulatory molecules, thereby enhancing antigen presentation by infected B cells (65). This provides a plausible mechanism through which host variants affecting CD40 or related B-cell activation pathways could modify EBV-driven immune responses in MS (19).

IL2RA and IL7R: cytokine receptor loci shaping T-cell regulation

Non-HLA MS risk loci also include cytokine receptor genes such as IL2RA and IL7R, which influence T-cell activation, survival, and immune tolerance. IL2RA encodes the alpha chain of the IL-2 receptor and is important for regulatory T-cell biology, while IL7R encodes the IL-7 receptor alpha chain and contributes to T-cell development and homeostasis. Variants in these genes may alter the balance between effector and regulatory immune responses, potentially lowering the threshold for autoreactive T-cell activation after EBV-driven antigen presentation (66).

The connection between IL2RA/IL7R and EBV-associated MS is likely indirect rather than EBV-specific. Unlike CD86, where a direct EBV interaction has been reported in pediatric-onset MS, IL2RA and IL7R are better understood as part of a broader immune susceptibility background. These variants may not determine EBV infection itself, but they may influence how the immune system responds to persistent antigenic stimulation, including stimulation derived from EBV-infected B cells (14).

Integrative interpretation

Taken together, non-HLA loci appear to modify EBV-associated MS risk by shaping the immune context in which EBV is controlled. CD86 provides the most direct current example of a non-HLA gene–EBV interaction, particularly in pediatric-onset MS, where the CD86 rs2255214 GG genotype interacted with childhood EBV infection. CD40, IL2RA, and IL7R are also biologically relevant because they regulate B-cell activation, T-cell costimulation, and cytokine-dependent immune homeostasis, although their EBV-specific effects are less directly established (63).

Thus, the non-HLA contribution to EBV-associated MS should be interpreted as a polygenic immune-amplification background. In genetically susceptible individuals, EBV-infected B cells may provide antigenic stimulation, while variants in APC and costimulatory pathways may enhance B/T-cell interactions, sustain chronic immune activation, and increase the probability of autoreactive responses. This model complements the HLA-centered framework by showing that antigen presentation is not determined only by peptide-binding HLA alleles, but also by the broader genetic regulation of APC function and lymphocyte activation (63).

Figure 3 illustrates how non-HLA loci involved in antigen-presenting cell pathways, including CD86, CD40, IL2RA, and IL7R, may modify immune responses to EBV and influence MS susceptibility.

Polygenic risk score and EBV-positive risk stratification

Polygenic risk score (PRS) and shared genetic architecture of EBV activity and MS

Recent large-scale genetic research has begun to illuminate how polygenic risk influences both EBV activity and MS susceptibility. A new study developed PRSs for EBV DNA positivity using genome-wide association data from over 600,000 individuals and applied these scores to large population cohorts, including the UK Biobank. Individuals in the top decile of the EBV DNA PRS showed a significantly higher prevalence of MS compared with those in the bottom decile, and most of this association was attributable to variants within the MHC region. These findings suggest that genetic variants that influence EBV activity also contribute to MS risk, supporting the concept of a shared genetic architecture between EBV persistence and MS susceptibility (67).

This work provides some of the strongest evidence to date that polygenic risk related to EBV biology can stratify individuals by MS risk, particularly when MHC (including classical HLA risk and protective alleles) is included. It supports the idea that PRS can capture genetic variation influencing EBV–host interactions and highlights the potential utility of PRS in research settings to identify subgroups at elevated EBV–MS risk (67).

Integration of PRS, HLA, and EBV serology in risk models

While the EBV DNA PRS mentioned above is based on EBV activity rather than solely on traditional MS GWAS, it aligns with the broader observation that MHC variation drives much of the overlap between EBV-related genetic signals and MS genetic susceptibility. In the UK Biobank analysis, PRS constructed from variants inside the HLA region showed a stronger association with MS risk than PRSs based on non-MHC variants, suggesting that HLA-mediated effects are especially critical for stratified genetic risk in EBV-positive individuals (67).

Several earlier epidemiological studies have also explored the combination of HLA genotypes and EBV serology for MS risk discrimination. For example,

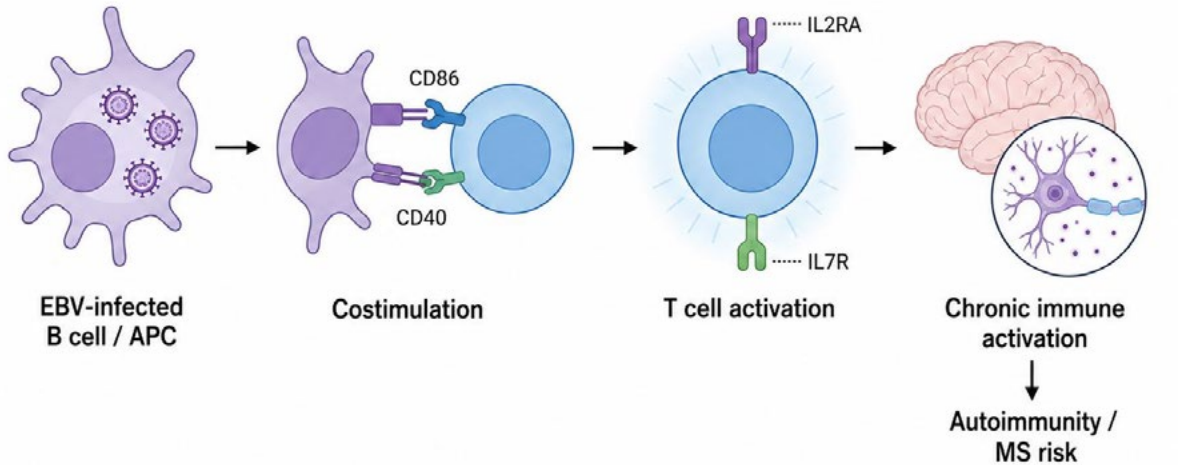


Fig 3. Non-HLA loci shaping EBV-driven immune activation in MS. EBV-infected B cells act as antigen-presenting cells. Genetic variants in CD86 and CD40 modulate B/T-cell costimulation, while IL2RA and IL7R influence T-cell survival and regulatory pathways. Together, these non-HLA variants create a polygenic background that can amplify EBV-associated immune responses and increase the risk of autoreactive CNS-directed immunity.

case control analyses indicated that higher levels of anti-EBV antibodies together with risk HLA-DR alleles discriminate MS cases from controls more effectively than either measure alone, which implies that host genetics and EBV immune markers can be jointly informative (68). Such studies provide conceptual support for multivariate risk models that incorporate HLA, anti-EBV serology, and broader genetic risk scores.

Limitations and current research status

Despite these promising research findings, the use of PRS combined with HLA and EBV serology for individual MS risk prediction remains largely experimental. Several key limitations still constrain clinical application: PRS models depend heavily on population ancestry and large reference datasets; the effect sizes of many non-HLA variants are modest; and EBV infection is nearly universal, reducing the specificity of simple seroprevalence for risk prediction. Therefore, integrated risk stratification using PRS + HLA + EBV serology is currently a research tool that may enrich study cohorts or elucidate biological mechanisms, but it is not yet ready for routine clinical use (67).

Future studies with larger and more diverse cohorts, refined EBV-related genetic scores, and longitudinal validation will be needed to determine if this combined approach can achieve sufficient predictive accuracy for clinical risk stratification. Researchers are also exploring whether additional biomarkers, such as EBV DNA load or multiplex antibody signatures, can further improve integrated risk models.

Proposed high-risk genetic profiles

This review supports a model in which EBV infection acts as a major upstream trigger for MS, but disease risk becomes substantially enriched only in individuals with a permissive immunogenetic background. Because EBV infection is highly prevalent in the general population, EBV seropositivity alone has limited predictive value. A more informative approach is to define risk-enrichment profiles that integrate EBV exposure, HLA risk and protective alleles, EBV-specific immune responses, and selected non-HLA immune loci. These profiles should not be interpreted as diagnostic categories, but as biologically plausible frameworks for stratifying EBV-positive individuals in research settings (34).

Core EBV–HLA risk profile

The most established high-risk profile is EBV-positive / HLA-DRB1*15:01-positive, especially when the individual also belongs to the broader HLA-DR15 haplotypic background. HLA-DRB115:01 is the strongest genetic risk allele for MS, and its relevance in EBV-associated MS is biologically coherent because it can shape CD4+ T-cell responses to EBV-derived antigens. In this model, EBV-infected B cells provide the antigenic source, while HLA-DRB115:01 determines how viral and potentially cross-reactive peptides are presented to CD4+ T cells (34).

This core profile becomes more compelling when combined with evidence of enhanced EBV-specific humoral immunity, particularly high anti-EBNA1 IgG titers. Anti-EBNA1 responses have been associated with MS risk, and EBNA1 is mechanistically relevant because EBNA1-directed immunity can cross-react with CNS-associated antigens. Therefore, the profile EBV-positive / HLA-DRB1*15:01-positive / high anti-EBNA1 IgG represents one of the most biologically supported EBV-associated MS risk configurations (69).

Loss of HLA class I protection

A second layer of risk involves the absence of protective HLA class I alleles, particularly HLA-A*02:01. In contrast to HLA-DRB115:01, which increases MS susceptibility, HLA-A02:01 has been repeatedly described as protective. In EBV-associated models, this protection may reflect more effective CD8+ T-cell-mediated immune surveillance of EBV-infected B cells. Therefore, the absence of HLA-A*02:01 may remove an important protective mechanism and contribute to a higher-risk state (43).

The combined profile HLA-DRB115:01-positive / HLA-A02:01-negative may therefore be more informative than either allele considered alone. In this configuration, class II-mediated presentation may favor EBV-specific or cross-reactive CD4+ T-cell activation, while reduced class I protection may allow less efficient control of EBV-infected B cells. This profile can be conceptualized as a combination of "increased pathogenic antigen presentation" and "loss of protective antiviral surveillance (43, 70)."

Infectious mononucleosis and HLA-E*01:01

A tertiary risk-enrichment profile includes infectious mononucleosis and HLA-E*01:01. Infectious mononucleosis serves as a clinical indicator of symptomatic primary EBV infection and has historically been linked to a heightened risk of multiple sclerosis. Recent population-centric research suggests that individuals with HLA-E*01:01 who have had infectious mononucleosis have a markedly elevated risk of MS, irrespective of other HLA alleles linked to MS. This indicates that HLA-E*01:01 could delineate a subpopulation where symptomatic EBV infection is followed by diminished or dysregulated immunological response to EBV (12).

This HLA-E profile is particularly important because it expands the EBV–MS model beyond classical HLA-DR and HLA-A alleles. HLA-E regulates NK-cell and

CD8+ T-cell responses, and the 2025 findings support the hypothesis that non-classical HLA variation may influence how efficiently EBV infection is controlled after infectious mononucleosis. Thus, IM history + HLA-E*01:01, especially when combined with HLA-DRB115:01 or absence of HLA-A02:01, represents an emerging high-risk configuration that deserves further replication and functional validation (12).

Non-HLA immune amplification profile

Beyond HLA, non-HLA immune loci may amplify EBV-associated immune activation. The clearest current example is CD86, a costimulatory molecule involved in antigen-presenting cell and T-cell interactions. In pediatric-onset MS, a study reported interactions between childhood EBV infection and both DRB1*15 and the GG genotype of the CD86 risk variant rs2255214, supporting a role for antigen-presenting cells in EBV-associated pediatric MS risk (63).

Other non-HLA loci, including CD40, IL2RA, and IL7R, may contribute less specifically by shaping B-cell activation, T-cell costimulation, cytokine signaling, and immune tolerance. These genes may not be EBV-specific risk factors, but they can create a polygenic immune environment in which EBV-infected B cells are more likely to drive persistent T-cell activation or autoreactive responses. Therefore, a broader risk profile may include high non-HLA immune genetic burden, particularly involving antigen-presenting cell and B/T-cell costimulatory pathways (43).

Proposed tiered model of EBV-associated MS risk

Based on the evidence reviewed above, EBV-positive individuals can be conceptually stratified into three research-oriented risk categories. A lower-risk EBV-positive profile would include EBV seropositivity without HLA-DRB115:01, presence of protective HLA-A02:01, no history of infectious mononucleosis, low or moderate anti-EBNA1 titers, and low polygenic immune risk. In these individuals, EBV latency may remain effectively controlled without progression toward CNS autoimmunity (43).

An intermediate-risk profile may include EBV seropositivity with either HLA-DRB115:01 positivity or absence of HLA-A02:01, together with elevated anti-EBNA1 IgG or a history of infectious mononucleosis. This category reflects partial enrichment of risk, where one or more immunogenetic features may increase EBV-driven immune activation but the full high-risk configuration is not present (12).

