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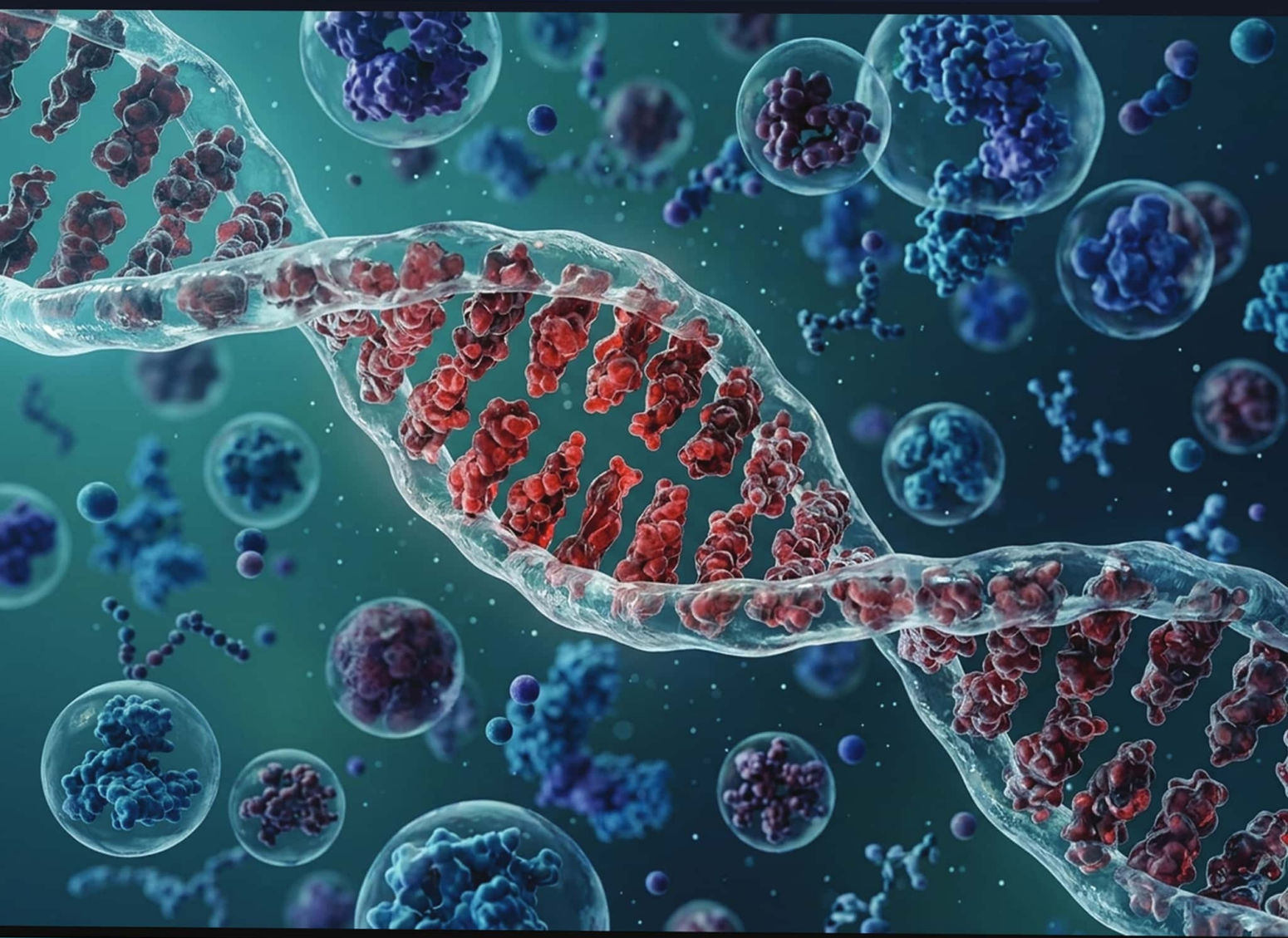

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Association of FGFR2 Gene Polymorphisms (rs2981582 and rs1219648) with Breast Cancer Susceptibility in Iranian Women: A Case-Control Study with Haplotype and Expression Analysis

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ABSTRACT

Background: Breast cancer is the most common malignancy among women in Iran, characterized by a relatively early age of onset and a rising incidence rate. The single-nucleotide polymorphisms rs2981582 and rs1219648, located in intron 2 of the *FGFR2* gene, have been linked to breast cancer susceptibility in genome-wide association studies (GWAS). Nevertheless, their significance in the Iranian population has not been extensively investigated.

This study investigates the association of *FGFR2* polymorphisms (rs2981582 and rs1219648) with breast cancer risk in Iranian women, alongside haplotype interactions and gene expression profiling.

Methods: A case-control study was conducted with 160 participants (80 cases and 80 age-matched controls). *FGFR2* SNPs were genotyped with PCR-RFLP. Chi-square tests were used to analyze associations of haplotypes. *FGFR2* expression was evaluated in breast cancer subtypes using GEO (GDS2635, GDS3853) and Expression Atlas datasets. Statistical analyses were carried out using SPSS version 22.0 (IBM Corp., Armonk, NY, USA), with statistical significance defined as $P<0.05$. Hardy–Weinberg equilibrium (HWE) was verified for both SNPs in the control group ($P>0.05$).

Results: The TT genotype of rs2981582 was significantly associated with increased breast cancer risk ($P=0.00$; OR=3.566). No independent association was found for rs1219648 ($P>0.05$). Haplotypes AC and AT were significantly associated with elevated risk ($P=0.004$ and $P=0.001$, respectively). *FGFR2* expression was upregulated in lobular carcinoma and downregulated in ductal carcinoma compared to healthy controls ($P<0.05$).

Conclusion: The rs2981582 TT genotype and specific haplotypes (AC, AT) are associated with increased breast cancer risk in Iranian women, supporting *FGFR2* as a potential biomarker for early detection and personalized risk assessment in this population.

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INTRODUCTION

Breast cancer continues to be the most commonly diagnosed malignancy and the primary cause of cancer-related mortality among women globally, with more than 2.3 million new cases and nearly 685,000 deaths reported annually according to GLOBOCAN 2020.

Despite improvements in screening and treatment worldwide, the burden of this disease is increasing rapidly in low- and middle-income countries, including Iran, where it represents the most prevalent cancer among women and shows a concerning pattern of earlier onset compared to Western populations (1).

In recent years, growing attention has been directed toward genetic susceptibility factors associated with the risk of Breast cancer, particularly single-nucleotide polymorphisms (SNPs). Intronic variants in the *FGFR2* gene have been identified as significant contributors in several population-based genome-wide association studies (GWAS) (2, 3). The *FGFR2* gene encodes a receptor tyrosine kinase involved in regulating cell proliferation, differentiation, and angiogenesis, and disruption of these processes can contribute to tumor development. Notably, the SNPs rs2981582 and rs1219648, located within intron 2 of the *FGFR2* gene, have been associated with alterations in transcription factor binding and gene expression, thereby increasing susceptibility to breast cancer (2).

Despite a vast number of association studies being conducted around the world, the Iranian population has not been notably represented in genetic epidemiology studies. Initial studies from regional locations, such as Northern and Azeri Iranian subpopulation studies, provided inconsistent or insufficient evidence for a role of *FGFR2* SNPs in susceptibility to breast cancer (4, 5).

Furthermore, there is a paucity of data integrating SNP analysis with gene expression profiles to explore the functional consequences of these variants.

This study aims to investigate the role of relevant *FGFR2* polymorphisms (rs2981582 and rs1219648) in breast cancer risk in an Iranian female population. This study also analyses the expression patterns of *FGFR2* in different histological subtypes of breast cancers by way of bioinformatics datasets. Our findings can provide insight into the understanding of genetic risk factors for Iranian women and can help develop population-specific markers for early detection.

MATERIALS AND METHODS

Study Design and Participants

A case-control study was conducted with 160 Iranian women (80 breast cancer cases and 80 age-matched controls) recruited from hospitals in Markazi province. The cases were histologically confirmed breast cancer patients, and the controls were asymptomatic women with no personal or family history of cancer. Inclusion criteria for cases included a confirmed diagnosis of primary breast cancer, while controls were excluded if they had any history of malignancy or family history of breast cancer. Ethical approval was obtained from the Ethics Committee of Arak University (IR.ARAKMU.REC.1395.288), and written informed consent was provided by all participants.

Sample Collection and DNA Extraction

Peripheral blood samples (5 mL) were obtained and collected into EDTA-containing tubes. Genomic DNA was isolated using the YTA Genomic DNA Extraction Mini Kit (Yekta Tajhiz Azma, Iran) in accordance with the manufacturer's instructions. DNA concentration and purity were evaluated using UV spectrophotometry (Nanodrop), and its integrity was confirmed by electrophoresis on a 2% agarose gel.

Genotyping of *FGFR2* SNPs

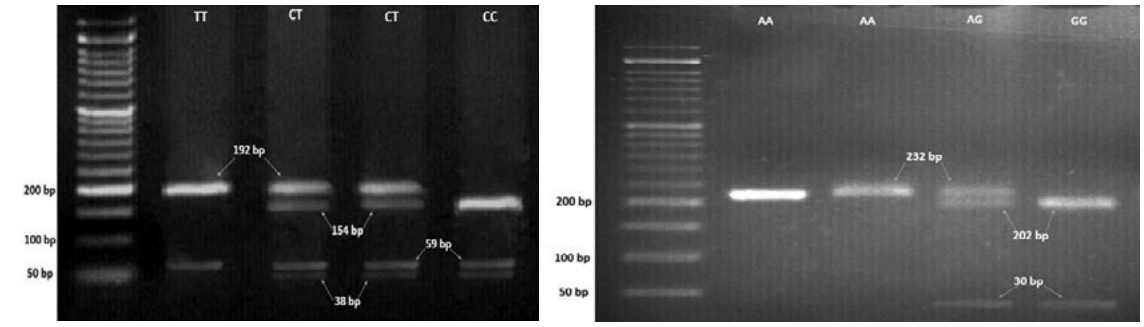
SNPs rs2981582 (T>C) and rs1219648 (A>G) in intron 2 of *FGFR2* were genotyped using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP). PCR amplification was performed in a 25 µL reaction volume containing genomic DNA, primers, dNTPs, buffer, $MgCl_2$, and Taq polymerase. Thermal cycling was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of 95°C for 30 seconds, 58°C (rs2981582) or 60°C (rs1219648) for 30 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 7 minutes. Primers (Table 1) were validated using the NCBI Primer-BLAST. PCR products were digested with *AciI* (rs2981582) and *LguI* (rs1219648) at 37°C for 16 hours, then resolved on a 2% agarose gel and visualized under UV illumination (Figure 1). Genotype distributions in the control group were assessed for Hardy–Weinberg equilibrium using a chi-square test.

Gene Expression Analysis

Public bioinformatics platforms yielded gene expression data that allows for *FGFR2* expression to

Table 1. Sequence of primers used in the amplification of the FGFR2 gene polymorphisms

Gene	SNP	Primer Seq	Product size (bp)
FGFR2	rs1219648	F: 5'-ACGCCTATTTTACTTGACACGC R: 5'- GCTGGACAGGTCATTGTGGTG	232
FGFR2	rs2981582	F: 5'- CCCTTTGGAGACAACGTGAGC R: 5'- GCACGAGATGTGTTCCCAGAG	251



(A) rs2981582: TT genotype yields an uncut 192 and 59 bp band; TC genotype shows 192, 154, 59, and 38 bp bands; CC genotype shows 154, 59 and 38 bp bands.

(B) rs1219648: AA genotype remains uncut at 232 bp; AG genotype shows 232, 202, and 30 bp bands; GG genotype displays 202 and 30 bp bands.

Fig 1. Agarose gel electrophoresis patterns of sample RFLP-PCR products for SNPs in the FGFR2 gene

be characterized across different histological subtypes of breast cancer.

This was accomplished by obtaining GEO Datasets (GDS2635 and GDS3853) through NCBI GEO, and also from the Expression Atlas (EBI) for profiling *FGFR2* expression in normal and tumor breast tissues.

Expression levels were compared in ductal and lobular subtypes and in healthy reference individuals.

Statistical Analysis

Genotype and allele distributions were compared using chi-square tests. Odds ratios (ORs) along with 95% confidence intervals (CIs) were calculated to evaluate the strength of associations. Multivariate logistic regression analysis was applied to control for potential confounding factors, including age, menopausal status, family history, smoking, and oral contraceptive use. Haplotype frequencies were estimated using Haploview. Power analysis performed with G*Power indicated that the sample size (n=160) achieved 80% power to detect an OR of 3.5 at α=0.05. Statistical analyses were conducted using SPSS version 22.0 (IBM Corp., Armonk, NY, USA), with P<0.05 considered statistically significant.