A high-risk EBV-associated MS profile can be proposed as: EBV-positive, especially with infectious mononucleosis history; HLA-DRB115:01/HLA-DR15 positive; HLA-A02:01 negative; high anti-EBNA1 IgG; HLA-E*01:01 carrier; and elevated non-HLA immune risk involving APC or costimulatory pathways such as CD86. This profile integrates the strongest established genetic risk factor, loss of a protective HLA class I allele, EBV-specific humoral dysregulation, emerging HLA-E evidence, and non-HLA immune amplification (12).

Interpretation and clinical caution

These proposed profiles should be interpreted as risk-enrichment models, not clinical prediction tools. At present, no combination of EBV serology and genetic markers can determine whether an individual EBV-positive person will develop MS. The profiles are most useful for organizing mechanistic evidence, designing prospective studies, enriching high-risk research cohorts, and developing future preventive strategies such as EBV vaccination trials or immune monitoring in genetically susceptible groups (53).

In summary, the most plausible high-risk configuration for EBV-associated MS is not defined by a single genetic variant, but by the convergence of HLA class II risk, loss of HLA class I protection, EBV-specific humoral activation, non-classical HLA-mediated antiviral control, and non-HLA immune costimulatory pathways. This integrated model helps explain why EBV infection is common, but MS is rare, and why only a genetically susceptible subset of EBV-infected individuals progresses toward CNS-directed autoimmunity (43).

Table 2 summarizes the proposed EBV-associated MS risk profiles, integrating EBV exposure, HLA class I and II alleles, EBV-specific humoral responses, and non-HLA immune loci into a tiered research-oriented framework.

Knowledge gaps and future directions

Despite major progress in defining the relationship between EBV, host genetics, and MS, several important knowledge gaps remain. First, most available evidence supports EBV as a major upstream trigger, but the precise sequence of immunological events between EBV infection and clinical MS onset is still incompletely understood. The interval between EBV seroconversion and MS diagnosis may span years, indicating that future studies need to focus on the preclinical phase of MS. Large longitudinal cohorts with repeated sampling before and after EBV infection are needed to track EBV serology, EBV DNA load, neurofilament light chain, HLA genotype, polygenic risk score, and immune-cell phenotypes over time. Such studies would clarify whether EBV-specific immune dysregulation precedes neuroaxonal injury, follows early CNS inflammation, or evolves in parallel with other risk factors (3).

A second major limitation is the lack of ethnic and ancestral diversity in many genetic studies of MS. Most HLA and polygenic risk findings have been

Table 2. Proposed EBV-associated MS risk profiles

Risk category	EBV-related features	HLA profile	Non-HLA / immune features	Interpretation
Lower-risk EBV-positive profile	EBV seropositive; no IM history; low/moderate anti-EBNA1	HLA-DRB115:01 negative; HLA-A02:01 positive	Low polygenic immune burden	EBV latency likely controlled; lower likelihood of CNS autoimmunity
Intermediate-risk profile	EBV seropositive; elevated anti-EBNA1 or IM history	HLA-DRB115:01 positive or HLA-A02:01 negative	Some immune risk variants	Partial enrichment of EBV-associated immune risk
High-risk EBV-associated MS profile	EBV positive; IM history; high anti-EBNA1 IgG	HLA-DRB115:01/HLA-DR15 positive; HLA-A02:01 negative; possible HLA-E*01:01	CD86 risk genotype or high non-HLA immune PRS	Strongest proposed risk-enrichment profile; research use only
Pediatric-onset enriched profile	Childhood EBV infection	HLA-DRB1*15 positive	CD86 rs2255214 GG genotype	Supports APC/costimulation involvement in EBV-associated pediatric MS
Emerging IM–HLA-E profile	Infectious mononucleosis history	HLA-E*01:01 carrier	Possible weaker NK/CD8 EBV control	Emerging profile requiring replication

Note: These profiles are proposed for mechanistic and research stratification. They should not be used as stand-alone clinical predictors of MS.

generated in European-ancestry populations, yet HLA allele frequencies, linkage disequilibrium patterns, EBV infection timing, infectious mononucleosis prevalence, and MS incidence vary across populations. This limits the generalizability of proposed profiles such as HLA-DRB115:01-positive / HLA-A02:01-negative / high anti-EBNA1. Future studies should include diverse ancestries and underrepresented populations to determine whether the same EBV–HLA interactions apply globally or whether population-specific HLA haplotypes and EBV immune-response patterns modify risk differently (71).

Functional genomics will be essential for moving beyond association toward mechanism. GWAS and PRS identify risk loci, but they do not explain how specific variants alter EBV control, B-cell activation, antigen presentation, or CNS-directed autoimmunity. Future research should integrate transcriptomics, epigenomics, chromatin accessibility, proteomics, and CRISPR-based perturbation studies in EBV-infected B cells, dendritic cells, CD4+ T cells, CD8+ T cells, and NK cells. This would help determine whether variants in CD86, CD40, IL2RA, IL7R, and other immune loci directly alter responses to EBV or instead act as broader autoimmune susceptibility modifiers (71).

Single-cell technologies represent another important direction. MS is immunologically heterogeneous, and bulk profiling may obscure rare but pathogenic EBV-reactive or autoreactive lymphocyte populations. Single-cell RNA sequencing, paired TCR/BCR sequencing, spatial transcriptomics, and single-cell epigenomics could identify EBV-specific B-cell and T-cell clones, define their activation states, and determine whether they are enriched in individuals carrying high-risk HLA profiles. These approaches could also clarify whether EBV-reactive immune cells migrate into the CNS, acquire tissue-resident phenotypes, or participate in intrathecal antibody production and meningeal inflammation (72).

HLA peptidomics is likely to become particularly important for this field. The central hypothesis of this review is that genetically determined antigen presentation shapes EBV-associated MS risk, but the exact viral and self-peptide repertoires presented by risk HLA molecules remain incompletely defined. Recent work showing that EBV infection can reprogram the transcriptome and immunopeptidome of HLA-DR15-positive B cells provides a strong rationale for larger studies using immunopeptidomics across different HLA backgrounds, EBV latency states, and immune-cell types. Such studies could identify the EBV-derived and CNS-derived peptides most relevant to molecular mimicry, epitope spreading, and autoreactive CD4+ T-cell activation (35).

Another key challenge is translating integrated risk models into clinically meaningful tools. Combining PRS + HLA genotype + EBV serology is conceptually attractive, but current models are not ready for individual-level prediction. EBV infection is too common, MS is relatively rare, and many high-risk genetic or serological markers have limited positive predictive value when used alone. Future work should test whether integrated models can reliably identify enriched-risk groups for research enrollment, longitudinal monitoring, or prevention trials. These models should include not only HLA-

DRB115:01, HLA-A02:01, HLA-E variation, and non-HLA immune loci, but also dynamic biomarkers such as anti-EBNA1 domain-specific antibodies, EBV DNA load, neurofilament light chain, and possibly single-cell immune signatures (73).

Finally, EBV vaccination and EBV-targeted prevention strategies represent one of the most important future directions. If EBV is a near-necessary upstream trigger for MS, preventing primary EBV infection or reducing symptomatic infectious mononucleosis could theoretically lower MS incidence. However, EBV vaccine-based MS prevention trials face major practical challenges, including the long delay between EBV infection and MS onset, the need for very large cohorts, and the difficulty of selecting appropriate endpoints. Recent discussions emphasize that such trials may require long-term follow-up and risk-enriched recruitment strategies, potentially using HLA genotype, EBV serology, and PRS to identify individuals most likely to benefit from monitoring or preventive intervention (73).

Overall, the field is moving from epidemiological association toward mechanistic and translational stratification. The next generation of studies should combine longitudinal sampling, diverse populations, functional genomics, single-cell immune profiling, HLA peptidomics, and EBV vaccine research. This integrated approach will be necessary to determine whether EBV-associated MS can be predicted, prevented, or intercepted before irreversible CNS injury occurs.

CONCLUSION

EBV is a major trigger for MS, but infection alone is not sufficient; host genetic background determines who progresses to CNS autoimmunity. HLA-DRB115:01 enhances presentation of EBV antigens and promotes autoreactive immune responses, while absence of HLA-A02:01 and carriage of HLA-E*01:01 may reduce antiviral control and further elevate risk. Non-HLA loci such as CD86, CD40, IL2RA, and IL7R can amplify EBV-driven immune activation, creating a permissive environment for autoimmunity. The highest-risk research profile combines EBV positivity, especially with infectious mononucleosis or high anti-EBNA1 titers, HLA-DRB115:01, absence of HLA-A02:01, possible HLA-E*01:01, and increased non-HLA immune burden. These profiles are currently useful for research and mechanistic studies rather than clinical prediction. Future longitudinal, functional, and single-cell studies, along with HLA peptidomics and EBV vaccine trials, will be critical to clarify why only a subset of EBV-infected individuals develop MS and to guide potential preventive strategies.

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Nanoparticles in the Treatment of Anemia: A Narrative Review

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ABSTRACT

Background: Anemia is a common haematological disorder characterised by reduced haemoglobin concentration and impaired oxygen transport. Although conventional iron supplementation remains a standard therapeutic approach, its effectiveness may be limited by poor absorption and gastrointestinal adverse effects. Nanoparticle-based systems have therefore emerged as potential platforms for improving iron delivery and therapeutic efficacy.

Methods: A narrative review was conducted using PubMed, Scopus, and Web of Science to identify relevant publications published between 2010 and 2026. The search included terms related to anemia, nanoparticles, iron supplementation, targeted drug delivery, and haematological therapies. Preclinical, animal, in vitro, and relevant review studies were considered, and findings were synthesised qualitatively.

Results: Preclinical and experimental evidence suggests that several nanoparticle-based systems, particularly iron-containing nanocarriers and iron oxide nanoparticles, may improve iron delivery, bioavailability, sustained release, and treatment tolerability. Some animal and in vitro studies have also reported improvements in haemoglobin and other haematological parameters, together with potential antioxidant and anti-inflammatory effects. However, these findings are largely derived from experimental models, and evidence from human clinical studies remains limited. In addition, certain nanomaterials have demonstrated potential oxidative, genotoxic, or systemic toxicities, highlighting the importance of safety evaluation.

Conclusion: Nanoparticle-based technologies show promising preclinical potential for improving iron delivery and addressing selected mechanisms associated with anemia . However, current evidence is insufficient to establish their clinical efficacy or long-term safety. Well-designed clinical studies are required to determine whether the benefits observed in experimental models can be translated into effective and safe therapies for patients with anemia.

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INTRODUCTION

Chronic diseases, including cancer, iron-deficiency anaemia, and other long-term health conditions, represent major public health challenges, causing significant physical and psychological suffering, impairing quality of life, and placing a substantial burden on patients, their families, and healthcare systems (1, 2). Anemia is one of the most common haematological disorders worldwide and is characterised by a reduction in haemoglobin concentration, a decreased

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red blood cell (RBC) count, or impaired oxygen-carrying capacity of the blood (3). It affects hundreds of millions of individuals globally, particularly young children, pregnant women, older adults, and patients with chronic illnesses, making it a major public health concern (3). The condition is associated with fatigue, generalised weakness, impaired cognitive performance, delayed growth and development, reduced physical productivity, and an increased risk of morbidity and mortality (3). Anemia arises from a wide range of underlying causes, including iron deficiency, vitamin B12 and folate deficiencies, chronic inflammatory disorders, inherited haematological diseases, chronic kidney disease, malnutrition, and infectious diseases (4). Given its high prevalence and substantial clinical burden, the development of safe, effective, and targeted therapeutic strategies remains an important priority in modern medicine (3, 4).

The pathophysiological mechanisms underlying anemia vary according to its aetiology and generally involve impaired erythrocyte production, accelerated destruction of circulating red blood cells, or excessive blood loss (4). Iron deficiency anemia develops when depleted iron stores compromise haemoglobin biosynthesis, thereby reducing tissue oxygenation (5). In anemia associated with chronic inflammation, increased production of pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), stimulates hepcidin expression, resulting in reduced intestinal iron absorption and impaired mobilisation of stored iron (6). Furthermore, excessive oxidative stress and the accumulation of reactive oxygen species (ROS) can damage erythrocyte membranes, disrupt erythropoiesis, and shorten red blood cell lifespan (5). In hereditary disorders such as thalassaemia and sickle cell disease, genetic abnormalities alter the structure or function of haemoglobin, further contributing to disease pathogenesis (6). The multifactorial nature of these mechanisms highlights the need for therapeutic approaches capable of addressing multiple pathological pathways simultaneously.