RESULTS

Demographic Characteristics of Study Participants

Demographic analysis (Table 2) showed that cases had a slightly higher mean age (49.8 ± 9.1 years) than controls (47.2 ± 8.7 years; P=0.07). Cases were more likely to be postmenopausal (75.0% vs. 60.0%, P=0.03), have a family history of breast cancer (33.8% vs. 13.8%, P=0.004), lower education levels (≤ high school: 82.5% vs. 56.3%, P=0.001), smoking history (18.8% vs. 7.5%, P=0.03), and oral contraceptive use (65.0% vs. 50.0%, P=0.05).

Genotypic and Allelic Frequencies

Genotype frequencies for rs2981582 and rs1219648 were in Hardy-Weinberg equilibrium in controls (P=0.13 and P=0.65, respectively). The TT genotype of rs2981582 was significantly more frequent in cases

Table 2. Investigating the relationship between clinical variables and the risk of breast cancer

Variable	Cases (n=80)	Controls (n=80)	P-value
Mean Age (years)	49.8 ± 9.1	47.2 ± 8.7	0.07
Postmenopausal (%)	60 (75.0%)	48 (60.0%)	0.03
Family History of BC (%)	27 (33.8%)	11 (13.8%)	0.004
Education Level ≤ High School (%)	66 (82.5%)	45 (56.3%)	0.001
Smoking (%)	15 (18.8%)	6 (7.5%)	0.03
Oral Contraceptive Use (%)	52 (65.0%)	40 (50.0%)	0.05

(52.5%) than controls (26.3%), with a strong association with breast cancer risk (P=0.001; OR=3.566, 95% CI: 1.519-8.005). After adjusting for confounders (age, menopausal status, family history, smoking, and oral contraceptive use), the association remained significant (adjusted OR=3.20, 95% CI: 1.40-7.80, P=0.002). No significant association was observed for rs1219648 genotypes (P>0.05). Allelic analysis showed that the T allele of rs2981582 was significantly associated with increased breast cancer risk, while the G allele of rs1219648 showed no significant difference (Table 3). Analysis of recessive and dominant models for rs2981582 confirmed a stronger association in the recessive (TT vs. TC+CC: OR=3.56, 95% CI: 1.58- 8, P=0.002) and Dominant (CC vs. TC + TT: OR=3.8, 95% CI: 1.92-7.54, P=0.00) models. Agarose gel electrophoresis patterns of PCR-RFLP products are shown in Figure 1.

In Figure 1, agarose gel electrophoresis patterns of sample RFLP-PCR products for SNPs in the *FGFR2* gene are shown.

Haplotype Analysis

Haplotype frequencies were estimated using Haploview software. Four haplotypes (AC, GC, AT, GT) were identified, with AC (frequency: 0.42 in cases vs. 0.28 in controls) and AT (frequency: 0.35 in cases vs. 0.20 in controls) showing significant associations with increased breast cancer risk (P=0.004 and P=0.001, respectively; Table 4). The overall haplotype distribution differed significantly between cases and controls (χ^2=21.914, df=3, P=0.00).

Table 3. Study of genotypic and allelic frequencies of the FGFR2 gene SNPs in patient and control groups

SNP	Genotype	Cases (n=80)	Controls (n=80)	OR (95%CI)	P-value	HWB
rs2981582	CC	18 (22.5%)	42 (52.5%)	-	-	0.13
	TC	35 (43.8%)	28 (35%)	2.91 (1.38-6.13)	**0.004**	
	TT	27 (33.7%)	10 (12.5%)	3.566 (1.519-8.005)	**0.00**1	
	TT vs	27 (22.5%)	10 (12.5)	3.56 (1.58- 8)	**0.002**	
	TC + CC	53 (77.5%)	70 (87.5%)			
	CC vs	18 (22.5%)	42 (52.5%)	3.8 (1.92-7.54)	**0.00**	
	TC + TT	62 (77.5%)	38 (47.5%)			
rs1219648	AA	33 (41.3%)	36 (45%)	-	-	0.65
	AG	36 (45%)	34 (42.5%)	1.15 (0.59-2.24)	0.67	
	GG	11 (13.7%)	10 (12.5%)	1.057 (0.593-2.063)	0.757	
	GG vs	11 (13.7%)	10 (12.5%)	1.11 (0.44-2.79)	0.81	
	AG + AA	69 (86.3%)	70 (87.5%)			
	AA vs	33 (41.3%)	36 (45%)	1.16 (0.62-2.17)	0.63	
	AG + GG	47 (58.7%)	44 (55%)			
	Allele					
rs2981582	T	89 (55.6%)	112 (70%)	2.925 (1.864-4.633)	**0.000**	**-**
	C	71 (44.4%)	48 (30%)	-	-	
rs1219648	A	102 (63.8%)	106 (66.3%)	-	-	
	G	58 (36.2%)	54 (33.7%)	0.860 (0.554-1.335)	0.501	

Table 4. Investigating the association between haplotypes of two SNPs studied in two groups and the risk of breast cancer

Haplotype	Chi-square	DF	P value
AC	8.205	1	0.004
GC	5.117	1	0.024
AT	11.809	1	0.000
GT	4.56	1	0.033
Total	21.914	3	0.000

DF: Degree of freedom

FGFR2 Gene Expression Analysis

Data on the expression of *FGFR2* from GEO datasets and Expression Atlas indicated a higher expression in lobular carcinoma samples than in healthy tissue, while they showed lower expression in ductal carcinoma samples. The difference was statistically significant (P=0.000 for lobular vs control; P<0.05 for ductal vs control).

DISCUSSION

Breast cancer is a multifactorial disorder shaped by both environmental and genetic factors and continues to be the most prevalent malignancy among women in Iran (5). Investigating the influence of genetic polymorphisms on cancer susceptibility is essential for enhancing early detection, risk assessment, and tailored treatment approaches. Polymorphisms in the *FGFR2* gene, specifically rs2981582 and rs1219648, are among the most extensively researched genetic markers in this area.

In the current research, we examined these two *FGFR2* polymorphisms in an Iranian female cohort and found a significant association between the TT genotype of rs2981582 and increased risk of breast cancer (P=0.001; OR=3.566). In contrast, rs1219648 was not significantly associated with the disease. Notably, haplotype analysis demonstrated a significant relationship between specific haplotypes and susceptibility to Breast cancer, indicating a potential synergistic effect of these SNPs on disease risk.

Much research has looked at the link between *FGFR2* SNPs, especially rs2981582 and rs1219648, and the risk of breast cancer in different groups of people. Most of these studies have found similar results; however, certain differences are important for each group. Jia et al. performed a meta-analysis of over 50,000 participants and established that rs2981582 and rs1219648 were significantly linked to the susceptibility of breast cancer in Caucasian and Asian populations (6). Zhang et al. also established that several intronic SNPs, especially rs2981582, were more strongly correlated when comparing East Asian individuals with Europeans due to the possibility of variations in the frequency of alleles as well as linkage disequilibrium structure (7). The results indicate a significant correlation between the rs2981582 TT genotype and heightened breast cancer susceptibility in Iranian women. This aligns with regional studies, including Zhang et al. (2016), which identified rs2981582 as a significant *FGFR2* variant associated with breast cancer in a pooled analysis of more than 35 studies (2). Cui et al. noted that even within Asia, populations such as Chinese and Korean women exhibited variable risk estimates, highlighting the necessity for population-specific studies, such as the one conducted in Iran (8). Interestingly, we observed a discrepancy in our findings from some studies conducted in the U.S. and some studies conducted in European populations. For instance, Zanna et al. observed a potential survival benefit in male breast cancer patients linked to the rs2981582 mutation, suggesting a possible gender or subtype-specific effect (9). Similarly, Wang et al. indicated a modifying effect of hormone receptor status on the *FGFR2*-breast cancer association. With regard to rs1219648, our study found no significant association in isolation, though haplotype analysis did show that combinations involving this SNP (e.g., TG) significantly increased disease risk (10). This aligns with the findings of Yang et al. in Chinese populations, where individual SNP effects were modest but haplotypic combinations conferred higher predictive power (11). Differences between studies can be attributed to multiple factors: Genomic architecture by ethnicity and allele frequency differences, Differences in environmental exposure and lifestyle choices, including smoking and contraceptive medication use, Study design heterogeneity includes heterogeneity in sample size, use of different genotyping methods, and adjustment for confounding variables. The difference in hormone receptor subtypes may also contribute to the interaction with *FGFR2* expression pathways (10). Overall, these studies have shown that rs2981582 is

robustly and consistently associated with breast cancer risk globally and exemplifies how local haplotype-based analysis is needed with underrepresented populations such as women of Iranian origin.

The molecular basis for the link between *FGFR2* polymorphisms and breast cancer has been partly explored. *FGFR2* is a member of a receptor family exhibiting tyrosine kinase activity, implicated in cell proliferation and differentiation, and encodes a receptor that executes this function. Single nucleotide polymorphisms (SNPs), including rs2981582, are found in intron 2 of the *FGFR2* gene - a region shown previously to harbor cis-regulatory elements - and these polymorphic variations are thought to enhance the binding affinity of transcription factors, including Oct-1, Runx2, and CP2, and upregulate *FGFR2* expression in breast epithelial cells (1). This dysregulation may promote mitogenic signaling, resistance to apoptosis, and eventually carcinogenesis.

Our gene expression analysis with publicly available datasets indicated that *FGFR2* was overexpressed in lobular carcinoma subtypes, while it was downregulated in ductal carcinoma. This finding highlights the heterogeneity of breast cancer and suggests that *FGFR2* expression may be restricted to certain subtypes. These patterns have already been described in other regional series, including the Markazi province study (12), which also highlighted the inconsistent risk conferred by *FGFR2* based on the subtype of tumor.

In clinical practice, identification of high-risk genotypes, especially rs2981582 TT and their associated risk haplotypes, can provide useful opportunities for the creation of population-specific genetic screening programs, especially in high-incidence populations of world regions such as Iran for early-onset breast cancer. *FGFR2* has also been investigated as a potential target for therapy, and the pattern of expression can aid in predicting the response to tyrosine kinase inhibitors or chemotherapy regimens (13).

Limitations: The sample size (n=160) was modest, potentially limiting statistical power for detecting smaller effects. The study did not assess hormone receptor subtypes (ER/PR/HER2), which may influence *FGFR2* associations. Lack of a replication cohort limits generalizability.

Future Directions: Larger studies, functional assays (e.g., luciferase assays for rs2981582), and integration of transcriptomic/epigenetic data are needed to elucidate *FGFR2*'s role in Iranian breast cancer patients.

CONCLUSION

This study suggests a strong association between the TT genotype of *FGFR2* rs2981582 and an increased risk of Breast cancer among women in Iran. Additionally, the rs1219648 genotype was effective at modulating the risk via haplotype. All of these results are consistent with those seen internationally and emphasize the role of the *FGFR2* variants in breast cancer predisposition. Because the intronic SNPs potentially possess regulatory function, this research validates the use of *FGFR2* genotyping as a population-level biomarker for early screening and risk, specific to the given population. Future studies ought to explore further the functional analyses and the role that the functional analyses play, besides clinical and hormonal parameters, to enhance precision oncology, especially in the local context.

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Ethics Approval and Consent to Participate

The study protocol was approved by the Ethics Committee of Arak University under the ethical approval code: IR.ARAKMU.REC.1395.288. Informed consent was obtained from all participants prior to inclusion in the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

Not applicable.

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Pituitary hormones Profile, Cholesterol Levels, and Steroidogenic Genes Expression are Useful Information in Prostate Cancer

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ABSTRACT

Objective: To investigate the relationship between changes in the level of Pituitary hormones, cholesterol levels, and the expression of genes involved in the biosynthesis of androgens, this study was designed.

Methods: In this study, the amount of changes in the levels of LH, FSH, and PRL hormones, as well as the level of cholesterol as a precursor of androgens, LDL and HDL lipoproteins, and the expression level of two genes, CYP17A1 and CYP11A1, in 120 people with prostate cancer as a case group and 120 people with BPH as a control group by RT-qPCR.

Results: The statistical analysis demonstrated that serum levels of testosterone, LH, and TSH were significantly higher in the malignant group compared to the benign group. PRL levels were also elevated in the Prostate cancer (PCa) group; however, this difference did not reach statistical significance. No significant difference was observed in serum PSA levels between the two groups. Prostate volume was significantly greater in the benign group than in the malignant group. Serum cholesterol levels were significantly higher in the PCa group compared to the Benign prostatic hyperplasia (BPH) group. In contrast, serum levels of LDL and HDL lipoproteins showed no significant differences between the groups. Additionally, the expression levels of CYP11A1 and CYP17A1 genes were significantly increased in the PCa group relative to the BPH group.

Conclusion: The results of this study showed that monitoring the hormonal profile and cholesterol level can play an important role in predicting the course of the disease.

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INTRODUCTION

Prostate cancer (PCa) is the second most frequently diagnosed malignancy and the sixth leading cause of cancer-related mortality among men worldwide. The global burden of this disease is projected to reach nearly 2.3 million new cases (1). Age-related enlargement of the prostate, known as Benign prostatic hyperplasia (BPH), is commonly associated with symptoms such as polyuria in men over 60 years of age (2). Owing to similarities in histopathological and molecular features, BPH is regarded as a potential stage in the initiation of prostate tumors; however,

the precise mechanisms underlying the progression from BPH to prostate cancer remain unclear (3-4). The incidence of PCa is higher in developed countries, largely due to the widespread use of prostate-specific antigen (PSA) testing for diagnosis (5). Advances in medical science in recent years, particularly in developed regions, have led to notable improvements in patient survival and prognosis. Major risk factors for prostate tumor development include age, race, genetic background, family history, obesity, and smoking (6). When treatment fails, PCa may progress to Castration-resistant prostate cancer (CRPC), which represents a significant clinical challenge (7).

Androgens are essential for the growth of PCa. They stimulate proliferation in both normal and malignant prostate cells through binding to and activating the androgen receptor (AR), a protein expressed in these cells (8). AR functions as a steroid receptor transcription factor for testosterone and dihydrotestosterone (DHT) and belongs to the nuclear receptor superfamily, sharing structural similarities with the estrogen, progesterone, glucocorticoid, and thyroid hormone receptors (9). Overexpression of AR allows PCa to progress even at castration levels of androgens. Tumor cells with AR amplification can persist during androgen deprivation therapy and advance to Castration-resistant prostate cancer (CRPC) (10).

Testosterone and dihydrotestosterone are the predominant androgens in men. Circulating testosterone levels are correlated with PSA levels, Gleason score, and AR expression in newly diagnosed PCa patients. In a retrospective study, men with low baseline testosterone exhibited reduced levels of LH, FSH, and estradiol, along with lower PSA levels, but a higher mean Gleason score. In these individuals, AR expression was elevated compared to groups with normal or high testosterone levels (11). AR demonstrates high affinity for both testosterone and 5α-dihydrotestosterone (DHT), which serve as its principal physiological ligands. The majority of circulating testosterone in healthy adult males is produced by the testes, while approximately 5–10% originates from the adrenal glands (12).

PCa cells are also capable of synthesizing small amounts of testosterone directly from cholesterol via sequential enzymatic activity involving CYP11A1, CYP17A1, HSD3B1 or HSD3B2, and AKR1C3, through a pathway typically active in the adrenal glands and testes (13). Previous studies have suggested that cholesterol biosynthesis may be elevated in CRPC (14-15); however, whether CRPC cells can generate physiologically meaningful levels of androgens de novo from cholesterol remains unclear. Investigations, primarily in LNCaP cells and xenograft models, have shown that key enzymes required for de novo steroidogenesis, including CYP11A1 and CYP17A1, are expressed and may be upregulated in castration-resistant sublines (16-17).

FSH and LH are produced by the pituitary gland. It's located at the base of your brain, and it's responsible for many functions in the body. FSH is necessary for sperm production (spermatogenesis). LH stimulates the production of testosterone, which is necessary to continue the process of spermatogenesis (18). Several studies have implicated dysregulation of the FSH and luteinizing hormone (LH) system as a whole in both the initial development and progression of prostate cancer, and the development of CRPC (19). Testosterone is produced from cholesterol in testicular Leydig cells under the regulation of pituitary gonadotropin LH; accordingly, several studies have reported an association between cholesterol levels and the risk of Prostate cancer (20-21). Hormonal markers may also be applied alongside prostate-specific antigen to improve its predictive performance (22).

In the present study, serum levels of testosterone, LH, FSH, prolactin, and cholesterol were assessed in individuals with prostate cancer and Benign prostatic hyperplasia. Since CYP11A1 and CYP17A1 enzymes play roles in testosterone biosynthesis from cholesterol, their expression levels were examined in patients with prostate cancer and benign prostatic hyperplasia. Furthermore, the association between changes in the expression of these genes and the levels of LH and FSH hormones, as well as cholesterol as a precursor, was analyzed. This study aimed to investigate the relationship between hormonal profile, cholesterol levels, and alterations in the expression of genes involved in the androgen synthesis pathway in patients with prostate cancer and benign prostatic hyperplasia.

MATERIALS AND METHODS

The population studied in this research included 120 people with prostate cancer and 120 people with benign prostatic hyperplasia, referred to Khatam Al-Anbia Hospital in Tehran between 2020 and 2021. After receiving informed consent from the patients, their information and files were received from the hospital. The conditions for entering the study were having a pathology confirmation based on benign and malignant prostate hyperplasia, as well as having a complete list of hormonal tests before receiving any medication. Also, in order to investigate the changes in CYP11A1 and CYP17A1 gene expression, a FFPE block was obtained from the prostate tissue in the pathology department of the hospital. All samples were taken from patients before the start of treatment.

Hormonal information, including total testosterone level, Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), prolactin (PRL), histopathological information, including prostate size, tumor stage, Gleason score, demographic information, including

patient age, marital status, and education. Also, the levels of prostate surface antigen, cholesterol, LDL, and HDL were collected.

Then, the RT-qPCR method was used to investigate the changes in the expression of the androgen receptor (AR) gene and the relationship between the expression changes of this gene and hormonal, biochemical, and pathological factors. For this purpose, RNA extraction from FFPE section was performed as previously described (22), Briefly, under appropriate conditions, tissue samples were deparaffined by xylene, then protein digestion was performed by proteinase K and its buffer (Tris-HCl 100mM + NaCl 200mM + EDTA 2mM + SDS1%). After that, 1 mL Trizol solution was added to samples. Continue the steps according to the manufacturer's instructions. RNA samples were quantified using a spectrophotometer and then subjected to electrophoresis on a 1.5% agarose gel to assess RNA integrity. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, cat. K1622) according to the manufacturer's protocol.

Expression levels of the gene were measured by real-time PCR using the RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). Real-time PCR was carried out on a RotorGene Q real-time PCR system (Qiagen, Germany). Signals were normalized to the "house-keeping" gene GAPDH as an endogenous internal control. Primer sequences were used include: CYP17A1 forward primer: 5'-CCGTAAGGGTATCGCCTTCG-3', CYP17A1 reverse primer: 5'-CCATCCTTGAACAGGGCAAAG-3' (NM_000102.4); CYP11A1 forward primer: 5'-GCTGAAGTGGAGCAGGTACA-3', CYP11A1 reverse primer: 5'-CTTTGACCAGGACTGAGCGT-3'(NM_000781.3) and GAPDH forward primer: 5'-GTCTCCTCTGACTTCAACAGCG-3', GAPDH reverse prime:5'-ACCACCCTGTTGCTGTAGCCAA-3' (NM_002046.7). The optimal annealing temperature for each primer pair was established prior to analyzing the experimental samples. Real-time PCR reactions were performed in triplicate for each sample. The mean Ct values obtained from each reaction were normalized against GAPDH. Relative gene expression levels were then determined using the 2^-ΔΔCt method.

All statistical analyses were conducted using SPSS for Windows, version 26.0 (IBM, SPSS, Chicago, IL, USA). Normality of data distribution was assessed using the Kolmogorov–Smirnov test. Comparisons between the two groups were performed using the Mann–Whitney U test. Correlations were evaluated using Pearson's correlation coefficient for continuous variables and Spearman's correlation coefficient for ordinal and discrete variables.

RESULTS

The population studied in this research includes 120 people with prostate carcinoma and 120 people with benign prostatic hyperplasia. The demographic characteristics these patients are shown in Supplementary Table 1. As shown, the malignant and the benign groups were well matched according to age (median 61.45 years for the malignant group and 66.4 years for benign group) In the comparison between the two groups, no significant relationship with age was reported $p>0.05$. In the benign group, 118 people were married and only 2 people were single, and in the benign group, 116 people were married and only 4 people were single, $p>0.05$. In terms of education level, no significant relationship was reported between the two groups $p>0.05$ (The details of the level of education in the two groups can be found in supplementary Table 1).

The levels of testosterone, LH, TSH and prolactin hormones in the serum of the two groups were compared as shown in Table 1. The serum testosterone level was significantly higher in the benign group compared to the malignant group (mean level in BPH was 428.01 ng/dl, compared with PCa 382.84 ng/dl, $p<0.05$). The level of LH (6.14 mIU/ml in PCa Vs. 5.12 mIU/ml in BPH; $p<0.05$) and TSH (7.96 mIU/ml in PCa Vs 6.82 mIU/ml in BPH; $p<0.05$) in the malignant group was significantly higher than the benign group. The PRL level was also higher in the PCa group (8.18 ng/ml) than in the BPH group (97.98 ng/ml) but this difference was not statistically significant ($p>0.05$).

As can be seen in Supplementary Table 2, no significant difference was observed in serum PSA level in the two groups (level means in PCa 6.34 Vs. 6.89 ng/ml in BPC; $p>0.05$). The prostate volume was compared in the groups; it was significantly larger in the benign group than in the malignant group (55.37 ml in BPH Vs 47.07 ml in PCa; $p<0.05$).

Also, as can be seen in Supplementary Table 3, the level of LH in stage II of the disease is higher than in stage III, and the level of FSH in stage III is higher than in stage II. However, no significant changes were observed between testosterone and prolactin levels in stage II and III of the disease.

Cholesterol level as one of the precursors of testosterone synthesis, was also measured in two groups; as can be seen in Table 2, the level of serum cholesterol was significantly higher in the PCa group than in the BPH group (level means in PCa 217.5 Vs. 189.8 mg/dl in BPC; $p<0.05$). Also, the levels of LDL (level means in PCa 94.2 Vs. 90. 3 mg/dl in BPC; $p>0.05$) and HDL (level means in PCa 49.02 Vs. 45.87 mg/dl in BPC; $p>0.05$) lipoproteins in the

Table 1. The relationship between patients› demographic information and the occurrence of benign prostatic hyperplasia (BPH) and prostate carcinoma.

parameters	BPH (mean±SD)	Pca (mean±SD)	Sig ≤0.05*
Testosterone	428.01 (56.19)	382.84 (81.67)	**<0.05**
LH	5.12 (0.58)	6.14 (0.81)	**<0.05**
FSH	6.82 (0.81)	7.96 (0.53)	**<0.05**
PRL	7.98 (0.53)	8.18 (0.76)	>0.05

*Mann-Whitney U test. Testosterone normal range: 300-1000 ng/dl. LH normal range: 20-70 yeas between 1.3-8.0 mIU/ml. FSH normal range: between 1.5-12.4 mIU/ml. PRL normal range: between 2-17 ng/ml

Table 2. Evaluation of the relationship between prostate volume indices and PSA levels with benign prostatic hyperplasia and prostate carcinoma.

Parameters	BPH (mean±SD)	Pca (mean±SD)	Sig ≤0.05*
cholesterol	189.8 ± 36.85	217.5 ± 46.97	**<0.05**
LDL	90.3 ± 11.23	94.2 ± 10.91	>0.05
HDL	45.87 ± 10.10	49.02 ± 8.89	>0.05

*Mann-Whitney U test. Cholesterol normal range: 125-200 mg/dl. LDL normal range: <100 mg/dl. HDL normal range ≥40 mg/dl.

Table 3. The relationship between testosterone, LH, FSH, and prolactin hormone levels and stage II and III of the disease.

parameters	Stage II (mean±SD)	Stage III (mean±SD)	Sig ≤0.05*
Testosterone	366.42 (49.81)	381.09 (34.11)	>0.05
LH	6.42 (0.33)	5.19 (0.74)	**<0.05**
FSH	7.41 (0.41)	8.01 (0.32)	**<0.05**
PRL	7.93 (0.14)	8.24 (0.49)	>0.05

serum of the subjects were measured and compared in two groups, As can be seen, there was no significant relationship between the serum levels of these two lipoproteins in the two groups.

In another phase of this study, the level of cytochrome P450 family 11 subfamily A member 1 gene (CYP11A1; NCBI ID 1583) and cytochrome P450 family 17 subfamily A member 1 (CYP17A1; NCBI ID 1586) expression at the mRNA level in two groups was measured by the relative qRT-PCR method. As can be seen in figures 1 and 2, the expression level of these genes in the PCa group was significantly higher than in the BPH group ($p<0.05$). Then, the expression changes of these genes were compared with hormonal, pathological, and biochemical factors of patients in two groups. Pearson's correlation test showed a significant relationship between these genes expression changes and testosterone, LH, FSH, and PRL levels ($p<0.05$); However, no significant relationship was reported between the expression changes of this gene and serum PSA level, prostate volume, and disease stage ($p>0.05$). Among the biochemical factors, only a significant relationship was observed with cholesterol level ($p<0.05$), but no significant relationship was observed with LDL and HDL levels ($p>0.05$).