Current therapeutic options for anemia include oral and intravenous iron supplementation, erythropoiesis-stimulating agents, blood transfusion, vitamin replacement therapy, and, in selected cases, haematopoietic stem cell transplantation (7). Although these interventions have substantially improved patient outcomes, each possesses important limitations. Oral iron preparations often exhibit poor bioavailability, limited intestinal absorption, gastrointestinal intolerance, and poor patient adherence, whereas parenteral iron administration may be associated with iron overload, infusion-related reactions, and the need for repeated clinical visits (8). Common gastrointestinal adverse effects including nausea, constipation, abdominal discomfort, and dyspepsia remain major barriers to long-term compliance with oral iron therapy (9). Moreover, patients with chronic inflammatory disorders frequently demonstrate suboptimal responses to conventional iron supplementation because inflammatory pathways interfere with iron metabolism. These limitations have stimulated considerable interest in developing advanced drug delivery systems capable of improving iron absorption while reducing systemic toxicity and treatment-related adverse effects (8, 9).

Recent advances in nanotechnology have opened new avenues for the diagnosis and treatment of haematological disorders. Owing to their nanoscale dimensions, large surface-area-to-volume ratio, tunable physicochemical properties, and versatile surface functionalisation, nanoparticles have emerged as highly promising drug delivery platforms (10, 11). These nanocarriers can enhance pharmaceutical stability, improve intestinal absorption, provide sustained and controlled drug release, and facilitate targeted delivery to specific tissues or cell populations (10, 11). Furthermore, nanoparticle-based formulations protect therapeutic agents from premature degradation, thereby improving the bioavailability of iron, vitamins, erythropoiesis-stimulating agents, and other bioactive molecules involved in erythropoietic regulation (10, 11).

A broad range of nanoparticle systems including iron nanoparticles, polymeric nanoparticles, lipid nanoparticles, liposomes, nanoemulsions, dendrimers, and nanocomposite materials has been investigated for the treatment of anemia (12). Iron-based nanoparticles have attracted particular attention because they demonstrate superior bioavailability, enhanced gastrointestinal absorption, and fewer gastrointestinal adverse effects than conventional iron supplements (13). Experimental studies have shown that these nanocarriers facilitate more efficient intestinal iron uptake while providing sustained and controlled iron release (12, 13). In addition, several nanoparticle platforms have been engineered to deliver erythropoietin, antioxidants, and anti-inflammatory agents directly to target tissues, thereby enhancing erythropoiesis through attenuation of oxidative stress and inflammatory responses (13, 14).

Nanotechnology also offers promising therapeutic opportunities for complex forms of anemia, including thalassaemia, sickle cell disease, and anemia of chronic disease (15). Nanoparticle-mediated delivery systems have been explored for gene therapy applications, including the transport of small RNA molecules and genome-editing components designed to correct inherited haemoglobin disorders (16). Beyond therapeutic delivery, multifunctional nanoplatforms have demonstrated considerable potential as theranostic systems by integrating diagnostic imaging with targeted treatment within a single platform (16). Magnetic

nanoparticles have likewise shown considerable value in blood cell separation, targeted drug delivery, and the enhancement of diagnostic procedures, further expanding the clinical applications of nanomedicine in haematology (15, 16).

Despite these encouraging advances, several important challenges continue to limit the clinical translation of nanoparticle-based therapies (17). Potential cytotoxicity, long-term tissue accumulation, nanoparticle-induced oxidative stress, unintended immune activation, and uncertainties regarding long-term biosafety remain major concerns (17). Additional obstacles include the lack of standardised manufacturing protocols, high production costs, formulation stability issues, and the limited availability of robust clinical evidence supporting routine therapeutic use (18). Consequently, comprehensive evaluation of nanoparticle biocompatibility, toxicological profiles, pharmacokinetics, and long-term safety remains essential before widespread clinical implementation can be considered (19).

Given the rapid expansion of research in nanomedicine and haematology, a comprehensive synthesis of the available evidence is both timely and necessary. Although numerous studies have investigated nanoparticle-based approaches for the treatment of anemia, current evidence remains fragmented, particularly regarding their mechanisms of action, therapeutic efficacy, safety profiles, existing limitations, and future clinical prospects. Therefore, this review aims to critically evaluate the current evidence on the therapeutic applications of nanoparticles in the treatment and management of anemia, with particular emphasis on their underlying biological mechanisms, clinical potential, safety considerations, and future directions for research and clinical translation.

METHODS

This study was conducted as a narrative review of the therapeutic applications of nanoparticles in anemia. A literature search was performed in PubMed, Scopus, Web of Science, SID, and ISC for publications published from January 2010 to 2025.

The search combined keywords related to anemia, iron deficiency anemia, nanoparticles, nanomedicine, nanocarriers, iron supplementation, drug delivery, targeted therapy, and erythropoiesis using the Boolean operators AND and OR. A representative search strategy was: (Anemia OR Iron Deficiency Anemia) AND (Nanoparticles OR Nanomedicine OR Nanocarriers) AND (Iron Supplementation OR Drug Delivery OR Targeted Therapy).

Relevant original studies, preclinical and in vitro investigations, animal studies, and pertinent narrative or systematic reviews were considered. Publications were excluded if they were duplicates, lacked sufficient relevant information, were unavailable in full text, or focused exclusively on nanoparticle toxicity without therapeutic relevance to anemia.

Relevant publications were screened for their relevance to the objectives of the review, and key information was extracted regarding nanoparticle type, experimental model, therapeutic target, delivery strategy, mechanism of action, efficacy, safety, and study limitations. Because this was a narrative review, no formal meta-analysis or quantitative statistical synthesis was performed. Findings were qualitatively synthesised according to nanoparticle type, iron delivery, erythropoiesis, antioxidant and anti-inflammatory mechanisms, iron deficiency anemia, chronic disease-associated anemia, and emerging nanomedicine approaches.

The literature selection and synthesis were intended to provide a critical overview of the current evidence rather than a systematic estimation of treatment effects.

RESULTS

The included studies evaluated several nanoparticle-based approaches for anemia , predominantly in animal and other preclinical models. Iron-based nanoparticles and nanoformulations, including Fe_3O_4, ferric pyrophosphate, ferrihydrite, and solid lipid nanoparticles, were mainly investigated for improving iron delivery, absorption, bioavailability, and controlled release. Several studies reported improvements in haemoglobin concentration, red blood cell indices, and other haematological parameters in experimental models.

Other nanoparticle systems, including gold, tin, and gadofullerene nanoparticles, were investigated for their effects on oxidative stress, inflammation, and erythropoiesis. Reported findings included reduced reactive oxygen species and oxidative damage, modulation of antioxidant and inflammatory pathways, and improved erythropoietic responses. Advanced platforms such as albumin-based nanoparticles and folate-targeted systems were also investigated for targeted or enhanced delivery of iron and other therapeutic agents.

The evidence was predominantly preclinical, while clinical evidence was limited. Some studies also reported formulation-dependent toxicity, oxidative stress, or genotoxic effects, indicating variability in the biological effects of different nanomaterials. Overall, the findings were heterogeneous with respect to nanoparticle type, experimental model, therapeutic target, and measured outcomes.

A detailed summary of the characteristics and principal findings of the studies included in this review is presented in Table 1 and 2.

A total of 25 publications were initially identified through the literature search. Following assessment of

Table 1. Representative animal and experimental studies investigating nanoparticle-based approaches for anemia.

Author	Nanoparticle / Delivery System	Active Agent	Animal/Experimental Model	Main Mechanism	Main Findings
Goorani et al. (2020)(20)	Biogenic gold nanoparticles (AuNPs)	Plant extract	In vivo/in vitro; mouse	Antioxidant and anti-inflammatory effects; ROS reduction	Improved haematological parameters in experimental models
Jia et al. (2020)(22)	Gadofullerene nanoparticles (GFNPs)	Gadofullerene	Mouse model of aplastic anemia	ROS scavenging; erythrocyte maturation	Enhanced erythropoiesis and red blood cell maturation
Ahmeda et al. (2020)(23)	Green tin nanoparticles (SnNPs)	Plant extract	Mouse model of haemolytic anemia	Antioxidant and anti-inflammatory effects	Reduced oxidative damage and improved haematological indices
Fathy et al. (2019)(25)	Lipid matrix nanoparticles (LMNPs)	$FeSO_4$	Mouse model of IDA	Enhanced iron bioavailability	Improved iron absorption compared with conventional formulations
Hashem et al. (2018)(26)	Fe_3O_4 nanoparticles	Iron	Mouse model of IDA	Folate-mediated targeting	Improved targeted iron delivery and correction of iron deficiency
Elsayed et al. (2014)(27)	Magnetite nanoparticles	Iron	Mouse model of IDA	Controlled iron release	Improved therapeutic efficiency through sustained iron delivery
Hosny et al. (2015)(28)	Iron-loaded solid lipid nanoparticles (Fe-SLNs)	Iron	In vivo pharmacokinetic model	Enhanced intestinal absorption	Improved pharmacokinetic profile and treatment tolerability
Shafie et al. (2016)(29)	Iron nanoparticles	Iron	Mouse model of haemolytic anemia	Anti-inflammatory activity	Reduced inflammation and improved haematological parameters
Szudzik (2021)(31)	Sucrosomial® iron	Iron	Guinea pig model	Sustained gastrointestinal absorption	Efficient iron uptake with prolonged absorption
Bahal et al. (2016)(32)	γPNA nanoparticles	Gene-editing construct	Mouse model of β-thalassaemia	Targeted β-globin gene correction	Demonstrated gene-editing and therapeutic potential
Wang et al. (2019)(34)	Fe_3O_4@APS nanoparticles	Iron with polysaccharide coating	Mouse model	Haematopoietic stimulation	Demonstrated therapeutic and potential theranostic functions
El-Bahr et al. (2021)(35)	Plant-derived iron oxide nanoparticles	Iron	Mouse model	Antioxidant activity	Reduced oxidative stress and improved blood parameters
Darwish et al. (2021)(36)	BSA–Fe nanoparticles	Iron + folic acid	Mouse model of IDA	Albumin-based nanoencapsulation	Improved iron delivery and therapeutic effectiveness
Srinivasu et al. (2015)(37)	Ferric pyrophosphate nanoparticles (FePP NPs)	Iron	Mouse model of IDA	Increased bioavailability	Enhanced iron absorption compared with conventional preparations
Powell et al. (2014)(38)	Ferrihydrite nanoparticles	Iron	Mouse model	Ferritin-mimetic iron storage	Efficient physiological iron delivery and favourable bioavailability

relevance and eligibility, 7 publications were excluded. Consequently, 18 publications were included in the final narrative synthesis. The characteristics and principal findings of the included studies are summarised in Tables 1 and 2

Based on the studies summarized above, no single nanoparticle can be considered universally the most effective, as the investigated anemia models and therapeutic mechanisms differ substantially. Nevertheless, gadolinium-based fullerene nanoparticles (GFNPs) have shown notable efficacy in a murine model of chemotherapy-induced aplastic anemia, primarily through ROS scavenging and enhanced erythrocyte maturation, whereas in iron-

Table 2. Representative review studies and secondary evidence on nanoparticle-based approaches to anemia.

Author	Nanoparticle / Approach	Evidence Type	Main Focus	Principal Conclusions
Alphandéry (2019)(21)	Iron oxide nanoparticles (IONPs)	Narrative review	Iron delivery and magnetic targeting	Discussed the potential of IONPs for targeted iron delivery and therapeutic applications
Kumari & Chauhan (2022)(24)	Iron nanoparticles	Narrative review	Iron bioavailability and absorption	Summarised evidence regarding nanoparticle-based iron delivery and improved absorption
Pai & Garba (2012)(39)	Ferumoxytol	Clinical	Intravenous	Reported clinical evidence supporting intravenous

deficiency anemia (IDA), formulations such as FePP, Fe_3O_4, and iron-loaded solid lipid nanoparticles (Fe-SLNs) have mainly improved hematological outcomes by enhancing iron absorption and bioavailability. A major limitation of these studies is their predominantly preclinical nature, together with differences between animal models and human disease, limited information on the long-term safety and tissue accumulation of nanoparticles, and interspecies differences in dosage and pharmacokinetics. Therefore, findings from animal studies cannot be directly extrapolated to humans. Among the approaches reviewed, ferumoxytol is distinct in that it is an iron-based nanoparticle formulation supported by clinical evidence, particularly in patients with chronic kidney disease and iron deficiency, although safety considerations, including hypersensitivity reactions, remain important.

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DISCUSSION

Anemia remains one of the most prevalent nutritional and haematological disorders worldwide and continues to impose a substantial clinical and socioeconomic burden through its adverse effects on physical development, cognitive performance, work productivity, and overall quality of life (41). Although oral and intravenous iron preparations remain the cornerstone of treatment, particularly for iron deficiency anemia, their therapeutic effectiveness is frequently compromised by limited gastrointestinal absorption, poor bioavailability, treatment-related adverse effects, prolonged administration, and suboptimal patient adherence (41). The findings synthesised in this review indicate that nanoparticle-based drug delivery systems have the potential to overcome many of these limitations by improving iron transport, optimising pharmacological delivery, and enhancing therapeutic efficacy.