DISCUSSION

This study was conducted in three phases, which include 1. Prostate cancer-related hormone profiling phase 2.The phase of checking the levels of cholesterol and its derivatives 3. The phase was to investigate the expression of the testosterone biosynthesis pathway genes from the precursor of cholesterol. Among previous prospective investigations examining hormones and prostate cancer, although substantial overall evidence suggests that androgens and pituitary hormones contribute to the etiology of Prostate cancer, no significant associations between hormone levels and prostate cancer risk have been consistently demonstrated. Stattin et al. reported a reduced risk of prostate cancer in individuals with higher circulating total testosterone levels (23), consistent with the

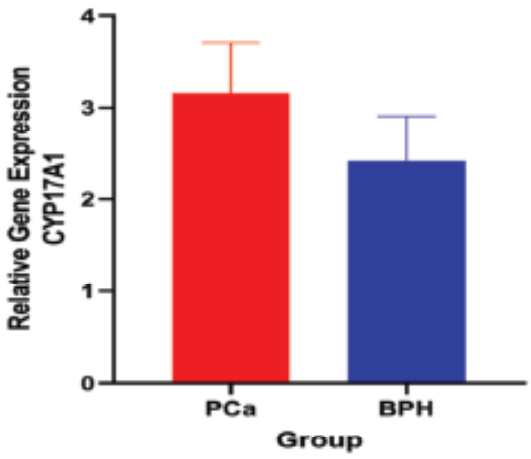

Fig 1. Significant increase in CYP17A1 gene expression in the PCa group compared to the BPH group (p<0.05).

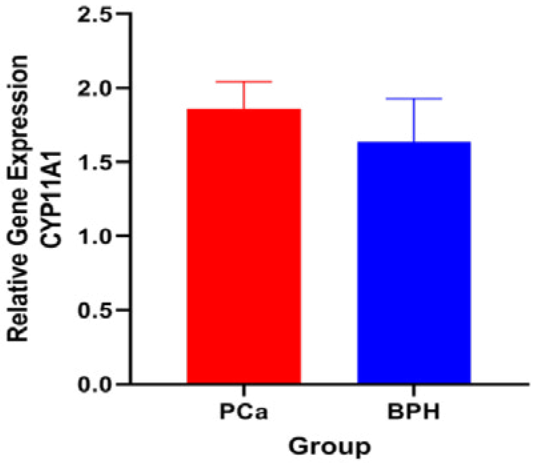

Fig 2. Significant increase in CYP11A1 gene expression in the PCa group compared to the BPH group (p<0.05).

findings of the present study. In rodent tumor models, initiation and progression of prostate cancer have been observed following exposure to exogenous androgens (24). However, the short-term androgen exposure used in animal models may not accurately reflect the chronic, mildly elevated androgen levels typically observed in men.

The results of present study showed that the levels of LH, FSH and PRL hormones were significantly higher in the malignant group than in the benign group; but the level of testosterone in the malignant group was lower than the benign group.

Prostate cancer suppresses testosterone production, likely through local secretion of inhibin or other factors that trigger negative feedback on the hypothalamic–pituitary axis; accordingly, testosterone levels have been reported to increase markedly following radical prostatectomy (25). In addition, because prostate cancer cells offer more binding sites for testosterone than Benign prostatic hyperplasia (BPH) cells, they may function as a reservoir for androgens, thereby reducing circulating testosterone concentrations. Other studies have also shown that low pre-treatment testosterone levels are associated with poorer prognosis (26). Mean testosterone levels decline progressively as the disease advances from organ-confined to metastatic stages (27). Therefore, baseline testosterone levels may be valuable for inclusion alongside other well-established prognostic indicators.

In men, LH stimulates testosterone production from the interstitial cells of the testes (Leydig cells). FSH stimulates testicular growth and enhances the production of an androgen-binding protein by the sertoli cells (28). Some studies have shown, higher LH levels in older men may have an effect on the development of benign prostatic hyperplasia or prostate carcinoma (29). Exposure of cancer cell lines to LH has been linked to upregulation of intratumoral steroidogenesis (30). Enhanced steroidogenic activity is frequently observed in Castration-resistant prostate cancer (CRPC) cells, where it is associated with resistance to androgen deprivation therapy (31). In patients with Prostate cancer, LH levels have been reported to carry prognostic value; however, this observation is inconsistent with findings from other studies, which often show considerable variability. In studies of the hypothalamic–pituitary axis, Harper et al. proposed that low baseline testosterone levels together with elevated LH are indicators of poor prognosis (32), a conclusion also supported by Chen et al. in patients with metastatic prostate cancer, where poor outcomes were found to be independent of tumor grade (33). In the present study, a significant association was observed between disease stage and the levels of LH and FSH.

There may be regulatory interactions between the prostate gland and the pituitary gland. Alterations in the prostate, such as the development of Prostate cancer, can disrupt this interaction and lead to changes in hormone levels, including FSH, LH, testosterone, and free testosterone. In the present study, serum FSH levels were higher in patients with prostate cancer compared to those with Benign prostatic hyperplasia (BPH); however, other similar studies have not identified a significant association between serum FSH levels and the risk of prostate cancer diagnosis. The elevated serum FSH levels observed in cancer patients in this study may be explained by two possible mechanisms: (1) a direct effect of prostate cancer cells on FSH secretion, and (2) the influence of promoter proteins produced by cancer cells that enhance FSH secretion and are associated with reduced testosterone levels.

Limited information is available regarding the role of prolactin (PRL) in Prostate cancer. PRL contributes to

the regulation of genes responsible for the production of PRL-inducible protein in both normal prostate tissue and prostate cancer; transcript levels of this protein have been reported to be significantly higher in carcinoma compared with benign prostate epithelium (33). In the present study, PRL levels were also elevated in the PCa group relative to the Benign prostatic hyperplasia (BPH) group, although this difference did not reach statistical significance.

The cholesterol and its derivatives profile has been of importance in many disease conditions. Its role in BPH and PCa is being examined herein. The results of the present study showed a significant increase in cholesterol levels in the PCa group compared to the BPH group. But there was no correlation between the level of HDL and LDL lipoproteins and prostate cancer.

Cholesterol serves as a fundamental precursor for androgen biosynthesis; therefore, it may contribute to the growth of PCa through its role in steroidogenesis. Raftopoulos et al. demonstrated that cholesterol acts as a critical substrate for de novo steroidogenesis in prostate cells. Their findings indicate that the proliferation of androgen-independent prostate cancer cells can be affected by extracellular lipid levels and the availability of LDL-cholesterol. Moreover, the uptake of extracellular cholesterol via endocytosis of LDL-derived cholesterol followed by its transport and storage as cholesteryl esters within lipid droplets is necessary to sustain prostate cancer cell growth (34). These observations provide further insight into the interplay between extracellular cholesterol, intracellular cholesterol metabolism, and prostate cancer progression, as well as the potential mechanisms linking hypercholesterolemia to more aggressive disease.

Animal model study has shown that high cholesterol levels are significantly related to increased tumor size and increased intracellular testosterone levels. Therefore, it is expected that the expression level of steroids steroidogenic enzymes will increase in cancer cells (35).

In this study, we investigated the expression level of CYP11A1 and CYP17A1, which are enzymes that initiate the conversion of cholesterol to androgens, and the results showed a significant increase in the expression of these two genes compared to the BPH group. This result suggests that cholesterol acts not only as an essential precursor, but also as a pathway agonist, stimulating the upregulation of steroidogenic gene expression. These results are in accord with those of others who demonstrated that proteins responsible for cholesterol regulation are altered during disease progression to increase the pool of available cholesterol, coincident with an increase in androgens to physiologically relevant levels (36). Based on these results, it can be concluded that cholesterol can be used as a therapeutic target in prostate cancer. On the other hand, the results of expressive studies have shown that monitoring steroidogenic genes in patients with PCa may provide useful information for therapy intervention.

In Prostate cancer, tumor cells may produce the androgens testosterone and DHT from adrenal progestagens or dehydroepiandrosterone, provided that CYP17A1, HSD17β3, and HSD3β are expressed within the tumor. Alternatively, when intratumoral androgen production originates from cholesterol, the presence of CYP11A1 is also required. These four enzymes CYP11A1, CYP17A1, HSD3β, and HSD17β3 are recognized as key contributors to the conversion of cholesterol into testosterone. In the present study, increased expression of CYP11A1 and CYP17A1 genes was observed, and in our previous study, elevated expression of HSD3β family genes in prostate cancer was also reported (37).

CONCLUSION

Androgens promote the proliferation of Prostate cancer cells, as well as metastasis and progression to CRPC. In addition to androgenic hormones, pituitary hormones are also involved in the initiation and progression of prostate cancer. LH, FSH, and PRL either independently or in combination with androgens have physiologically important functions in the normal prostate. Their role in the development of Benign prostatic hyperplasia and prostate carcinoma remains an important topic for investigation. In the present study, LH and FSH levels were found to be associated with the occurrence of prostate cancer. Additionally, cholesterol levels, as a precursor for androgen synthesis, were higher in patients with prostate cancer. Consistent with this, the expression levels of genes encoding enzymes involved in androgen biosynthesis from cholesterol were significantly increased. Overall, these findings suggest that monitoring hormonal profiles and cholesterol levels may have an important role in predicting disease progression.

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Declarations

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analyzed during

the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Examining the Autoimmune Disorder Rheumatoid Arthritis and the Genetic Determinants Contributing to its Genesis

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ABSTRACT

Rheumatoid arthritis (RA) is an irreversible systemic autoimmune disorder. The advancement of the illness results in joint deformity and associated functional impairment, which profoundly impacts the standard of life of those affected. This review offers an overview of rheumatoid arthritis (RA), including a broad introduction to the illness, its epidemiology, associated risks, and pathogenesis. It also emphasizes advancements in fundamental research and the many mechanisms of signaling and molecular processes, including genetic variables. Summary of previous studies: In recent decades, researchers have garnered more interest in rheumatoid arthritis. Aberrant signaling pathways in rheumatoid arthritis (RA) constitute a significant area of study for identifying and treating the condition, offering crucial insights for comprehending this complex illness and formulating relevant therapies. The etiology of rheumatoid arthritis is associated with several signaling pathways. Research has repeatedly examined the etiology of rheumatoid arthritis (RA), revealing that both environmental and genetic variables play significant roles in its onset. Additionally, several research indicates that the susceptibility and severity of rheumatoid arthritis (RA) may correlate with the HLA-DRB1 variant, which exhibits the most significant genetic relationship with RA.

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INTRODUCTION

Rheumatoid arthritis (RA) stands as a prevalent systemic inflammatory condition. Its nomenclature is derived from Greek roots translating to "swollen bones" (1, 2). Historical records indicate that the first clinical delineation of this pathology occurred in 1880, when French physician Augustine Jacob Lander-Bois characterized its primary manifestations under the term "asthenic gout." However, the specific terminology "rheumatoid arthritis" was formally introduced in 1859 by the British rheumatologist Alfred Baring Garrod (2). The disease typically emerges during the fourth or fifth decade of life and exhibits a female predominance, with incidence rates three to five times higher in women than in men (2). Predilection sites include the joints of the hands, wrists, feet, and knees. Early clinical presentations are marked by erythema, edema, localized hyperthermia, pain, and diminished joint function (3).

To standardize diagnosis, the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) have established revised classification criteria. These guidelines integrate data on joint involvement patterns, specific serological markers (such as Rheumatoid Factor or Anti-Citrullinated Protein Antibodies), symptom

duration, and levels of acute-phase reactants. As the disease progresses, patients experience increased joint stiffness and deformity. In advanced stages, RA leads to varying degrees of osseous damage, muscle atrophy, synovial infiltration into articular cartilage, subchondral bone erosion, and compromised ligamentous and tendinous integrity (4). Beyond joint pathology, RA significantly impairs quality of life and can induce extra-articular complications affecting the eyes, nervous system, skin, kidneys, lungs, liver, heart, and skeletal system (5, 6, 7).

While the exact etiology of RA remains elusive, it is widely believed to result from a complex interplay between genetic predisposition and environmental triggers. The core pathogenic mechanism involves an autoimmune response where the immune system erroneously targets joint tissues, leading to inflammation and synovial capsule thickening, which subsequently damages cartilage and bone. Diagnosis relies heavily on clinical evaluation and physical symptom assessment (8, 9).

It is crucial to distinguish RA from other arthritic conditions. Arthritis is broadly classified into non-inflammatory types, such as osteoarthritis, and inflammatory types. Inflammatory arthritis may stem from crystal deposition disorders (including gout, pseudogout, and calcium phosphate deposition), infectious agents (such as *Staphylococcus aureus*, *Neisseria gonorrhoeae*, Lyme disease, parvovirus, or enterovirus), or autoimmune pathways. This autoimmune spectrum encompasses a diverse range of rheumatic diseases, including systemic lupus erythematosus (SLE), Sjögren's syndrome, adult-onset scleroderma, spondyloarthritis (SpA), psoriatic arthritis (PsA), and polymyositis (PM) (10). Due to the overlapping symptoms among these rheumatic conditions, precise differential diagnosis is essential (11).

Despite advances in understanding biomolecular pathways, the root cause of RA is not fully elucidated. A leading hypothesis suggests that dysregulated citrullination processes drive the production of anti-citrullinated protein antibodies (ACPAs). The clinical course of RA is characterized by fluctuating disease activity with intermittent flare-ups. Without appropriate therapeutic intervention, symptoms tend to worsen progressively, resulting in irreversible joint destruction and substantial physical and cognitive impairment (10, 11). Furthermore, the burden of symptoms and associated comorbidities reduces life expectancy by several years (12). Current statistical analyses underscore that RA represents not only a significant medical challenge but also a major public health concern (13, 14).

Epidemiological overview

In the last 30 years, several experts have thoroughly examined the alterations in the frequency and incidence of rheumatoid arthritis (RA). These studies indicate that rheumatoid arthritis (RA) is a worldwide affliction, prevalent across all races, genders, ethnicities, nationalities, and ages (13, 14).

Prevalence of RA in epidemiological studies

Epidemiological research indicated the incidence of rheumatoid arthritis in various European, Asian, North American, and South American nations from 1990 to 2005. Prevalence rates in Serbia (0.18%) (15), China (0.28%) (16), France (0.31%) (4), Italy (0.33%) (5), and the United States (0.41%) were documented (16). Higher prevalence rates were seen in Japan (1.7%) and Argentina (1.97%). The prevalence ratio was greater in Japan (1.7%) and Argentina (1.97%), with all studies indicating that RA prevalence was three to five times higher in women compared to males. The greatest disparity was seen in Argentina (females 3.2%, men 0.6%), whilst the minimal values were recorded in Serbia (females 0.29%, males 0.09%) (16). The incidence of RA has consistently risen since 1990 (3, 16).

Risk factors and symptoms

The pathogenesis of rheumatoid arthritis (RA) is deeply rooted in specific signaling cascades. Consequently, contemporary pharmacological research has prioritized the development of inhibitors targeting these RA-specific pathways. This review aims to clarify the risk factors and underlying mechanisms of RA, with a specific emphasis on the signaling networks that drive the disease. It provides a comprehensive overview of currently approved therapeutic agents, as well as emerging candidates undergoing clinical and preclinical evaluation, including advanced targeted therapy modalities (10, 16). Extensive research over the past few decades has highlighted the critical roles of both genetic and ecological factors in RA development. Key genetic susceptibility loci identified include *HLA-DRB1*, *TNFRSF14*, and *PTPN22*. Among these, specific alleles of *HLA-DRB1* exhibit the strongest association with RA onset and disease severity. Notably, the prevalence of these risk alleles varies across different ethnic and geographic populations. A central concept in this genetic framework is the "shared epitope" theory. This hypothesis suggests that certain *HLA-DRB1* alleles, which share a conserved sequence of five amino acids, contribute to RA pathogenesis by altering how antigen-presenting cells process and display antigens. This dysregulated antigen presentation is believed to disrupt normal immune tolerance, leading to T cell-mediated autoimmune responses that directly fuel the inflammatory process (10, 16).

Beyond genetics, environmental influences such as smoking, dietary patterns, and hygiene standards

are pivotal in RA etiology. These external factors can directly modify gene expression through post-transcriptional mechanisms or indirectly affect susceptibility genes via epigenetic modifications (17). The complex interaction between environmental triggers, epigenetic changes, and genetic vulnerabilities leads to alterations in the expression levels of various encoded proteins. This molecular imbalance is thought to exacerbate autoimmune tolerance breakdown, thereby promoting the development and progression of rheumatoid arthritis (Figure. 1).

Rheumatoid arthritis (RA) is characterized by the production of autoantibodies targeting joint tissues. Although the disease primarily affects the musculoskeletal system, it possesses significant systemic potential, frequently involving the eyes, skin, lungs, heart, liver, and bones. The hallmark clinical features include joint inflammation, edema, fever, pain, and stiffness. These symptoms predominantly originate in the small joints of the hands and feet but can also extend to larger articulations such as the shoulders and knees (19, 20, 21). The pain experienced in RA is distinctly articular, arising directly from the site of inflammation, rather than neuropathic in nature. Persistent inflammation can restrict the range of motion, lead to joint deformity, and cause localized osteoporosis surrounding the affected areas (22, 23). Furthermore, these systemic processes highlight the heightened susceptibility of RA patients to chronic conditions and cardiovascular diseases (24, 25). Uncontrolled chronic inflammation may also precipitate severe complications, including renal amyloidosis, cutaneous rheumatoid nodules, and interstitial lung disease (ILD) (26-31). Ocular manifestations often present as episcleritis, while hepatic involvement may manifest as autoimmune hepatitis (32-35). Additionally, carpal tunnel syndrome is a common complication, resulting from wrist edema that compresses the median nerve, leading to peripheral neuropathy (36).

In recent years, air pollution has emerged as a critical environmental factor in the etiology of RA. Atmospheric pollutants, comprising particulate matter (PM) of various sizes and gases such as nitrate, ozone, sulfur dioxide, and carbon monoxide, originate from both anthropogenic sources (e.g., fossil fuel combustion, industrial chemical manufacturing, agriculture) and natural events (e.g., volcanic eruptions, wind-blown dust). While the impact of these pollutants is often studied in the context of respiratory diseases—where ozone exposure is known to damage alveoli and induce secondary lung injury via enzymatic interactions recent epidemiological evidence links them directly to RA (20, 36). Studies conducted in the United States, Canada, and Sweden have established a correlation between air pollution levels and RA incidenceSpecifically, Al-Saber et al. (2020) identified nitrates and sulfur dioxide as significant risk factors for RA onset (20, 36). A cross-sectional study involving 888 RA patients further demonstrated that higher exposure to air pollution correlates with elevated C-reactive protein (CRP) levels. Since CRP levels reflect disease severity and resistance to biological therapies, these

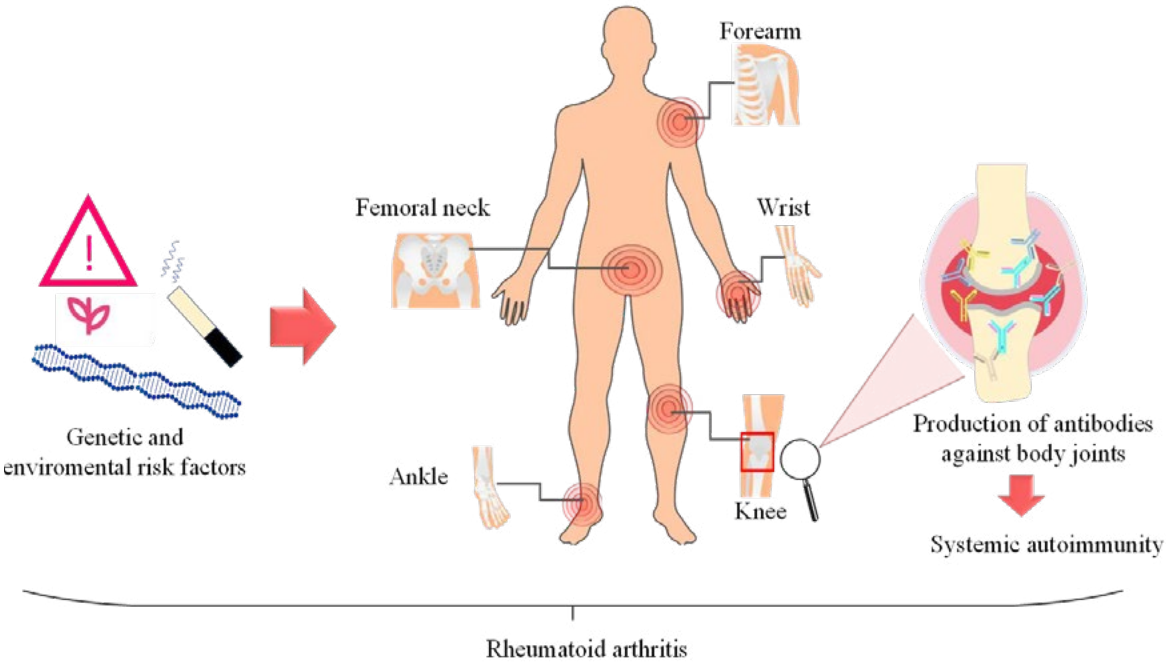

Fig1. Genetic and environmental risk factors in an asymptomatic individual may begin to activate the immune system against the joints. The joints that are most affected by RA are shown in the figure.

findings suggest a direct link between environmental pollutants and RA disease activity (37).

The molecular mechanisms underlying the association between air pollution and RA are multifaceted. Inhalation of particulate matter triggers the generation of reactive oxygen species (ROS), which activate nuclear factor kappa B (NF-κB). This activation stimulates T helper type 1 (Th1) cells to secrete pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). These cytokines promote the maturation of monocytes into dendritic cells, which then present self-antigens to autoreactive T lymphocytes. This process drives T cell migration to target tissues, exacerbating joint inflammation and bone erosion. Concurrently, ROS-induced citrullination of arginine residues generates citrullinated peptides, worsening chronic lung pathology and systemic inflammation. The resulting anti-citrullinated protein antibodies (ACPAs) bind to Fc receptors and activate the complement system, leading to joint inflammation and osteoclast-mediated bone resorption (20, 37).

Beyond air pollution, other environmental and biological factors contribute to RA pathogenesis. Reduced exposure to ultraviolet B (UVB) light decreases the cutaneous synthesis of 1,25-dihydroxy vitamin D3, a potent immunomodulator acting through the vitamin D receptor (VDR). This deficiency may impair immune regulation, thereby increasing RA risk (38). Equally important is the role of the gut microbiota, the most densely populated bacterial ecosystem in the human body (39). Intestinal dysbiosis in RA patients is associated with the activation of specific autoimmune pathways, including toll-like receptor (TLR) or NOD-like receptor (NLR) stimulation of antigen-presenting cells, molecular mimicry, increased intestinal permeability, altered T cell differentiation, and enhanced mucosal inflammation (40). Comparative studies reveal significant differences in the gut microbial composition of RA patients versus healthy individuals, with specific bacterial shifts correlating with disease states (41). These gastrointestinal microbes likely influence RA onset by modulating immune responses via specialized immunomodulatory cells located within the intestinal mucosa.

Epigenetic regulation in the RA signaling pathway

Epigenetics is heritable changes in gene expression without changing the DNA sequence. Epigenetics regulates the activation or deactivation of genes. The primary processes involved in this process are histone modification, DNA methylation, and non-coding RNA pathways. These alterations delineate certain gene expression patterns. Genetic and environmental variables, particularly smoking, that influence gene expression are intricately linked to the pathophysiology of rheumatoid arthritis (RA). Fortunately, these epigenetic alterations are reversible, and the associated enzymes that regulate histone or DNA modifications may also be reverted. Methylation is now suggested as a therapeutic target for rheumatoid arthritis (RA) (3, 41).

Epigenetic Regulation in Rheumatoid Arthritis: The Role of Histone Modifications and Deacetylases

Histones are fundamental proteins responsible for organizing DNA into nucleosomes, which further condense to form chromosomes within the cell nucleus. The N-terminal tails of these histones undergo various post-translational modifications, including ubiquitination, acetylation, methylation, phosphorylation, and ADP-ribosylation (42). These epigenetic marks play a critical role in regulating gene expression, either by silencing or activating specific genetic pathways. Consequently, histone modifications have become a focal point in medical research, particularly in oncology, where they offer new avenues for therapeutic intervention.

In the context of rheumatology, significant differences in histone lysine methylation profiles have been observed between osteoarthritis articular fibroblasts (OASFs) and rheumatoid arthritis articular fibroblasts (RASFs) (43). These studies have identified distinct patterns in the expression of histone lysine methyltransferases (HKMTs) and histone lysine demethylases (HKDMs) at the mRNA level, suggesting that histone lysine methylation (HKM) is a key regulator of gene expression in RASFs.

Among the enzymes involved in histone modification, Sirtuin 1 (SIRT1), a nuclear-localized, NAD-dependent deacetylase belonging to the class III histone deacetylase family, is extensively studied. SIRT1 plays a multifaceted role in the pathogenesis of rheumatoid arthritis (RA). Its overexpression has been linked to the production of pro-inflammatory cytokines and the inhibition of apoptosis in RA synovial cells, thereby promoting disease progression (44).