One of the most consistently reported advantages of iron-containing nanoplatforms is their ability to improve iron bioavailability and gastrointestinal uptake. The reviewed studies demonstrated that iron oxide nanoparticles, iron-loaded nanocapsules, and other engineered nanostructures can protect iron from premature degradation or precipitation within the gastrointestinal tract while facilitating its transport across intestinal epithelial cells (42). Consequently, a greater proportion of administered iron becomes available for systemic circulation and erythropoiesis. These improvements have been reflected by significant increases in haemoglobin concentration, red blood cell counts, and serum ferritin levels across numerous experimental models (42). Such findings suggest that nanotechnology may substantially enhance the therapeutic efficiency of iron replacement therapy compared with conventional formulations.

Controlled drug release and tissue-specific targeting represent additional strengths of nanoparticle-based therapeutic systems. Several advanced nanocarriers have been designed to deliver iron or erythropoiesis-stimulating agents in a sustained manner, thereby maintaining more stable circulating iron concentrations and reducing fluctuations associated with conventional supplementation (42). This controlled-release profile may improve therapeutic efficiency while potentially reducing transient exposure to high levels of iron and, consequently, the risk of iron-related oxidative stress (43). Furthermore, the physicochemical properties of some nanoparticle-based delivery systems may offer opportunities for preferential iron delivery to hematopoietic tissues; however, direct targeting of the bone marrow or erythroid progenitor cells has not been sufficiently established and requires further validation in appropriate in vivo models (43). Such approaches may therefore have potential to enhance erythropoietic support while limiting unnecessary systemic exposure, although their targeting efficiency, biodistribution, and long-term safety remain to be determined.

Beyond serving as delivery vehicles, numerous

nanoparticle formulations possess intrinsic biological activities that may further contribute to anemia management. Chronic inflammation and oxidative stress play pivotal roles in the pathogenesis of several forms of anemia, particularly anemia associated with chronic disease. Evidence reviewed in this study indicates that certain nanoplatforms exhibit antioxidant and anti-inflammatory properties capable of reducing reactive oxygen species, suppressing inflammatory mediators such as C-reactive protein (CRP), and protecting haematopoietic stem and progenitor cells from oxidative injury. These complementary mechanisms may create a more favourable microenvironment for erythropoiesis and could be particularly advantageous in patients whose anemia is driven by persistent inflammatory processes.

Another important observation emerging from the current literature is the rapid development of multifunctional nanoplatforms that integrate therapeutic and diagnostic capabilities within a single system. Iron oxide-based magnetic nanoparticles, for example, have demonstrated considerable promise not only as iron delivery vehicles but also as contrast agents for medical imaging and treatment monitoring. In parallel, advances in nanoparticle-mediated gene delivery have expanded opportunities for transporting therapeutic genes, regulatory nucleic acids, and genome-editing components aimed at correcting inherited disorders of haemoglobin synthesis. Although these approaches remain largely experimental, they represent promising directions for the future treatment of genetically determined and treatment-resistant forms of anemia.

Despite these encouraging advances, important safety concerns continue to limit the clinical translation of nanoparticle-based therapies. Several studies have reported that certain nanoparticles, particularly when administered at high doses or following prolonged tissue accumulation, may induce oxidative stress, disrupt cellular membrane integrity, impair mitochondrial function, and produce genotoxic effects. Titanium dioxide nanoparticles and selected iron oxide formulations have been identified among the materials associated with such adverse biological responses under specific experimental conditions. These findings emphasise that therapeutic efficacy must always be balanced against potential toxicological risks through comprehensive preclinical safety assessment and rigorous optimisation of nanoparticle design.

In addition to toxicological considerations, several practical challenges remain before nanoparticle-based therapies can be routinely incorporated into clinical practice. Most available evidence has been generated from in vitro experiments and animal models, whereas high-quality clinical data evaluating long-term safety, pharmacokinetics, biodistribution, and therapeutic efficacy in humans remain limited. Moreover, substantial variability in nanoparticle composition, synthesis methods, particle size, surface modification, dosing strategies, and outcome measures complicates direct comparisons across studies. Establishing standardised manufacturing protocols and conducting well-designed multicentre clinical trials will therefore be essential for translating these promising technologies into evidence-based therapeutic options for patients with anemia.

CONCLUSION

The evidence reviewed in this study indicates that nanoparticle-based drug delivery systems represent a highly promising strategy for improving the management of anemia. By enhancing iron absorption and bioavailability, promoting erythropoiesis, reducing oxidative stress and inflammatory responses, and enabling sustained, targeted drug delivery, nanotherapeutic platforms have the potential to address several limitations associated with conventional treatment modalities. Nevertheless, successful clinical implementation will require a more comprehensive understanding of nanoparticle–host interactions, optimisation of biocompatible and safe nanocarriers, and robust evidence from large-scale, well-controlled clinical trials. Continued advances in nanomedicine, precision drug delivery, and biomaterial engineering are expected to accelerate the development of safer, more effective, and increasingly personalised therapeutic approaches for patients with anemia.

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Author Contributions

Saeed Razavi-Dizaji was responsible for the conceptualization, methodology, investigation, data collection, analysis, interpretation of results, and preparation of the manuscript. The author read and approved the final version of the manuscript.

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Availability of Data and Materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the Ethics Committee of Urmia University of Medical Sciences (Ethics approval code: [insert code, if applicable]). All

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Consent to Publication

Not applicable.

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Convergence with Global Trends in the Knowledge Structure and Leading Subfields of Personalized Medicine Research in Iran

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ABSTRACT

Background: Driven by artificial intelligence (AI) and genomics, personalized medicine (PM) is rapidly expanding. Mapping its conceptual structure through co-word analysis identifies research trends, thematic gaps, and priorities. This study mapped the conceptual structure and thematic evolution of PM in Iran from 2005 to 2025 using Web of Science (WoS) data.

Methods: Keyword co-occurrence and network analysis were performed on 623 WoS-indexed documents. Cluster analysis in VOSviewer and strategic diagrams in Excel identified thematic sub-domains and their relationships.

Results: Cluster analysis revealed nine distinct thematic clusters: (1) AI and machine learning in medical imaging and diagnosis; (2) gene editing, omics technologies, and the microbiome's role in treatment; (3) personalized medicine and pharmacogenomics; (4) biomarkers and liquid biopsy in cancer diagnosis; (5) nanomedicine, biosensors, and targeted cancer therapy; (6) tissue engineering, regenerative medicine, and novel drug delivery systems; (7) early detection and management of chronic, inflammatory, and metabolic diseases; (8) nanoparticles, epigenetics, and integrative medicine approaches, including traditional medicine and digital health; and (9) cancer immunotherapy and tumor microenvironment analysis.

Conclusion: Personalized medicine in Iran has a dynamic, interdisciplinary, and evolving structure. Pharmacogenomics serves as the key infrastructure and driving engine, supporting the driving triangle of biomarkers and liquid biopsy, AI in medical imaging, and regenerative medicine with novel drug delivery. Cancer immunotherapy is a specialized, mature domain, while gene editing (CRISPR), nanomedicine, and the microbiome shape future frontiers. Clinical translation requires accelerating mature technologies, establishing national genomic databases, and strengthening interdisciplinary synergies to achieve precise, patient-centered, preventive treatments.

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INTRODUCTION

Personalized medicine (PM), first described conceptually in 1999, is a novel and revolutionary approach to healthcare aimed at fine-tuning diagnosis, prognosis, and treatment for each patient individually (1). This field represents a fundamental paradigm shift from the traditional one-size-fits-all model to a targeted healthcare approach, incorporating periodic, individualized, participatory, and predictive measures based on individuals' unique genetic, environmental, and lifestyle characteristics (2, 3). Nowadays, personalized medicine is expanding into most areas of

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clinical management, becoming a crucial standard for the adoption of new interventions; an approach that has generated high hopes for enhancing patients' quality of life, improving treatment effectiveness, reducing adverse effects, and optimizing costs and time in healthcare delivery (1, 2).

In the scientific literature and knowledge structure of this field, the terms personalized medicine and precision medicine are often used interchangeably, and considering both concepts is essential for a comprehensive elucidation of this new scientific direction (4, 2). Although personalized medicine initially emerged from pharmacogenetic and pharmacogenomic knowledge, a tangible shift towards the use of the term precision medicine gradually occurred (4). By employing specialized terms such as phenotype, endotype, and biomarker, precision medicine seeks to describe diseases more precisely and divide a heterogeneous disease into more homogeneous subgroups, thereby facilitating the provision of specific and targeted treatments (5). Therefore, in exploring the knowledge structure of this domain, both terms, personalized medicine and precision medicine, constitute complementary dimensions of a single paradigm in healthcare.

Rapid advancements in fourth industrial revolution technologies, such as big data, artificial intelligence (AI), machine learning (ML), deep learning (DL), and radiomics, have been the primary drivers of the ever-increasing growth and structural evolution of personalized medicine (6, 7). The integration of advanced computational methods, genomic analysis, and multi-omics data integration has created a profound transformation in clinical decision support systems, diagnostic imaging, and drug discovery (8, 9). The scope of application for this approach is expanding rapidly; so much so that, beyond the dominance of oncology-related topics and decision-making for individualized cancer treatments (10, 3), it has extended into emerging fields such as personalized regenerative medicine and cell therapy utilizing various stem cell sources, including induced pluripotent stem cells (iPSCs) (4), and even prevention and therapy in fetal medicine via prenatal organoids (1).

A review and analysis of global bibliometric literature indicates extraordinary dynamism and exponential growth in research outputs within this field over the past two decades (8, 3). Williams et al. reported an exponential growth of publications in this domain, primarily focusing on genetics and specific treatments. This upward trend has accelerated unprecedentedly in recent years (3); Virk et al. also confirmed the growth of research publications in this field (10). Furthermore, Adam et al. noted a 34.3 % increase in publications in this field solely in the year 2024 (8). In studies focusing on AI and ML in personalized medicine, Al-Dekah demonstrated a staggering annual growth rate of 42.1 % (6). In more specialized areas such as early breast cancer diagnosis, Pedük confirmed a clear acceleration in research post-2020, along with increased international collaboration and a proliferation of open-access publications (8).

Understanding the intellectual structure, identifying core topics, and tracking the evolution of keywords constitute a significant part of scientometric and semantic analyses in this context (8, 11). To accomplish this mapping of knowledge structures, a review of the literature demonstrates that co-word analysis and network analysis have been utilized as effective tools across various fields of science, including information literacy (12), financial literacy (13), digital literacy (14), Throbbing Headaches (15), general concepts of literacy and decision-making (16, 17), and neuroscience (18) to map knowledge structures. Applying these same proven methodologies to the medical domain, through a 10-year analysis of Web of Science (WoS) data (2009 to 2018) using co-word analysis, Lyu et al. demonstrated that the intellectual structure of precision medicine encompasses broad themes that are overall unbalanced. They identified recurring core keywords, including biomarkers, genomics, cancer, therapy, genetics, drug, targeted therapy, pharmacogenomics, pharmacogenetics, and molecular as the core areas and main body of knowledge (19). In this regard, Adam et al. revealed three main clusters: diagnostics and medical imaging, genomic and multi-omics data integration, and personalized treatment strategies (8). Additionally, Khakimova et al. showed that citing articles expand the key terms of the cited articles, creating a stable terminological map for identifying knowledge development trends (11). However, Williams et al. highlighted a significant thematic gap, demonstrating that despite the dominance of genetic and oncological research, little attention has been paid to social and environmental determinants of health and health disparities (3).

As the review of the research background indicates, international research in the fields of personalized and precision medicine is rapidly moving towards maturity, the integration of AI technologies, and comprehensive biological analyses (6, 9, 19). Nevertheless, despite the comprehensive mapping of global scientometric landscapes (7, 10, 11), assessing the engagement, orientation, and extent of participation of Iranian researchers with this knowledge wave requires further investigation. Analyzing Iranian indexed publications in the Web of Science (WoS) database can determine whether domestic research has evolved in alignment with international hotspots such as deep learning, biomarkers, and multi-omics data or suffers from imbalances and knowledge gaps. The insights derived from this structural evaluation provide an evidence-

based knowledge framework to guide policy decisions, optimize the allocation of research budgets, and prioritize interdisciplinary collaborations toward the practical implementation of personalized medicine in the country (8, 19). Therefore, the present study aims to analyze the conceptual structure, identify core and emerging areas, and map the thematic evolution of Iranian research in the field of personalized medicine indexed in the WoS database from 2005 to 2025.

METHODOLOGY

Study Design and Data Retrieval Strategy

This study adopted a bibliometric, co-occurrence, and network analysis design to investigate the scientific landscape of personalized medicine. Data retrieval was conducted on August 25, 2026, using the advanced search section of the Web of Science (WoS) database. The search strategy incorporated the following query:

TS=("Personalized medicine" OR "Precision medicine" OR "Personalized healthcare" OR "Personalized Treatment") AND CU=(Iran) AND PY=(2005-2025)

The document types were limited to original research articles, review articles, proceeding papers, and early access publications. A total of 1,970 documents met these predefined inclusion criteria and were retrieved for analysis. Following data retrieval, the bibliographic records were exported in Excel format, and the required fields were extracted with full records and cited references in plain text format for subsequent data preprocessing.