Another critical family of enzymes is the histone deacetylases (HDACs). Research highlights the involvement of HDAC1 in the synthesis of inflammatory mediators. Notably, the deletion of HDAC1 in T cells has been shown to provide a protective effect in murine models of collagen-induced arthritis (45). Furthermore, the activation of fibroblast-like synoviocytes (FLS) and the upregulation of HDAC1 and HDAC2 are prominent features of the RA synovium. The concentration of FLS in rheumatoid arthritis synovial fluid (RA-SF) is significantly higher than that of OASFs (46). In animal models of adjuvant-induced arthritis, HDAC6 protein levels are notably

elevated in synovial tissues. Therapeutic inhibition of HDACs in these models has demonstrated efficacy in reducing joint swelling and synovial inflammation, thereby alleviating RA symptoms. These findings suggest that HDAC inhibitors represent a promising therapeutic strategy for RA (47, 48).

Additionally, recent investigations into platelet-derived growth factor (PDGF)-stimulated FLS have revealed that the histone demethylase JMJD3 is upregulated via the Akt signaling pathway. This upregulation enhances the migratory and proliferative capabilities of FLS, further contributing to the aggressive nature of RA synovial hyperplasia.

Epigenetic Regulation in Rheumatoid Arthritis: The Role of DNA Methylation

DNA methylation, a pivotal epigenetic mechanism, is catalyzed by DNA methyltransferases (DNMTs). These enzymes transfer a methyl group from S-adenosylmethionine (SAM) to the cytosine bases within CpG dinucleotides, primarily located in gene promoter regions, leading to the formation of 5-methylcytosine (5mC) and subsequent gene silencing (49). Due to its reversible nature, DNA methylation has emerged as a promising therapeutic target in rheumatology.

While global DNA methylation levels in the synovial fluid of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) show no significant disparity, distinct alterations are observed in peripheral blood mononuclear cells (PBMCs). Specifically, PBMCs from RA patients exhibit reduced global DNA methylation levels compared to healthy controls (50). This hypomethylated state is particularly pronounced in T cells and monocytes, suggesting that systemic immune dysregulation in RA is closely linked to epigenetic modifications.

The restoration of methylation patterns is often associated with high cellular proliferation rates and depends on the availability of SAM as a methyl donor. Hypomethylation in these contexts leads to the overproduction of various molecular components, including extracellular matrix proteins, growth factors, receptors, matrix-degrading enzymes, and adhesion molecules. Consequently, methylation status serves as a critical biomarker for assessing cell proliferation in tissue samples.

The inflammatory microenvironment significantly influences these methylation patterns. Experimental activation of fibroblast-like synoviocytes (FLS) with pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), reveals that RA-FLS exhibit significantly lower methylation levels compared to OA-FLS. Furthermore, while the intracellular concentration of 5-methylcytosine is elevated in RA-FLS, the methylation status of specific promoter regions, such as that of the T-box 5 (TBX5) transcription factor, differs markedly from that in OA-FLS. These findings underscore that even subtle changes in DNA methylation can profoundly impact the behavior of multiple cell types, driving the pathogenesis of rheumatoid arthritis (51, 52).

MicroRNAs in the Pathogenesis of Rheumatoid Arthritis

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play a critical role in post-transcriptional gene regulation. The biogenesis of miRNAs begins in the nucleus, where a primary miRNA transcript is synthesized and subsequently cleaved by the enzyme Drosha to form a precursor miRNA (pre-miRNA) (53, 54). This precursor is then exported from the nucleus to the cytoplasm via the transport protein Exportin-5. In the cytoplasm, the enzyme Dicer processes the pre-miRNA into a mature miRNA duplex. One strand of this duplex is loaded into the RNA-induced silencing complex (RISC), where it guides the complex to complementary messenger RNA (mRNA) targets, leading to their degradation or translational repression (55, 56). Dysregulation of these miRNAs has been implicated in the inflammatory and autoimmune processes characteristic of rheumatoid arthritis.

Therapeutic Strategies for Rheumatoid Arthritis

The primary objectives of rheumatoid arthritis (RA) management are to alleviate pain, reduce joint inflammation, prevent or slow down joint destruction, minimize disability, and preserve the patient's functional capacity. Although there is currently no cure for RA, early and aggressive intervention can significantly reduce the risk of irreversible joint damage and improve overall quality of life (3,56). Recent pharmacological advancements have introduced a diverse array of therapeutic options, although the complex molecular mechanisms governing antibody fate remain a challenge for drug development.

Effective management relies on early diagnosis and a comprehensive approach that combines non-pharmacological strategies with pharmacological interventions. Regular monitoring of treatment efficacy is essential to adjust therapies and minimize adverse effects (57). According to the American College of Rheumatology (ACR), pharmacological agents are classified into traditional synthetic disease-modifying antirheumatic drugs (tsDMARDs), biologic DMARDs (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) (58). These medications aim to modify the disease course rather than merely treating symptoms.

In clinical practice, nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (GCs) are frequently used as adjunctive therapies to provide

Table 1. Drugs and treatments used in RA.

Nonsteroidal anti-inflammatory drugs (NSAIDs)		
Name	**The mechanism**	**Ref**
Naperlan (naproxen sodium) **Mobic (meloxicam)** **Doxys (ibuprofen and famotidine)**	They block cyclooxygenase (COX) activity, thus obstructing the production of prostaglandins (PGs) and eliciting antipyretic and analgesic actions used to alleviate symptoms of rheumatoid arthritis discomfort.	(60,61)
Traditional disease-modifying anti-rheumatic drugs (DMARDs)		
Sulfasalazine	Inhibits inflammatory chemokines and cytokines and changes adenosine metabolism.	(66)
Leflunomide	Inhibits metabolisms of dihydroorotate dehydrogenase and pyrimidine.	(67)
Hydroxychloroquine	Modulation of TLR7 and TLR9 activity and perhaps stabilisation of macrophage lysosome.	(68,69)
Methotrexate	It prevents the formation of purines and pyrimidines by inhibiting some factors and thus prevents the proliferation of inflammatory cells.	(70)
Biologic DMARDs		
Inhibition of T cells	By attaching an inhibitor to the CD80 and CD86 receptors of T lymphocytes, the activation of these cells can be prevented	(68)
Inhibition of B cells	By binding the inhibitor to the CD20 receptor, it prevents antigen presentation and antibody production.	(69)
TNF	TNF regulates gene expression via the modulation of methylation and acetylation. Hypomethylation has been shown to correlate with heightened expression of genes implicated in the development of rheumatoid arthritis.	(71)
Inhibition of Cytokine	Disruption of cytokine networks in RA disease pathogenesis	(71)
Inhibition of interleukin-6	Inhibition of interleukin 6 leads to non-differentiation of B lymphocytes and inhibition of leukocyte activity.	(72)
Inhibition of Interleukin-1	Inhibition of interleukin 1 prevents the activation of leukocytes, endothelial cells and osteoclasts.	(73)

rapid symptom relief and reduce inflammation (59). However, they do not alter the underlying disease progression. Therefore, the cornerstone of RA treatment remains the use of DMARDs, which include both non-biological and biologic agents, alongside emerging targeted therapies (3, 59). Table 1 summarizes key elements of these therapeutic categories. Despite these advances, the incomplete control of symptoms in some patients highlights the ongoing need for more effective and personalized treatment strategies.

Pharmacological Management of Rheumatoid Arthritis: Approved Therapies and Mechanisms

The clinical management of rheumatoid arthritis (RA) relies on a combination of symptomatic relief and disease-modifying agents. Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently prescribed to alleviate pain and inflammation. Commonly used agents include naproxen sodium (Naperlan), meloxicam (Mobic), and ibuprofen (often formulated with famotidine in products such as Duexis) (60,61). However, the prolonged or excessive use of NSAIDs is associated with significant adverse effects, including gastrointestinal complications such as abdominal pain, peptic ulcers, and potential hemorrhage, as well as hematological issues like leukopenia and thrombocytopenia. To mitigate gastric toxicity, some formulations combine NSAIDs with proton pump inhibitors or H2 blockers like famotidine. Nevertheless, these medications carry risks of hepatic and renal impairment (60, 61).

Glucocorticoids (GCs) also play a role in symptom control. Delayed-release formulations, such as Rayos (prednisone), are designed to mimic natural circadian rhythms, thereby improving efficacy and reducing side effects (62, 63, 75). Despite their rapid anti-inflammatory action, long-term glucocorticoid therapy is limited by severe systemic side effects, including endocrine dysfunction, osteoporosis, weight gain, and increased susceptibility to infections due to

immunosuppression.

Methotrexate (MTX) remains the cornerstone and first-line conventional synthetic disease-modifying antirheumatic drug (csDMARD) for RA treatment (64, 65, 74). Structurally similar to folic acid, MTX is an antifolate agent originally developed for cancer chemotherapy, now utilized at much lower doses for autoimmune conditions. Its therapeutic efficacy in RA is mediated through multiple interconnected mechanisms:

1.Adenosine Accumulation: MTX inhibits enzymes involved in purine metabolism, leading to the extracellular accumulation of adenosine. This adenosine acts as a potent anti-inflammatory mediator by binding to cell surface receptors, thereby suppressing the release of pro-inflammatory cytokines and inhibiting T-cell activation and the expression of adhesion molecules (64, 65, 74).

2.Immune Cell Modulation: MTX enhances the sensitivity of activated T cells to CD95-mediated apoptosis and promotes the targeted depletion of B cells. It also suppresses methyltransferase activity, which disrupts immune cell function.

3.Pyrimidine Synthesis Inhibition: At higher intracellular concentrations, MTX metabolites inhibit dihydroorotate dehydrogenase, a key enzyme in pyrimidine synthesis. This inhibition restricts the proliferation of activated lymphocytes, exerting a potent anti-inflammatory effect.

4.Reduction of Autoimmune Markers: By modulating these pathways, MTX reduces the production of autoantibodies and rheumatoid factors, thereby mitigating the immunopathological damage characteristic of RA (64, 65, 74).

These diverse mechanisms collectively contribute to the disease-modifying capabilities of MTX, making it an essential component of RA treatment protocols.

DISCUSSION AND CONCLUSION

Current evidence consistently demonstrates that rheumatoid arthritis (RA) exhibits a marked gender disparity, with incidence rates in women being three to five times higher than in men. As a systemic autoimmune pathology, RA is characterized by the immune system's aberrant attack on joint tissues, leading to synovial inflammation, capsular thickening, and progressive erosion of cartilage and bone. While the precise etiology remains elusive, it is widely accepted that the disease arises from a complex interplay between genetic predispositions and environmental triggers.

Genetic analysis highlights the *HLA-DRB1* allele as a primary susceptibility factor, with specific variations correlating to disease severity and clinical presentation. This genetic risk is often compounded by environmental exposures, particularly air pollutants such as nitrogen oxides and sulfur dioxide, which have been identified as significant contributors to RA onset. The pathogenic mechanisms involve dysregulated signaling pathways that drive autoimmune responses, resulting in clinical features such as joint stiffness, deformity, and localized osteoporosis.

Currently, there is no curative therapy for RA. Consequently, clinical management strategies prioritize symptom control, aiming to reduce pain and inflammation while halting or slowing structural joint damage. Disease-modifying antirheumatic drugs (DMARDs) and other conventional therapies remain the cornerstone of treatment, essential for preserving joint function and improving the long-term quality of life for patients. Future research must continue to elucidate the molecular underpinnings of RA to develop more targeted and effective interventions.

Ethical Statements and Declarations Supplementary Materials

This article does not include any supplementary data or files.

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Author Contributions

R.R. and R.Sh. contributed to the conceptualization and software development of the study. R.R. was primarily responsible for the methodology. All authors participated in the critical review and editing of the final manuscript.

Data Availability

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

Conflict of Interest

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

Ethical Approval and Consent

As this study is a review article and does not involve human participants or animal subjects, ethical approval and informed consent were not required.

Consent for Publication

Not applicable.

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A Personalized Medicine Approach to Microbiome Analysis Aimed at Characterizing the Gut Microbiome

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ABSTRACT

The human gut microbiome constitutes a highly diverse and unique ecosystem that plays a critical role in shaping host metabolism, immune function, and vulnerability to numerous diseases. Thanks to recent breakthroughs in high-throughput sequencing, shotgun metagenomics, and integrative multi-omics strategies, researchers can now achieve comprehensive profiling of microbial communities with strain-level precision and detailed functional insights. Specific microbial patterns have emerged as reliable predictive biomarkers for assessing disease risk, tracking progression, and determining treatment outcomes in various conditions, including metabolic syndrome, inflammatory bowel disease, autoimmune disorders, and cancer. By combining microbiome data with host genomics, metabolomics, and clinical metrics, precision medicine is enhanced, facilitating tailored interventions such as dietary changes, probiotics, prebiotics, and fecal microbiota transplantation. Sophisticated bioinformatics tools, alongside machine learning and artificial intelligence, streamline the analysis of complex, high-dimensional multi-omics data, helping to pinpoint crucial microbial taxa, functional pathways, and predictive markers. Nevertheless, significant hurdles persist regarding the standardization of sample collection, sequencing protocols, bioinformatic workflows, and reproducibility across different study cohorts. Additionally, ethical issues such as data privacy, informed consent, and fair access require careful attention. Future studies that integrate longitudinal multi-omics profiling, mechanistic investigations of host–microbe interactions, and robust clinical validation of microbial biomarkers are expected to propel microbiome-driven personalized medicine forward. Ultimately, a thorough characterization of the gut microbiome offers a revolutionary approach to proactive, patient-centric healthcare, shifting focus from general population-based models to precise, individualized strategies for prevention, diagnosis, and therapy.

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INTRODUCTION

The human intestinal microbiome constitutes a complex and evolving ecosystem that is vital for host physiology, immune system regulation, metabolic processes, and susceptibility to diseases. Recently, progress in high-throughput sequencing and computational biology has provided remarkable insights into microbial diversity and function, establishing microbiome analysis as a fundamental component of precision and personalized medicine frameworks (1). Personalized medicine focuses on customizing medical decisions and treatments based

on individual biological profiles, with gut microbial signatures increasingly identified as valuable biomarkers capable of forecasting disease risk, therapeutic response, and overall health outcomes (1). Notably, differences in the composition and functional potential of gut microbiota are associated with various conditions, such as inflammatory bowel disease, metabolic syndrome, and autoimmune disorders, highlighting the potential of microbiome profiling for personalized healthcare strategies (2). With continuous technological improvements enhancing resolution and accuracy, characterizing individual gut microbiome signatures has become a promising pathway for refining diagnoses and customizing interventions in clinical settings. Consequently, comprehending the application of analytical methods to gut microbiome data within personalized medicine is crucial for translating research discoveries into effective clinical practices.

Advancements in metagenomic and multi-omics strategies have further strengthened the field by allowing for more comprehensive and functionally detailed profiling of gut microbial communities, surpassing the limitations of conventional taxonomic methods (1). Sophisticated metagenomic approaches, including long-read sequencing and integrated bioinformatics pipelines, enable high-resolution characterization of strain-level diversity and functional capabilities, which are critical for elucidating microbe–host interactions and identifying precise biomarkers of health and disease (3). When combined with the principles of personalized medicine, these advanced analytical tools offer significant potential for enhancing disease stratification, predicting therapeutic outcomes, and guiding customized treatment plans. However, challenges persist in standardizing data acquisition, analysis, and interpretation, as well as in integrating microbiome-derived insights into clinical decision-making (4). Moreover, converting microbiome data into actionable clinical tools demands rigorous validation and integration with other biological datasets, such as host genomics and metabolomics, to fully encompass the complexity of individualized health states. Thus, a systematic evaluation and synthesis of current analytical methodologies are required to direct future research and optimize applications within the realm of personalized medicine.

Personalized Medicine and the Gut Microbiome

Personalized medicine involves customizing clinical decisions, therapeutic regimens, and preventive measures to fit individual patient traits, such as genetic, environmental, and biological factors, instead of depending exclusively on broad population statistics. Although initially based on genomics and host genetics, this field has quickly broadened to incorporate additional biological layers like epigenomics, proteomics, metabolomics, and increasingly, the human microbiome. This evolution recognizes that inter-individual variations in biological systems can significantly affect disease susceptibility, treatment response, and prognosis, suggesting that more comprehensive biological profiling may enhance health outcomes. Recent studies emphasize that integrating diverse data sources enables clinicians to better stratify patients, predict therapeutic efficacy, and reduce adverse effects, especially in complex diseases where traditional biomarkers are inadequate. Thus, personalized medicine signifies a paradigm shift in healthcare—moving from reactive, one-size-fits-all approaches to proactive, data-driven, and patient-centered strategies. The integration of multilayer biological data, particularly from high-throughput technologies, facilitates the implementation of this precision framework in both research and clinical environments. Consequently, medical practice is shifting toward a more holistic characterization of individual biological profiles to optimize care (5, 6).

The gut microbiome consists of trillions of microorganisms, including bacteria, archaea, viruses, and fungi, which collectively influence host metabolism, immunity, nutrient absorption, and neurological signaling, thereby serving as a central element of human biology. Dysbiosis defined as an imbalance in the gut microbial community has been associated with an expanding list of chronic conditions, such as metabolic syndrome, inflammatory bowel disease, cardiovascular disorders, and neurological dysfunction, highlighting its systemic impact. Crucially, the composition and function of the gut microbiome vary significantly among individuals, shaped by diet, lifestyle, medications, and environmental exposures, which directly affects host health and disease progression. This inter-individual variability positions the gut microbiome as both a contributor to disease risk and a potential target for intervention, with microbial signatures offering insights into disease mechanisms and prognostic indicators. Understanding these microbial patterns can thus improve diagnostic precision and intervention strategies, particularly in diseases where standard clinical markers fail to capture underlying complexity. Consequently, the gut microbiome is not merely a biomarker of health status but also a mechanistic determinant of disease outcomes (6, 8).

Characterizing the gut microbiome at the individual level provides essential insights into host–microbe interactions and offers the potential to uncover biomarkers linked to disease onset, progression, and treatment response. As sequencing technologies and bioinformatic tools have advanced, researchers can now profile microbial communities with high resolution, revealing individual-specific microbiome signatures that influence therapy effectiveness and disease trajectory. Such characterization supports precision medicine by enabling the stratification of patients into subgroups with shared microbial features that correlate with clinical outcomes, thereby guiding more targeted interventions and reducing trial-and-error approaches in treatment selection. Furthermore, leveraging microbiome profiles alongside host genomic and clinical data enhances predictive models, allowing for better risk assessment and tailored therapeutic design. For instance, microbiome-based diagnostics are showing promise in predicting responsiveness to

cancer immunotherapy and in tailoring nutritional and metabolic interventions to an individual's unique microbial composition. Despite these advances, challenges remain in standardizing analytical methods and translating microbiome insights into routine clinical practice, emphasizing the need for integrative studies and robust clinical validation (9, 10).

Composition and Diversity of the Human Gut Microbiome

The human intestinal microbiome constitutes a highly intricate ecosystem comprised of bacteria, archaea, viruses, and fungi, each of which contributes to host well-being through metabolic, immunological, and signaling mechanisms. Bacterial species are the predominant members of this community, with Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria forming the most abundant phyla; their relative abundance significantly impacts host metabolism and immune homeostasis. Although present in lower quantities, archaea play pivotal roles in processes such as methanogenesis and the production of short-chain fatty acids, thereby affecting energy extraction and gut physiology. Viruses, particularly bacteriophages, regulate bacterial populations and facilitate horizontal gene transfer, which in turn influences the structure and functionality of the microbial community. Fungal communities, while relatively sparse, interact with host immune responses and help maintain the integrity of the gut barrier. The interkingdom interactions among these taxa establish a dynamic equilibrium that is crucial for sustaining gut homeostasis and overall health. Recent advancements in multi-omics and sequencing technologies have deepened our understanding of these microbial components, uncovering previously uncharacterized species and functional capabilities (11, 12). A thorough comprehension of the composition of these microbial communities lays the groundwork for identifying microbial signatures linked to health and disease.

The composition and diversity of the gut microbiome exhibit considerable variation among individuals, shaped by factors such as host genetics, age, diet, lifestyle, medications, and environmental exposures. This inter-individual variability serves as a key determinant of health outcomes and differential responses to therapies, underscoring the necessity of personalized approaches to microbiome analysis. Variables including dietary patterns, antibiotic consumption, geographic location, and early-life exposures significantly mold microbial diversity, impacting both microbial abundance and functional potential. Age-related alterations in gut microbiota composition, such as shifts in the Firmicutes/Bacteroidetes ratio, can affect metabolic processes and disease susceptibility. Furthermore, host genetics interact with microbial populations to modulate immune responses, nutrient absorption, and metabolic pathways, highlighting the complex interplay between host and microbe. Grasping these sources of variability is vital for developing reliable biomarkers and tailoring interventions in precision medicine (13, 14). By characterizing individual-specific microbiome profiles, researchers can identify microbial patterns associated with disease risk, therapeutic response, and the maintenance of health.

Determinants of Gut Microbiome Profiles

Host genetic makeup and epigenetic alterations are fundamental in determining the structure and function of the gut microbiome. Variations in host genes can influence immune reactions, the integrity of the mucosal barrier, and the availability of nutrients within the gut, which in turn affect how microbes colonize and proliferate. Research involving twins and families has shown that specific bacterial taxa are heritable, indicating a genetic influence on the composition of the microbiome. Furthermore, epigenetic processes, such as DNA methylation, histone modifications, and non-coding RNAs, regulate host–microbe interactions by altering gene expression in intestinal epithelial and immune cells. These dynamics can impact metabolic pathways, immune tolerance, and the risk of conditions like inflammatory bowel disease, obesity, and diabetes. Gaining insight into the genetic and epigenetic factors that shape the microbiome helps explain individual variations in microbial profiles and their relevance to personalized medicine. Combining host genomic information with microbiome data improves the prediction of disease risk and the design of customized interventions (15, 16). These integrative strategies are vital for creating precise, patient-specific therapeutic plans based on unique microbiome traits.

Environmental elements, such as diet, lifestyle choices, and medication exposure, significantly determine the composition and functionality of the gut microbiome. Dietary habits, including diets high in fiber, fat, or protein, directly modify microbial abundance, diversity, and the production of metabolites, which subsequently affects host metabolism and immune modulation. Lifestyle aspects, including physical exercise, stress levels, and sleep quality, also impact microbial makeup through endocrine and immunological mechanisms. Changes in the gut microbiome associated with aging reflect shifts in development, hormones, and immune function, with elderly individuals often showing decreased diversity and altered functional profiles. Drugs, especially antibiotics, can severely disrupt microbial communities, resulting in both immediate and prolonged dysbiosis. Additional factors like geographic location, hygiene practices, and exposure to pollutants further contribute to differences between individuals. A thorough understanding of these determinants is essential for interpreting microbiome data, creating personalized interventions, and forecasting therapeutic responses (17, 18). By accounting for host genetics, lifestyle, environmental exposures, and medication history, precision medicine can more effectively incorporate microbiome profiles into patient care strategies.

Sample Collection and Preprocessing Techniques

Accurate characterization of the gut microbiome is highly dependent on appropriate sample collection

strategies, as sampling methods directly influence the ability to capture microbial diversity and functional potential. Stool samples are the most commonly used specimens due to their non-invasive collection, ease of handling, and ability to represent microbial communities of the distal gastrointestinal tract. Consequently, stool-based sampling is widely applied in large-scale population studies and clinical research. However, it is increasingly recognized that stool samples primarily reflect luminal microbiota and may not fully represent mucosa-associated microbial communities, which often play a critical role in host–microbe interactions and disease mechanisms (19).

To address these constraints, researchers utilize alternative sampling methods like intestinal biopsies and mucosal scrapings to gain localized insights into the gut lining microbiota. While these techniques provide superior spatial resolution and direct evaluation of host-associated microbes, their invasive nature limits their feasibility for routine clinical application or longitudinal monitoring. Recent technological progress, encompassing endoscopic sampling, capsule-based collection devices, and minimally invasive rectal swabs, has broadened the ability to obtain site-specific microbiome samples while reducing patient discomfort. Furthermore, multi-site sampling facilitates comparative analyses between luminal and mucosal compartments, yielding a more comprehensive view of gut microbial ecology and enhancing the biological relevance of microbiome studies within personalized medicine frameworks (20).

Notwithstanding advancements in sampling technologies, issues concerning sample handling, storage, and preprocessing persist as significant sources of technical variability in microbiome research. Gut microbial communities are highly susceptible to environmental factors, including temperature fluctuations, oxygen exposure, and processing delays, all of which can modify microbial composition and metabolic activity. Consequently, standardized protocols for sample preservation, such as immediate freezing or the application of validated stabilization buffers, are crucial for maintaining microbial integrity. Additionally, variations in nucleic acid extraction techniques, sequencing library preparation, and bioinformatic pipelines can compromise reproducibility across different studies. Overcoming these hurdles demands strict compliance with standardized and validated workflows, the implementation of quality control measures, and transparent reporting of methodological details to ensure reliable taxonomic and functional profiling for precision medicine applications (21, 22).