Data Preprocessing and Concept Standardization

Data processing and concept extraction were executed using BibExcel software. Natural language processing (NLP) techniques within the software were applied to extract core concepts based on author keywords, yielding an initial pool of 9,969 concepts. To ensure data integrity, standardization and unification of concepts were performed, leaving 5,395 unique concepts. To construct a representative co-occurrence matrix that captures the overall schema of the field's main concepts, a cut-off threshold of 5 was selected. This threshold was established using Bradford's one-third law (20). Applying Bradford's law to determine the cut-off point in the keyword co-occurrence matrix serves as a systematic, evidence-based method for screening massive textual data; it identifies and retains core keywords shaping the knowledge structure while filtering out low-frequency, peripheral keywords that obscure map interpretation. Following a series of software commands, a symmetric co-occurrence matrix in plain text format with dimensions of 229 × 229 was created, with the diagonal cell values set to zero.

Network Visualization and Cluster Analysis

The co-occurrence matrix was converted into a similarity matrix utilizing the association strength index and subsequently imported into VOSviewer software. Within VOSviewer, the data were transformed into a correlation matrix to measure the association strength between the retained concepts matching the threshold, providing a measure of concept similarity based on relative co-occurrence frequencies (21, 22). The software's default Louvain clustering algorithm, utilizing the standard Newman-Girvan modularity criterion along with the Visualization of Similarities (VOS) algorithm, was employed for network mapping and clustering.

To map the conceptual structure, a strategic diagram was drawn using the centrality and density scores of the extracted thematic clusters based on the framework of Callon et al (23). This standard framework defines the role of each thematic cluster across four strategic quadrants using centrality and density, enabling the identification of current thematic statuses and potential evolutionary paths. The strategic diagram describes internal relationships and correlations between different clusters, aligning with the approach introduced by Mellers et al. to enhance the visualization of maturity and cohesion within a research field (24).

RESULTS

Publication output trend

Figure 1 displays the publication output trend in the Personalized medicine domain within the Web of Science (WoS) database.

Figure 1 illustrates the trend of scientific document publication in the personalized medicine domain in the Web of Science database between 2005 and 2025. According to this diagram, the scientific output of this field, which underwent a slow process with a very low production volume in the first decade (2005–2014), gradually increased from 2015 and entered an exponential growth phase from 2021. The highest scientific output belongs to the years 2024 and 2025, indicating that personalized medicine has become one of the priorities and highly engaged research fields in the global health arena in recent years.

Frequency distribution of personalized medicine concepts based on keyword co-occurrence

The frequency distribution of the most used concepts in the studied period is shown in table 1.

Table 1 shows the frequency distribution of the most used concepts in the personalized medicine domain during the studied period and reveals the knowledge structure pattern of this realm. The concept of Personalized medicine, with a frequency of (373), has been the focus of research and has the highest repetition. In the subsequent ranks, the dominance of advanced computational technologies is evident;

Fig 1. Publication of personalized medicine between 2005 and 2025.

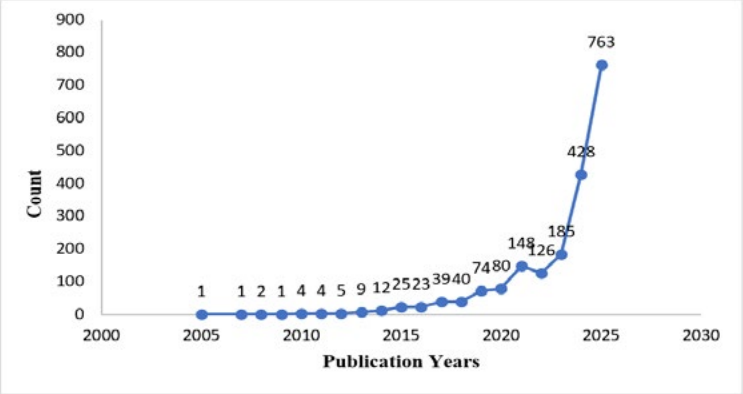


Table 1. Most used concepts in the personalized medicine domain by frequency.

NO.	Concept	Frequency	NO.	Concept	Frequency
1	Personalized medicine	373	11	Colorectal cancer	**45**
2	Artificial intelligence (AI)	125	12	Diagnosis	**43**
3	Machine learning (ML)	124	13	Pharmacogenomics	**40**
4	Biomarker	102	14	Nanoparticle	**37**
5	Cancer	100	15	Regenerative medicine	**34**
6	Drug delivery	72	16	Cancer therapy	**34**
7	Breast cancer	62	17	Tissue engineering	**33**
8	MicroRNA	62	18	Biosensor	**33**
9	Immunotherapy	61	19	3D printing	**33**
10	Deep learning	59	20	Targeted therapy	**32**

such that the concepts of Artificial intelligence (125), Machine learning (124), and Deep learning (59) collectively account for a major portion of knowledge production, indicating a transition in this field towards data-driven and automated analyses. Simultaneously, the biological-molecular foundation of this approach is strengthened by concepts such as Biomarker with a frequency of (102), MicroRNA with (62), and Pharmacogenomics with (40). From a clinical application perspective, the frequency pattern clearly highlights the dominance of neoplastic diseases; general and specific cancer concepts including Cancer (100), Breast cancer (62), and Colorectal cancer (45), alongside novel therapeutic approaches such as Immunotherapy (61), Targeted therapy (32), and Cancer therapy (34), all indicate the clinical focus of this field on precision oncology. In addition, the notable frequency of intertwined concepts of technology and treatment, such as Drug delivery (72), Nanoparticle (37), Regenerative medicine (34), Tissue engineering (33), 3D printing (33), and Biosensor (33), indicate the accelerated integration of engineering approaches and smart drug delivery with personalized medicine. Overall, this frequency distribution presents a cohesive picture of a field whose driving engine is artificial intelligence and biomarkers, and whose dominant applied context is the targeted diagnosis and treatment of cancer.

Knowledge topography: Nine conceptual clusters in the personalized medicine domain

To determine the number of clusters, the clustering analysis section of the VOSviewer software was used, the images of which are displayed in Figure 2 and Table 2. As shown in this image, the concepts of the personalized medicine domain in the studied time frame fall into nine clusters.

The results from Figure 2. The keyword co-occurrence map shows that the concepts in the personalized medicine domain are grouped into nine clusters.

Concept Trends in Iranian health policy research based on overlay visualization. Figure 3 presents the overlay visualization map showing concept evolution

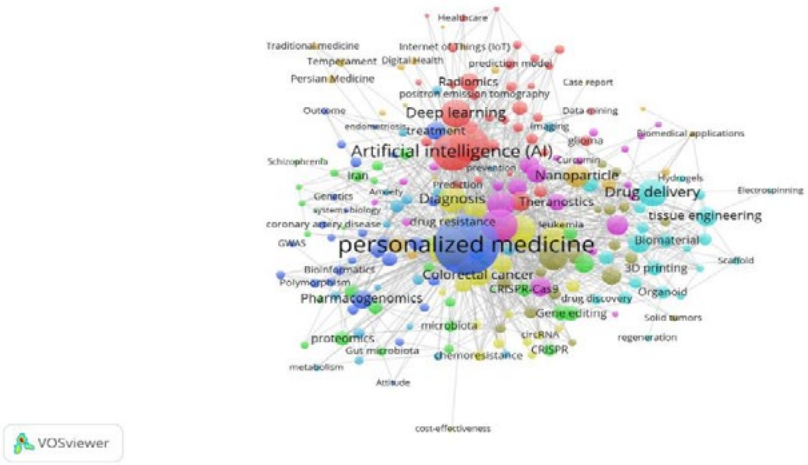


Fig 2. Co-word map of documents in the personalized medicine domain..

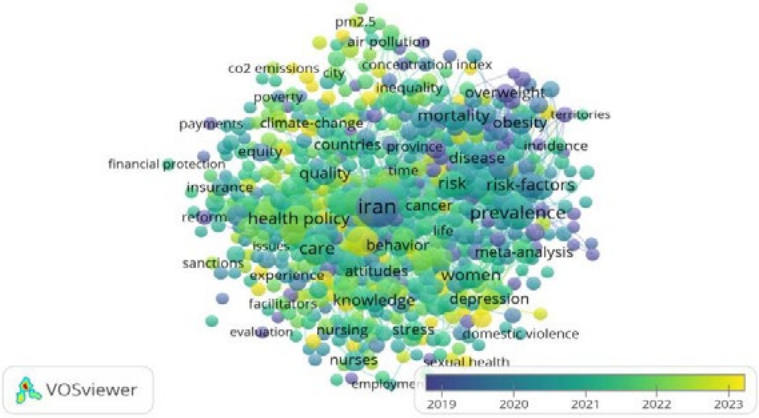


Fig 3. Co-word map of health policy literature based on overlay visualization.

over time. Overlay visualization is used to illustrate temporal developments in the field.

As shown in this map, nodes shifting toward yellow represent recent research trends (radiomics, liquid biopsy, extracellular vesicles, artificial intelligence, hydrogels, biomedical applications, infertility, nanomaterials, etc.). Conversely, concepts prominent in earlier decades appear in darker shades.

Table 2 displays the names of the clusters and their important sub-branches, along with the weight of each sub-branch.

Table 2 displays the nine main clusters and key sub-branches of the personalized medicine domain along with the weight of each concept, indicating the breadth and interconnectedness of this field. The main axis of this table is Cluster (3), i.e., Personalized medicine and Pharmacogenomics, which, with a weight of (1380) for the concept of Personalized medicine, emphasizes the importance of individualized treatments and the role of genetics in them. In the context of novel diagnostic technologies, Cluster (1) Artificial intelligence and machine learning in medical imaging and Cluster (4) Biomarkers and liquid biopsy in cancer diagnosis hold prominent positions. The molecular and cellular approach is also evident in Clusters (2) Gene editing, omics technologies, and the role of microbiome in treatment, and Cluster (8) Nanoparticles, epigenetics, and integrative medicine approaches. Furthermore, cancer treatment has been addressed from various angles, including through Nanomedicine, biosensors, and targeted cancer therapy (Cluster 5) and Cancer immunotherapy and tumor microenvironment analysis (Cluster 9). In the section on engineering and drug delivery technologies, the cluster Tissue engineering, regenerative medicine, and novel drug delivery systems (Cluster 6) is located. Finally, the cluster Early detection and management of chronic, inflammatory, and metabolic diseases (Cluster 7) complements this perspective. Overall, this table shows that personalized medicine is a profound intersection of artificial intelligence, nanotechnology, and genetics with clinical applications in the targeted diagnosis and treatment of diseases, especially cancer.

Strategic position of research clusters

After forming the matrix for each of the clusters and importing it into the UCINET software, the centrality

Table 2. Names of clusters and sub-branches of the personalized medicine domain.

No.	Cluster name	Main concepts & Concept weight
1	Artificial intelligence and machine learning in medical imaging and diagnosis	Artificial intelligence (AI) (562), Machine learning (ML) (502), Deep learning (304), Glioblastoma multiforme (GBM) (148), Radiomics (130), Magnetic resonance imaging (MRI) (78), Multiple sclerosis (MS) (66), Systematic review (64), Chemotherapy (56), Ovarian cancer (54), Brain cancer (48), Glioma (48), Positron emission tomography (44), Prediction model (44), Molecular imaging (40), Neural networks (40), Alzheimer's disease (38), Prediction (38), Internet of Things (IoT) (36), Convolutional neural network (CNN) (34), Survival (32), Classification (30), Parkinson's disease (30), Vaccine (30), Oral cancer (28), Overall survival (28), Radiotherapy (26), Data mining (24), Diagnostic imaging (24), Healthcare (22), Prostate cancer (20), Recurrence (20), Nuclear medicine (14), Adverse events (12), Artificial neural network (ANN) (12), Medical education (8),
2	Gene editing, omics technologies, and the role of microbiome in treatment	Cancer therapy (128), Gene editing (94), Gene Therapy (94), Proteomics (82), CRISPR-Cas9 (76), Metabolomics (72), Genomics (66), Clinical translation (64), CRISPR (60), Iran (60), Meta-analysis (54), Microbiota (54), Early diagnosis (44), Microbiome (38), Probiotics (38), Dysbiosis (34), Gut microbiota (34), Neurological disorders (26), Bipolar disorder (24), Neurodegenerative diseases (24), Transcriptomics (24), Heart failure (20), Attention-deficit/hyperactivity disorder (ADHD) (18), Metabolic syndrome (18), Sex differences (18), Neuroimaging (14), Stem cell therapy (12), Autism (8), Schizophrenia (8), Decision Making (6), Risk factors (6),
3	Personalized medicine and pharmacogenomics	Personalized medicine (1380), Pharmacogenomics (142), Treatment (130), Covid-19 (78), Next generation sequencing (NGS) (74), Diabetes mellitus (70), Single nucleotide polymorphism (60), Oncology (50), Epilepsy (40), Oxidative stress (40), Sars-cov-2 (40), Bioinformatics (36), Polymorphism (36), Drug repurposing (32), Genetic (32), Genetic variation (30), Coronary artery disease (28), Gene (28), Seizure (28), Variant (28), Exome sequencing (26), Gene expression (26), Outcome (22), Genetics (20), Gwas (18), Infertility (18), Endometriosis (12), Systems biology (12), Attitude (8),
4	Biomarkers and liquid biopsy in cancer diagnosis	Biomarker (548), MicroRNA (288), Diagnosis (262), Colorectal cancer (200), Exosome (170), Prognosis (152), Long non-coding RNAS (LNCRNAS) (122), Gastric cancer (102), Non-coding RNAS (98), Liquid biopsy (88), Extracellular vesicles (EVS) (84), Drug resistance (76), Circulating tumor cells (CTCS) (72), Hematologic malignancies (68), Chemoresistance (50), Leukemia (44), Pancreatic cancer (42), Therapeutic targets (34), Circrna (30), Multi-omics (30), Combination therapy (28), Acute myeloid leukemia (24), Diagnostic (22), Signaling pathways (20), Cost-effectiveness (8),
5	Nanomedicine, biosensors, and targeted cancer therapy	Cancer (422), Breast cancer (252), Nanomedicine (152), Targeted therapy (142), Biosensor (138), Non-small cell lung cancer (128), Nanotechnology (118), Theranostics (106), Cancer diagnosis (40), Point-of-care (30), Cancer immunotherapy (28), Detection (28), Tumor (28), Curcumin (24), Nanobiosensors (24), Cancer biomarkers (20), Doxorubicin (20), Nanomaterials (20), Nanosensors (20), Photothermal therapy (20), Biosensing (16), Photodynamic therapy (16), Melanoma (14),
6	Tissue engineering, regenerative medicine, and novel drug delivery systems	Drug delivery (288), Regenerative medicine (154), Tissue engineering (148), Microfluidics (92), 3D printing (84), Biomaterial (78), Stem cell (74), Organoid (68), 3D bioprinting (60), Cell therapy (58), Mesenchymal stem cells (52), Gene delivery (48), Organ-on-a-chip (46), Drug discovery (42), Chitosan (38), Bone tissue engineering (24), Drug screening (24), Hydrogels (24), Scaffold (24), Biocompatibility (22), Regeneration (18), Electrospinning (10),
7	Early detection and management of chronic, inflammatory, and metabolic diseases	Inflammation (70), Cardiovascular disease (66), Autoimmune disease (56), Early detection (54), Obesity (50), Clinical trials (48), Depression (44), Imaging (40), Inflammatory bowel disease (40), Type 2 diabetes (38), Natural language processing (32), Bariatric surgery (28), Mental health (28), Neoplasms (28), Therapeutics (28), Bibliometric analysis (24), Anxiety (22), Rheumatoid arthritis (22), Metabolism (20), Prevention (18), Psoriasis (18), Chronic obstructive pulmonary disease (16),
8	Nanoparticles, epigenetics, and integrative medicine approaches (traditional medicine and digital health)	Nanoparticle (204), Epigenetics (102), Targeted drug delivery (70), DNA methylation (36), Temperament (34), Controlled drug release (30), Persian Medicine (30), Digital Health (26), Surgery (24), Traditional medicine (22), Medicine (20), Qualitative research (18), Asthma (16), Biomedical applications (16), Assisted reproductive technologies (ART) (14), Male infertility (12), Stroke (12), Wound healing (12), Mhealth (8), Case report (6), Rehabilitation (6),
9	Cancer immunotherapy and tumor microenvironment analysis	Immunotherapy (274), Tumor microenvironment (124), Cancer treatment (86), Apoptosis (64), Angiogenesis (58), Immune checkpoint inhibitors (52), Metastasis (52), Triple-negative breast cancer (50), Single-cell sequencing (48), CAR-T cell (42), Therapy (42), Hepatocellular carcinoma (38), Autophagy (36), PD-L1 (34), Cancer vaccine (32), Nanocarriers (28), Tumorigenesis (26), Monoclonal antibodies (22), Solid tumors (20),

and density scores of the clusters were determined, and the strategic diagram was plotted. It should be noted that the origin of the diagram was set at 12.7 and 0.520 based on the average centrality and density of the clusters, respectively. The density and centrality scores of the clusters are shown in Table 3.

Cluster 4 has the highest centrality with a value of 23.36 and the highest density with a value of 0.973. This means that there is a strong relationship within the co-occurrence network of concepts in this cluster,

and also, the highest centrality in terms of influence, connection with other topics, and linkage exists in Cluster 4.

As seen in Diagram 1, the strategic diagram is divided into four parts, each forming a quadrant of the diagram. Clusters located in the first quadrant are cohesive and central to the researched field. These main clusters focus on a large part of the network. Clusters in the second quadrant are still cohesive but have lost centrality, each representing smaller, specialized parts of the researched field. In the third quadrant, clusters dissipate; the clusters in this quadrant are the emerging or declining parts of the network, and finally, the fourth quadrant contains clusters that have not yet matured but have the potential to become main parts (24, 25).

According to the strategic diagram, in the first quadrant, the clusters Artificial Intelligence and Machine Learning in Medical Imaging and Diagnosis (1), Biomarkers and Liquid Biopsy in Cancer Diagnosis (4), and Tissue Engineering, Regenerative Medicine, and Novel Drug Delivery Systems (6) are the main thematic clusters of this field. These clusters are cohesive, central to the researched field, and focus on a large part of the network.

The cluster Cancer Immunotherapy and Tumor Microenvironment Analysis (9) is located in the second quadrant of the strategic diagram. The clusters Gene Editing, Omics Technologies, and the Role of Microbiome in Treatment (2), Nanomedicine, Biosensors, and Targeted Cancer Therapy (5), Early Detection and Management of Chronic, Inflammatory, and Metabolic Diseases (7), and Nanoparticles, Epigenetics, and Integrative Medicine Approaches (Traditional Medicine and Digital Health) (8) are the emerging or declining parts of the network. The fourth quadrant displays clusters that have not yet matured but have the potential to become main parts; in this study, the cluster Personalized Medicine and Pharmacogenomics (3) is placed in this section.

DISCUSSION

Based on the data in Table 3 and the strategic

Table 3. Density and centrality of clusters resulting from the co-word analysis of the personalized medicine domain.

No.	Cluster name	Density	Centrality
1	Artificial Intelligence and Machine Learning in Medical Imaging and Diagnosis	21.56	0.602
2	Gene Editing, Omics Technologies, and the Role of Microbiome in Treatment	7.806	0.26
3	Personalized Medicine and Pharmacogenomics	13.793	0.493
4	Biomarkers and Liquid Biopsy in Cancer Diagnosis	23.36	0.973
5	Nanomedicine, Biosensors, and Targeted Cancer Therapy	10.957	0.498
6	Tissue Engineering, Regenerative Medicine, and Novel Drug Delivery Systems	18.636	0.887
7	Early Detection and Management of Chronic, Inflammatory, and Metabolic Diseases	4.909	0.234
8	Nanoparticles, Epigenetics, and Integrative Medicine Approaches (Traditional Medicine and Digital Health)	4.381	0.219
9	Cancer Immunotherapy and Tumor Microenvironment Analysis	9.368	0.52

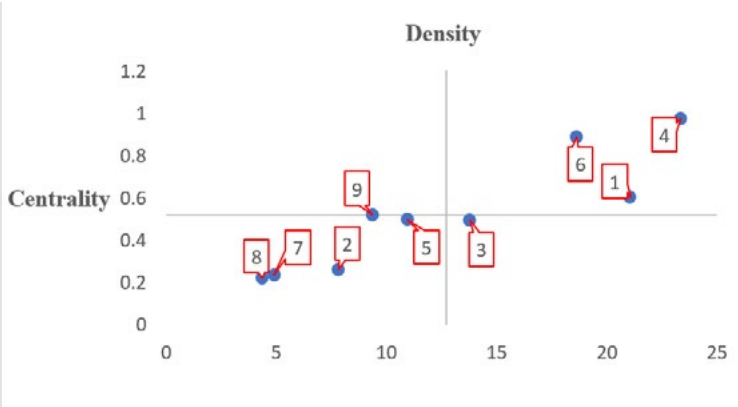


Diagram 1. Strategic diagram of the personalized medicine domain.

diagram, the analysis and discussion regarding the four quadrants are presented as follows:

The analysis of the strategic diagram shows that the first quadrant hosts three pivotal clusters: Artificial Intelligence and Machine Learning in Medical Imaging and Diagnosis (1), Biomarkers and Liquid Biopsy in Cancer Diagnosis (4), and Tissue Engineering, Regenerative Medicine, and Novel Drug Delivery Systems (6). The placement of these clusters in the first quadrant means that their density and centrality scores are above average, and as a result, these three domains are recognized as the cohesive, developed, and primary linking core in the co-word network of personalized medicine. Among these, Cluster 4, with the highest centrality and high density, is the most pivotal and cohesive research core of the entire field. The high weight of concepts such as Biomarker (548), MicroRNA (288), and Liquid biopsy (88) indicates that minimally invasive and molecular diagnostic methods, especially in colorectal and gastric cancers, form the main foundation of personalized medicine knowledge and have established the highest connection rate with other concepts in the network. On the other hand, Cluster 1, with a centrality of (21.056) and a density of (0.602), plays the computational and algorithmic arm role of this field. The prominent presence of concepts such as artificial intelligence, machine learning, deep learning, and Radiomics in this cluster is evidence that intelligent processing of imaging data (such as MRI and PET) in the diagnosis of intractable diseases has become a mainstream, mature, and structured stream in research. Finally, Cluster 6, with a density of 0.887 and a centrality of 18.636, completes the therapeutic and applied aspect of this quadrant. Concepts such as Drug delivery, Regenerative medicine, Tissue engineering, and emerging technologies like 3D bioprinting and Organ-on-a-chip demonstrate that therapeutic approaches based on personalized biological structures have achieved high theoretical and practical cohesion. In other words, the main foundations and drivers of personalized medicine rest on the triangle of advanced molecular diagnosis (Cluster 4), intelligent analysis of imaging data (Cluster 1), and targeted therapeutic design and tissue regeneration (Cluster 6), and these three domains constitute the mainstream drivers of knowledge in this arena.

The second quadrant only hosts Cluster 9, titled (Cancer Immunotherapy and Tumor Microenvironment Analysis). Its placement in this quadrant indicates that this cluster is in a good position in terms of internal cohesion and structural development, but compared to the clusters in the first quadrant, it has less centrality and linking power with other macro-domains of the network. In other words, this cluster represents a highly specialized, mature, and self-sufficient research domain in personalized medicine, where the topics within it are highly interdependent, However, its transversal connections with the entire network are not yet as extensive as the main clusters. Analyzing the prominent sub-branches of this cluster in Table 2 clearly elucidates the reason for this position. Concepts such as Immunotherapy, Tumor microenvironment, Immune checkpoint inhibitors, CAR-T cells, and PD-L1 indicate a very specific and focused knowledge chain on the molecular biology and immunology of cancer. These concepts form highly advanced and targeted therapeutic approaches that primarily focus on the mechanisms of inhibiting or stimulating the immune system against specific tumors. Although advanced technologies like Single-cell sequencing and cancer vaccines are present in this cluster, their function is more directed toward solving intra-domain problems of immunotherapy rather than linking disparate sections of the network. Therefore, Cluster 9 acts as a specialized and prominent niche in novel cancer treatments, which, while possessing a cohesive and well-established research literature, plays a specialized and separate part in the comprehensive map of personalized medicine.

The third quadrant encompasses clusters that have scored lower than the network average in both centrality and density. In bibliometric analyses, this quadrant represents emerging or declining topics; domains that, on the one hand, have not yet achieved internal and structural cohesion, and on the other hand, have not established extensive connections and influence over the entire personalized medicine network. In this study, four clusters (Clusters 2, 5, 7, and 8) are located in this quadrant, indicating the breadth of developing or interdisciplinary topics. The cluster Nanomedicine, Biosensors, and Targeted Cancer Therapy (5) is on the verge of entering the mature clusters in the first quadrant. The presence of widely used concepts such as Theranostics, Biosensor, and Nanotechnology indicates that this domain is a key and promising emerging technology that is rapidly growing and moving towards becoming one of the main pillars of the field. Cluster 2 (Gene Editing, Omics Technologies, and the Role of Microbiome in Treatment), despite the high appeal and importance of concepts such as Gene editing, CRISPR-Cas9, and Microbiome, has low density and limited centrality. This indicates that the clinical applications of CRISPR and the microbiome in personalized medicine have not yet turned into an integrated and structured paradigm, and research in this domain is scattered across numerous branches. Cluster 7 (Early Detection and Management of Chronic, Inflammatory, and Metabolic Diseases), due to covering a wide range of heterogeneous diseases (such as cardiovascular diseases, obesity, depression, and autoimmune diseases), has not been able to create high internal cohesion and is positioned as a general

and scattered domain on the periphery of the network. Cluster 8 (Nanoparticles, Epigenetics, and Integrative Medicine Approaches (Traditional Medicine and Digital Health)) has the weakest centrality and density scores in the entire network. The combination of heterogeneous topics like Epigenetics, Persian Medicine, and Digital Health in this cluster indicates a nascent, interdisciplinary, and peripheral domain that has not yet been able to find a clear structural position in the mainstream literature of personalized medicine. In other words, the third quadrant shows that advanced concepts such as gene editing, epigenetics, the microbiome, and nanomedicine, despite their abundant theoretical importance, are still in the development, testing, and emergence phase, and require integrative research and focus on specific clinical applications to become mainstream and cohesive streams of knowledge.

The fourth quadrant is dedicated to clusters that have a centrality higher than the average but a density lower than it. This quadrant represents basic, transversal topics with high potential. Due to their extensive transversal connections, these clusters play a linking and key role in the network. However, due to the breadth of their concepts and the diversity of their application domains, their intra-group relations have not yet reached complete cohesion. The clusters in this quadrant are the future drivers of the network, possessing a high potential to transfer to the first quadrant in the future. In the present study, Cluster 3, titled (Personalized Medicine and Pharmacogenomics), is positioned as the sole cluster in this strategic quadrant. This cluster has a very strong influence and linking power with other sections of the network, and its density is situated very close to the average. This cluster contains the most key and frequent concept of the entire research, namely the term personalized medicine itself. In addition, the foundational tools and concepts of Pharmacogenomics, Next generation sequencing (NGS), Single nucleotide polymorphism, and Bioinformatics are located in this cluster. These genetic and omics tools are infrastructural technologies upon which all other clusters, including AI diagnostics, liquid biopsy, and immunotherapy, depend to achieve their goals; therefore, this cluster is the heart of the network and the bridge connecting various concepts. Furthermore, the presence of highly diverse and heterogeneous clinical topics alongside foundational concepts, ranging from emerging infectious diseases like Covid-19 to metabolic diseases like Diabetes mellitus, neurological diseases such as Epilepsy, cardiovascular diseases (Coronary artery disease), Infertility, and even Drug repurposing, has caused the concepts within the cluster to be dispersed across multiple clinical branches, preventing them from forming a dense internal cohesion. Overall, the position of Cluster 3 in the fourth quadrant demonstrates that pharmacogenomics and basic genetics act as the foundation and main underlying driving engine of the entire personalized medicine domain. Given the very small gap between this cluster's density and the threshold, it is expected that with further integration of bioinformatics methods and the standardization of pharmacogenomics applications in the clinic, this cluster will soon transfer to the first quadrant and be recognized as the most established main pillar of this field.

CONCLUSION

The research results indicate that the personalized medicine domain possesses a dynamic, four-part, multidimensional, and evolving structure; such that pharmacogenomics and basic genetics (Cluster 3 in the fourth quadrant), as the foundation and underlying driving engine, feed the entire network and provide the necessary substrate for the establishment of the field's driving triangle, namely minimally invasive molecular diagnostics (Cluster 4), intelligent AI-based imaging analysis (Cluster 1), and regenerative medicine and novel drug delivery (Cluster 6) in the first quadrant. Simultaneously, the domain of immunotherapy and tumor microenvironment (Cluster 9 in the second quadrant), as a specialized, mature, and self-sufficient hub, undertakes the advancement of targeted cancer treatments, while highly promising domains such as nanomedicine, gene editing (CRISPR-Cas9), epigenetics, and the microbiome (Clusters 2, 5, 7, and 8 in the third quadrant), although currently having an emerging, developing, and somewhat scattered nature, shape the frontiers of knowledge and the future development paths of this field. This structural perspective proves that the future and main leap of personalized medicine relies on the interdisciplinary integration of the pharmacogenomics substrate with artificial intelligence algorithms and emerging diagnostic-therapeutic technologies, so that existing knowledge can transition from the research phase to tangible and integrated clinical applications.

Study Limitations

A key limitation of this study is its exclusive reliance on the Web of Science database for data retrieval. Consequently, relevant publications indexed in other major international and regional databases, such as PubMed, Scopus, and the Islamic World Science Citation Center (ISC), were not included, which may exclude pertinent domestic studies published outside WoS.

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Availability of data and materials

The data of study are available upon reasonable request.

Conflicts of interest

The author declares that there is no conflict of interest in this article.

Ethics Approval and Consent to Participate

This study was a bibliometric analysis based on publicly available secondary data extracted from the ISC database and did not involve human or animal subjects. Therefore, ethical approval and informed consent to participate were not required.

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Integrating Culture and Susceptibility Testing with Inflammatory Biomarkers for Personalized Antibiotic Protocols

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ABSTRACT

The escalating global crisis of antimicrobial resistance (AMR) necessitates a shift from empirical antibiotic therapy toward precision medicine integrating microbiological data with host inflammatory markers. This review evaluates personalized antibiotic protocols combining culture and susceptibility testing (CAST) with dynamic biomarkers, particularly procalcitonin (PCT) and C-reactive protein (CRP). Although CAST provides definitive susceptibility data, its turnaround time may delay treatment optimization. Biomarker-guided strategies offer real-time insights into host response and facilitate early de-escalation of broad-spectrum antibiotics.

We reviewed recent literature published between 2018 and 2026, focusing on high-impact studies. PCT-guided algorithms have reduced antibiotic duration by 2–5 days without increasing mortality or treatament failure across clinical settings, including community-acquired pneumonia and sepsis. Rapid molecular diagnostics and host-response transcriptomics further improve differentiation between colonization and active infection. Mathematical modeling and pharmacokinetic/pharmacodynamic (PK/PD) optimization enable individualized dosing, particularly in critically ill patients with altered drug clearance.

Clinical Decision Support Systems (CDSS) integrated with antibiotic stewardship programs enhance guideline adherence and reduce unnecessary antibiotic use. Despite challenges related to biomarker standardization, cost-effectiveness, and population validation, integrating artificial intelligence, genomics, and point-of-care diagnostics may enable continuous monitoring and proactive intervention. Ultimately, multimodal precision strategies can improve patient outcomes while reducing antimicrobial selective pressure and supporting sustainable AMR control.

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INTRODUCTION

The escalating global crisis of antimicrobial resistance (AMR) has necessitated a paradigm shift from empirical antibiotic therapy to precision medicine, emphasizing the integration of microbiological data with host inflammatory markers. While culture and susceptibility testing remain the gold standard for identifying pathogens and guiding targeted therapy, their inherent delays often compromise patient outcomes in critical care settings (1, 2). Consequently, contemporary clinical approaches increasingly advocate for the concurrent assessment of dynamic inflammatory biomarkers, such as procalcitonin and C-reactive protein, which provide real-time insights

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into the host's immune response and bacterial load (3). This multimodal strategy not only facilitates the early de-escalation of broad-spectrum antibiotics but also significantly reduces the selection pressure that drives resistant strain proliferation, thereby enhancing therapeutic efficacy and minimizing adverse drug events (4, 5).

Furthermore, the synthesis of microbiological susceptibility profiles with quantitative inflammatory markers offers a robust framework for optimizing antibiotic stewardship and improving clinical prognoses (6). Recent literature underscores that biomarker-guided algorithms can safely guide the duration of therapy, addressing the limitations of relying solely on culture results which may reflect colonization rather than active infection (7). By leveraging the predictive power of inflammatory kinetics alongside precise pathogen identification, clinicians can implement personalized regimens that balance rapid pathogen clearance with the preservation of the host microbiome (8). This integrative model represents a critical advancement in infectious disease management, promising to mitigate the morbidity and mortality associated with inappropriate empirical prescribing while fostering a more sustainable antimicrobial ecosystem (9, 10).

The Paradigm Shift from Empirical to Precision Medicine in Infectious Diseases

The global escalation of antimicrobial resistance (AMR) has rendered traditional empirical antibiotic therapy increasingly inadequate for managing severe infections. Growing evidence supports a transition toward precision medicine, where treatment decisions are driven by individual patient data rather than population averages (11). This shift is critical for reducing the selective pressure that drives the emergence of multidrug-resistant organisms, thereby preserving the efficacy of existing antibiotics. By moving away from "one-size-fits-all" approaches, clinicians can tailor interventions to specific pathogen profiles and host immune responses, potentially improving clinical outcomes in critically ill patients (12, 13).

Furthermore, the integration of advanced diagnostic technologies with clinical decision-making tools has accelerated this paradigm shift. Modern healthcare systems are increasingly adopting algorithms that combine real-time microbiological data with dynamic host markers to guide therapy. This approach not only enhances the accuracy of initial treatment but also facilitates rapid de-escalation once definitive results are available. Consequently, antibiotic stewardship is increasingly regarded as a core component of modern infectious disease management in the fight against resistant pathogens (14, 15).

Limitations of Traditional Culture and Susceptibility Testing

Despite being the gold standard, traditional culture and susceptibility testing (CAST) suffer from significant temporal delays that compromise patient care in acute settings. The process typically requires one to several days to yield definitive results, during which time patients remain on broad-spectrum empirical antibiotics. This delay increases the risk of adverse outcomes, including septic shock, multi-organ failure, and mortality, particularly in immunocompromised individuals or those with severe sepsis. Consequently, there is an urgent need for rapid diagnostic methods that can provide actionable data within hours rather than days to optimize therapeutic windows. In addition to time constraints, conventional CAST methods often fail to distinguish between colonization and active infection, leading to potential overtreatment. Many patients harbor bacteria asymptomatically (asymptomatic bacteriuria being the best-known example), yet antibiotics may be prescribed unnecessarily because of ambiguous clinical presentations. This ambiguity contributes to the indiscriminate use of broad-spectrum agents, exacerbating the global AMR crisis. Therefore, complementary diagnostic strategies that can quickly confirm active infection and guide targeted therapy are essential to overcome these inherent limitations of traditional microbiological workflows (16).

The Role of Procalcitonin in Guiding Antibiotic Therapy

Procalcitonin (PCT) has emerged as a pivotal biomarker for differentiating bacterial infections from viral or non-infectious inflammatory conditions. Its levels rise rapidly in response to systemic bacterial infections and decline quickly upon effective treatment, making it a useful dynamic marker for monitoring therapeutic response. Meta-analyses and most randomized trials have shown that PCT-guided algorithms can safely reduce antibiotic duration without increasing mortality or treatment failure rates, although results have not been uniform across settings. This biomarker provides clinicians with an objective measure to support the discontinuation of antibiotics, thereby minimizing unnecessary exposure and reducing the ecological impact of antimicrobial use (17, 18).

Moreover, PCT-guided strategies have been evaluated across various clinical settings, including acute respiratory tract infections, primary care, and sepsis in intensive care. These studies report roughly 25–35% shorter antibiotic exposure in hospital and intensive care trials and a markedly lower prescription rate in primary care, with no excess of adverse outcomes. By integrating PCT levels with clinical judgment, healthcare providers can make

more informed decisions about initiating, continuing, or stopping antibiotic therapy. This evidence-based approach enhances antibiotic stewardship efforts and contributes to the broader goal of reducing antibiotic-associated adverse effects and unnecessary antibiotic exposure (19) (Table 1).

C-Reactive Protein and Other Acute Phase Reactants

While procalcitonin is more specific for bacterial infections, C-reactive protein (CRP) remains a widely used acute phase reactant due to its availability and rapid response to inflammation. CRP levels increase within hours of an inflammatory stimulus and can provide valuable information when used in conjunction with other clinical parameters. However, its lower specificity compared to PCT necessitates careful interpretation, particularly in conditions with high baseline inflammation such as trauma or autoimmune disorders. Recent studies suggest that serial CRP measurements can be effective in monitoring treatment response, especially when combined with clinical stability criteri. Other novel inflammatory markers, such as soluble trigger receptor expressed on myeloid cells-1 (sTREM-1) and interleukin-6 (IL-6), are also gaining attention for their potential role in precision antibiotic therapy. These markers offer insights into specific immune pathways activated during infection, potentially allowing for more nuanced treatment strategies. However, the reported diagnostic accuracy of these markers has been variable, and to date none has been validated as a stand-alone guide for antibiotic therapy. Integrating these diverse inflammatory markers into clinical algorithms may provide a more comprehensive picture of the host-pathogen interaction, enabling better-informed decisions regarding antibiotic duration and intensity (20).

Rapid Molecular Diagnostics and Their Clinical Integration

The advent of rapid molecular diagnostics, such as multiplex polymerase chain reaction (PCR) panels and next-generation sequencing (NGS), has substantially improved the speed of pathogen identification. These technologies can detect specific resistance genes and virulence factors directly from clinical samples within hours, bypassing the need for prolonged culture periods. By providing immediate information on both the identity of the pathogen and its resistance profile, molecular diagnostics enable clinicians to initiate targeted therapy much earlier than traditional methods allow. This rapid turnaround time is crucial for improving outcomes in sepsis and other life-threatening infections .However, the integration of rapid molecular diagnostics into routine clinical practice faces challenges, including cost, interpretation complexity, and the risk of detecting colonizing organisms. It is essential to develop robust clinical algorithms that can distinguish between true infection and colonization when using these high-sensitivity tests. Molecular results therefore need to be interpreted in conjunction with clinical context to avoid overtreatment; combining them with inflammatory biomarkers is a logical extension of this approach. As these technologies become more accessible, their strategic implementation will be key to realizing the full potential of precision antibiotic stewardship in diverse healthcare settings (21, 22).

Host-Response Transcriptomics and Precision Profiling

Host-response transcriptomics represents a cutting-edge frontier in precision medicine, analyzing gene expression patterns to determine the nature and severity of infections. By examining the host's immune response at the molecular level, clinicians can distinguish bacterial from viral infections with high accuracy. This approach offers a comprehensive view of the host-pathogen interaction, providing insights that go beyond the presence of a specific pathogen. Recent studies have identified gene-expression endotypes in sepsis that are associated with differences in immune state and mortality, which may in future help guide treatment intensity (23, 24).

Furthermore, transcriptomic profiling may help to reserve antibiotics for patients with bacterial infection, supporting more personalized therapy. Machine learning algorithms are being developed to analyze these complex data sets and generate actionable recommendations for clinicians. This technology could reduce unnecessary antibiotic prescribing in patients with ambiguous presentations, although prospective clinical validation is still needed. If costs fall and prospective validation succeeds, host-response transcriptomics may become a standard tool in the management of severe infections (25).

Mathematical Modeling and Predictive Algorithms

Mathematical modeling and predictive algorithms are increasingly being used to optimize antibiotic dosing and duration based on patient-specific pharmacokinetic and pharmacodynamic (PK/PD) parameters. Model-informed precision dosing integrates pharmacokinetic models with patient characteristics and measured drug concentrations to individualize antibiotic doses. By predicting individual drug exposure, these tools allow clinicians to adjust therapy proactively rather than reactively. This quantitative approach enhances the precision of antibiotic stewardship and minimizes the risk of subtherapeutic dosing or unnecessary prolonged exposure (26, 27).

Additionally, artificial intelligence (AI) and machine

Table 1. Summary of Key Studies on Precision Antibiotic Stewardship Integrating Culture, Susceptibility, and Inflammatory Biomarkers (2007–2021).

Ref	Author (Year)	Study Design & Population	Intervention / Biomarker	Key Findings
4	Roberts et al. (2014)	Prospective multinational pharmacokinetic study, DALI (384 ICU patients enrolled, 68 hospitals)	Beta-lactam concentrations and PK/PD target attainment (50% fT>MIC)	16% of patients treated for infection did not reach the 50% fT>MIC target; they were 32% less likely to have a positive clinical outcome (OR 0.68, p = 0.009).
6	Cals et al. (2009)	Cluster randomized trial (primary care, lower respiratory tract infections)	Point-of-care CRP testing and/or communication skills training	Antibiotic prescribing at the index consultation was 31% with CRP testing vs. 53% without, 27% with training vs. 54% without, and 23% with both.
7	Briel et al. (2008)	Randomized trial (458 patients with acute respiratory tract infections, 53 primary care physicians)	PCT-guided approach vs. standard approach	Antibiotic prescription rate was 72% lower with PCT guidance, without an excess of restricted-activity days (mean increase 0.14 days, 95% CI −0.53 to 0.81).
13	Schuetz et al. (2018)	Individual patient data meta-analysis (acute respiratory infections; 6,708 patients)	Procalcitonin (PCT)-guided antibiotic treatment vs. usual care	30-day mortality was lower with PCT guidance (adjusted OR 0.83, 95% CI 0.70–0.99), and antibiotic exposure was shortened by about 2.4 days (8.1 to 5.7 days).
14	Schuetz et al. (2017)	Cochrane individual participant data meta-analysis (26 trials, 6,708 adults with acute respiratory tract infections)	PCT-guided initiation or discontinuation of antibiotics vs. usual care	Antibiotic exposure fell from 8.1 to 5.7 days; 30-day mortality was 8.6% vs. 10.0% (adjusted OR 0.83) and treatment failure did not differ significantly (23.0% vs. 24.9%).
17	Evans et al. (2021)	International guideline (adults with sepsis or septic shock)	PCT plus clinical evaluation to guide antimicrobial discontinuation	Suggests (weak recommendation, low quality of evidence) using PCT together with clinical evaluation, rather than clinical evaluation alone, to decide when to stop antimicrobials when the optimal duration is unclear.
18	Huang et al. (2018)	Multicenter randomized trial, ProACT (1,656 patients with suspected lower respiratory tract infection, 14 US hospitals)	PCT-guided care vs. usual care	No difference in antibiotic days (4.2 vs. 4.3) and no significant difference in adverse events (11.0% vs. 13.1%).
19	Abdul-Aziz et al. (2020)	Position paper / expert consensus (critically ill adults), endorsed by ESICM, ESCMID, IATDMCT and ISAC	Therapeutic drug monitoring (TDM) of antimicrobials	Recommends routine TDM for aminoglycosides, beta-lactams, linezolid, teicoplanin, vancomycin and voriconazole, noting that conventional dosing may risk clinical failure in critically ill patients.
20	Oliveira et al. (2013)	Randomized trial (94 patients with sepsis)	PCT- vs. C-reactive protein (CRP)-guided antibiotic therapy	Median antibiotic duration did not differ significantly (7.0 vs. 6.0 days, p = 0.13) and deaths were equal (21 per group); CRP was reported to be as useful as PCT in reducing antibiotic use.
26	de Jong et al. (2016)	Randomized, controlled, open-label trial (critically ill patients; 1,575 randomized)	PCT-guided vs. standard-of-care antibiotic duration	Median antibiotic treatment lasted 5 days with PCT guidance vs. 7 days with standard care; 28-day mortality was 20% vs. 25% (intention-to-treat).
27	Prkno et al. (2013)	Systematic review and meta-analysis (7 randomized trials, 1,075 adults with severe sepsis or septic shock in ICUs)	PCT-guided therapy	Antimicrobial treatment was shortened (HR 1.27, 95% CI 1.01–1.53; about 2 days) with no significant difference in 28-day mortality (RR 1.02, 95% CI 0.85–1.23).
35	Müller et al. (2007)	Diagnostic and prognostic accuracy study (community-acquired pneumonia)	High-sensitivity CRP vs. PCT	PCT predicted bacteremia better than CRP (AUC 0.85, p = 0.01), and PCT, unlike CRP, increased with increasing pneumonia severity.
36	Schuetz et al. (2009)	Randomized controlled trial, ProHOSP (lower respiratory tract infections)	PCT-based guidelines vs. standard guidelines	Mean antibiotic exposure was 5.7 vs. 8.7 days (−34.8%); composite adverse outcomes were similar (15.4% vs. 18.9%) and antibiotic-associated adverse effects were lower (19.8% vs. 28.1%).

learning algorithms are being trained on large clinical datasets to identify patterns that predict antibiotic resistance and treatment success. These systems can analyze vast amounts of electronic health record data to recommend optimal regimens tailored to individual patients. Recent systematic reviews show

that machine-learning models can predict sepsis onset with high accuracy on retrospective data, although few have been implemented clinically. The integration of these computational tools into clinical decision support systems may improve how antibiotic therapies are prescribed and monitored in real time (28, 29, 30).

Pharmacokinetic/Pharmacodynamic Optimization in Critical Care

Optimizing antibiotic dosing based on pharmacokinetic/pharmacodynamic (PK/PD) principles is essential for maximizing efficacy and minimizing toxicity, particularly in critically ill patients with altered physiology. Conditions such as sepsis, acute kidney injury, and hypoalbuminemia significantly affect drug distribution and clearance, leading to unpredictable drug levels. Therapeutic drug monitoring (TDM) allows for the adjustment of doses to achieve target PK/PD indices, ensuring adequate exposure at the site of infection. Recent studies emphasize the importance of individualized dosing strategies to overcome resistance mechanisms and improve clinical outcomes in severe infections.

Furthermore, the integration of rapid susceptibility data with PK/PD modeling enables the selection of the most effective antibiotic regimen for each patient. This approach considers factors such as minimum inhibitory concentration (MIC) values, drug half-life, and tissue penetration to optimize dosing intervals and amounts. Advanced monitoring techniques, including real-time bio-sensors and microdialysis, are being explored to provide more accurate measurements of drug concentrations at infection sites. These innovations represent a step toward more personalized antibiotic therapy (31).

Clinical Decision Support Systems and Implementation Strategies

The successful implementation of precision antibiotic protocols requires robust clinical decision support systems (CDSS) integrated into electronic health records. These systems provide real-time alerts and recommendations based on patient data, culture results, and guideline algorithms, assisting clinicians in making informed decisions. However, the effectiveness of CDSS depends on user acceptance, workflow integration, and the quality of underlying data. Recent studies highlight the importance of designing intuitive interfaces and providing ongoing education to ensure that healthcare providers utilize these tools effectively in their daily practice.

Moreover, organizational strategies such as antibiotic stewardship programs (ASPs) play a crucial role in supporting the adoption of precision medicine approaches. These programs involve multidisciplinary teams of physicians, pharmacists, and microbiologists who collaborate to monitor antibiotic use and provide feedback. Evidence suggests that computerised decision support and stewardship interventions can improve the adequacy of antibiotic coverage and adherence to prescribing policies, shorten antibiotic treatment, and do not appear to increase mortality. Successful implementation requires a culture of safety and continuous quality improvement, where data-driven insights are used to refine protocols and enhance the overall quality of care in infectious disease management (32).

Future Directions and Challenges in Personalized Antibiotic Therapy

Despite significant advancements, several challenges remain in the widespread adoption of personalized antibiotic protocols. Issues such as standardization of biomarker thresholds, cost-effectiveness, and equity in access to advanced diagnostics must be addressed. Additionally, there is a need for large-scale randomized controlled trials to validate new algorithms and technologies across diverse populations. Future research should focus on developing cost-effective rapid diagnostic tools and integrating multi-omics data to create comprehensive predictive models. Addressing these challenges will be essential for realizing the full potential of precision medicine in combating AMR (33).

Looking ahead, the convergence of artificial intelligence, genomics, and point-of-care diagnostics holds promise for transforming infectious disease management. Emerging technologies, such as CRISPR-based diagnostics and wearable biosensors, may enable continuous monitoring of infection status and treatment response. These innovations could facilitate a shift from reactive to proactive care, allowing for early intervention and prevention of complications. As the field evolves, collaboration between clinicians, researchers, and industry partners will be vital to developing sustainable solutions that improve global health outcomes and preserve the efficacy of antibiotics for future generations (34).

CONCLUSION

The transition from empirical to precision antibiotic therapy represents a critical advancement in combating the escalating threat of antimicrobial resistance. By synergizing the definitive diagnostic power of culture and susceptibility testing with the dynamic, real-time insights provided by inflammatory biomarkers such as procalcitonin and C-reactive protein, clinicians can achieve a more nuanced and accurate management of infectious diseases.This integrated approach not only facilitates the safe and early de-escalation of broad-spectrum antibiotics, thereby reducing the selective pressure

that drives resistant pathogen proliferation, but also optimizes patient outcomes by minimizing antibiotic-related adverse effects.

Furthermore, the incorporation of advanced technologies, including rapid molecular diagnostics, host-response transcriptomics, and artificial intelligence-driven clinical decision support systems, holds immense potential to overcome the inherent limitations of traditional diagnostic workflows. These innovations may enable personalized dosing strategies based on individual pharmacokinetic profiles, and shorter antibiotic exposure may help limit disruption of the host microbiome. Despite these promising developments, widespread implementation requires addressing challenges related to cost, standardization, and healthcare infrastructure. Future research must prioritize large-scale randomized trials to validate these multimodal algorithms across diverse clinical settings. Ultimately, fostering a collaborative environment among clinicians, microbiologists, and researchers is essential to sustainably integrate precision stewardship into routine practice, safeguarding the efficacy of existing antibiotics for future generations.

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Reyhaneh Mohammadi Gandom Abad conceived the review, performed the literature search and analysis, drafted the manuscript, and read and approved the final version.

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Ethics Approval and Consent to Participate

Not applicable. This review is based on previously published literature and did not involve human participants, human data, or animals.

Consent for Publication

Not applicable.

Competing Interests

The author declares that there are no competing interests.

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