Molecular and Omics Approaches for Microbiome Analysis

16S rRNA gene sequencing has become a cornerstone technique for profiling bacterial communities in the gut due to its cost-effectiveness, reproducibility, and ability to identify taxonomic composition at various phylogenetic levels. This method amplifies conserved regions of the bacterial 16S rRNA gene, allowing differentiation among bacterial taxa, though it is limited in resolution for strain-level identification and functional insights. In contrast, shotgun metagenomics sequences all genetic material in a sample, enabling comprehensive characterization of microbial diversity, including bacteria, archaea, viruses, and fungi, while also providing information on functional gene content. Shotgun metagenomics allows researchers to uncover microbial pathways involved in metabolism, immunity, and host interactions, offering deeper insights into microbiome function compared with 16S rRNA sequencing. Both techniques have been instrumental in large-scale microbiome studies, revealing correlations between microbial signatures and disease phenotypes, dietary influences, and therapeutic responses. Selection of sequencing method depends on study objectives, sample type, and desired resolution, with many researchers now integrating both approaches for complementary insights. Advanced bioinformatic pipelines are crucial for accurate taxonomic assignment, functional annotation, and downstream statistical analysis (23, 24). Table 1 provides a summary of key recent studies in this field.

While taxonomic profiling provides essential insights into microbiome composition, functional omics approaches such as metatranscriptomics and metabolomics are crucial for understanding microbial activity and host–microbe interactions. Metatranscriptomics measures gene expression in microbial communities, revealing active metabolic pathways and dynamic responses to environmental stimuli, including diet, medications, or disease states. Metabolomics complements this by profiling small molecules, metabolites, and signaling compounds produced by microbes, reflecting functional outputs that directly impact host physiology. Together, these approaches enable a more comprehensive understanding of microbiome function beyond mere presence or abundance of taxa, offering mechanistic insights into microbial contributions to health and disease. Integration of multi-omics datasets allows for correlation of microbial composition with functional capacity, enhancing the predictive power for personalized medicine applications. Challenges remain, including data integration, normalization, and interpretation of complex datasets, but these methods are rapidly advancing and becoming central to microbiome research (25, 26).

Bioinformatics and Data Analysis Pipelines

Bioinformatics pipelines are indispensable tools for transforming raw microbiome sequencing data into interpretable and biologically meaningful insights. They provide standardized workflows for processing, quality control, taxonomic classification, and functional annotation of microbial communities. Popular tools such as QIIME, MetaPhlAn, and HUMAnN have become widely adopted due to their versatility and reliability. QIIME (Quantitative Perspectives on Microbial Ecology) is primarily used for 16S rRNA gene sequencing data, offering comprehensive processing steps including sequence quality filtering,

Table 1. Key Recent Studies on Gut Microbiome Profiling and Personalized Medicine (2020–2025).

No.	Study (Authors, Year)	Population / Focus	Methods / Key Findings	Reference (APA)
1	Mousa & Al Ali (2024)	Patients with inflammatory bowel disease	Shotgun metagenomics identified microbial signatures associated with disease phenotype and therapeutic response; supports personalized diagnostics.	23
2	Zou et al. (2024)	Colorectal cancer patients	Multi-omic gut microbiome profiling revealed links between microbial composition, host genomics, and cancer progression.	24
3	Al Bataineh et al. (2025)	Middle Eastern CRC cohort	Identified strain-level microbial biomarkers for early detection and precision prevention strategies.	25
4	Piccinno et al. (2025)	Clinical colorectal cancer patients	Stool metagenomes revealed site-specific strain biomarkers for CRC progression and prognosis.	26
5	Song et al. (2025)	Acute pancreatitis	Shotgun metagenomics linked microbial composition and functional pathways to recovery and complications.	23
6	Human gut microbiome review (2025)	Clinical implementation	Review of metagenomic approaches, challenges, and clinical integration in precision medicine.	24
7	Multi-omics + Machine Learning (2025)	Precision profiling	Integration of multi-omics and machine learning enhanced biomarker discovery for personalized interventions.	25

taxonomic assignment, and alpha and beta diversity analyses. MetaPhlAn (Metagenomic Phylogenetic Analysis) focuses on shotgun metagenomics, enabling accurate species- and strain-level taxonomic profiling, while HUMAnN (The HMP Unified Metabolic Analysis Network) provides functional characterization by linking microbial taxa to metabolic pathways and biological processes. The combined use of these pipelines allows simultaneous assessment of microbial composition and functionality, enhancing reproducibility and comparability across studies, which is critical for applications in personalized medicine (37, 38).

Despite their practical value, the analysis of microbiome data presents distinct computational and statistical hurdles stemming from inherent characteristics like compositionality, sparsity, and high dimensionality. To reduce biases caused by variations in sequencing depth, normalization methods such as rarefaction, cumulative sum scaling (CSS), or centered log-ratio (CLR) transformation are frequently utilized. Feature selection techniques, encompassing differential abundance testing, machine learning algorithms, and network-based analyses, assist in pinpointing crucial microbial taxa or functional pathways linked to disease phenotypes or therapeutic results. These strategies enable researchers to concentrate on biologically significant signals amidst the complexity of microbial communities, thereby enhancing the reliability of subsequent interpretations (37, 38).

However, several persistent challenges remain, including batch effects, technical variability between sequencing runs, and limited reproducibility of results across different laboratories. Integrating multi-omics datasets, such as metagenomics, metatranscriptomics, metabolomics, and host genomic data, adds additional layers of complexity, requiring sophisticated statistical frameworks and computational workflows to ensure accurate and meaningful analyses. Addressing these challenges is essential for generating reliable microbial biomarkers and functional signatures that can be confidently applied in personalized medicine. Emerging bioinformatics platforms are now incorporating automated workflows, standardized pipelines, and interactive visualization tools to streamline data analysis and facilitate cross-study comparisons (39, 40).

By integrating high-quality sequencing data with reliable computational methods, bioinformatics pipelines allow researchers to derive detailed compositional and functional insights from complex microbiome datasetsThis integration not only enhances comprehension of host–microbe interactions but also facilitates the discovery of clinically relevant biomarkers, predictive models, and therapeutic targets. Ongoing development and refinement of bioinformatics tools will further enhance accuracy, reproducibility, and translational potential, thereby thereby ultimately closing the gap between microbiome research and the implementation of precision medicine strategies (37–40).

Microbial Biomarkers for Personalized Medicine

The identification of individual-specific microbiome signatures represents a fundamental pillar of personalized medicine, as it enables more accurate prediction of disease susceptibility, prognosis, and therapeutic response. Advances in high-resolution sequencing technologies, particularly shotgun metagenomics and integrated multi-omics approaches, have made it possible to detect strain-level microbial variations and functional potentials that differ markedly between individuals. These technologies allow researchers to move beyond broad taxonomic

classifications and instead focus on functionally relevant microbial traits that are directly linked to host physiology. Consequently, distinct microbial signatures can be utilized as biomarkers for a broad spectrum of conditions, encompassing metabolic, inflammatory, autoimmune, and infectious diseases (41, 42).

Personalized microbiome profiling also incorporates the substantial inter-individual variability driven by host genetics, dietary habits, lifestyle factors, medication use, and environmental exposures. By accounting for these sources of variability, microbiome-based biomarkers provide a more precise and biologically meaningful understanding of host–microbe interactions than population-averaged measures. Large-scale cohort studies have demonstrated consistent associations between specific microbial taxa, microbial-derived metabolites, and host health status, reinforcing their potential utility as individualized biomarkers. These findings support the use of microbiome profiles not only for disease characterization but also for patient stratification and risk assessment in clinical settings (41, 42).

Individual-specific microbiome signatures have been linked to disease risk and therapeutic outcomes across diverse clinical contexts, including cancer, metabolic disorders, autoimmune diseases, and infectious conditions. In particular, certain microbial taxa and metabolic pathways have been shown to predict responsiveness to immunotherapy, chemotherapy, and dietary interventions, enabling clinicians to tailor treatment strategies to individual patients. Furthermore, characteristic dysbiosis patterns may serve as early indicators of disease onset or progression, creating opportunities for preventive or early-stage interventions. Such applications highlight the growing role of the microbiome as a dynamic and informative biomarker system in precision medicine (43, 44).

Integrative analytical approaches that combine microbiome data with metabolomic, transcriptomic, and host genomic information further enhance predictive accuracy and provide mechanistic insights into host–microbe interactions. These multi-layered models support the concept of microbiome-guided precision medicine, in which clinical decisions and therapeutic interventions are informed by an individual's microbial composition and functional potential. Despite these advances, challenges remain in standardizing biomarker discovery pipelines, validating findings across diverse populations, and establishing clinical utility in routine practice. Nevertheless, continued methodological refinement and large-scale validation studies are expected to facilitate the integration of microbial biomarkers into patient care, ultimately improving therapeutic efficacy and reducing adverse outcomes (43, 44).

Clinical Applications of Gut Microbiome Characterization

The characterization of the gut microbiome has evolved into a potent and adaptable instrument for clinical diagnostics and prognostic evaluation across numerous disease states. A substantial body of research indicates that distinct microbial taxa, community architectures, and functional profiles are linked to ailments such as colorectal cancer, inflammatory bowel disease, metabolic disorders, cardiovascular conditions, and neurodegenerative diseases. Microbiome profiling facilitates non-invasive or minimally invasive disease detection, primarily via stool specimens, rendering it highly suitable for population screening and early diagnosis. Microbial biomarker panels extracted from these profiles can improve early disease detection and aid in patient stratification according to disease severity, progression risk, or anticipated clinical outcomes (45, 46).

Recent advances have shown that gut microbial signatures can complement traditional diagnostic tools, improving diagnostic accuracy and predictive performance. The integration of metagenomic, metatranscriptomic, and metabolomic data allows simultaneous assessment of microbial composition and functional activity, providing deeper insights into disease-associated pathways and mechanisms. Such integrative approaches enable identification of microbial metabolites and signaling pathways that are directly linked to disease progression and clinical outcomes. As a result, microbiome-informed diagnostics offer a more comprehensive and systems-level perspective compared to conventional biomarkers alone, supporting more informed clinical decision-making and risk assessment (45, 46).

Beyond diagnostics and prognosis, intestinal microbiome profiling plays a critical role in monitoring health status and evaluating treatment responses. Alterations in microbial composition and function can reflect patient responses to dietary modifications, pharmacological treatments, probiotics, prebiotics, and fecal microbiota transplantation. Longitudinal microbiome analyses allow clinicians to track dynamic changes in microbial diversity, stability, and metabolic activity over time, providing valuable information on therapeutic efficacy, treatment adherence, and potential relapse. Functional profiling, in particular, can reveal metabolic shifts associated with treatment response, while taxonomic changes may signal emerging dysbiosis or increased risk of adverse events (47, 48).

Integration of microbiome data with host clinical parameters, laboratory findings, and demographic information further enhances predictive modeling and supports personalized adjustments to therapeutic strategies. Continuous or repeated microbiome monitoring has the potential to guide individualized treatment plans, enabling timely intervention modifications and improving overall patient outcomes. Although technical, analytical, and standardization challenges remain, the development of reproducible biomarkers and standardized analytical pipelines is steadily advancing the clinical feasibility of microbiome-based applications. Together, these advancements highlight the increasing significance of intestinal microbiome characterization within precision medicine and its potential to enhance diagnostic accuracy, monitoring capabilities, and individualized patient management (47, 48). Table 2 presents recent studies highlighting the clinical applications of gut

Table 2. Recent Studies Highlighting Clinical Applications of Gut Microbiome (2020–2025).

No.	Study (Authors, Year)	Disease / Population	Clinical Application	Key Findings
1	Thomas et al., 2020	Various diseases	Diagnostic	45
2	Zeller et al., 2021	Colorectal cancer	Early detection	46
3	Li et al., 2022	Metabolic disorders	Monitoring treatment	47
4	Khalighi et al., 2023	Autoimmune disease	Therapeutic optimization	48
5	Yu et al., 2024	IBD patients	Prognosis	45
6	Chen et al., 2024	Cancer patients	Therapy response	46
7	Nakamura et al., 2025	Pediatric patients	Health monitoring	47

microbiome characterization.

Challenges, Limitations, and Ethical Considerations

Notwithstanding the rapid progress in gut microbiome research, substantial technical and methodological hurdles persist. Variations in sample collection, storage, and DNA extraction procedures can introduce biases that compromise reproducibility and comparability across different studies. Differences in sequencing platforms, library preparation techniques, and bioinformatics pipelines further contribute to inconsistencies in taxonomic and functional profiling. Therefore, standardizing protocols is crucial to ensure uniform data quality and reliable biomarker identification. Moreover, the extensive inter-individual variability of the gut microbiome complicates the interpretation of results and the creation of universal reference databases. The integration of multi-omics datasets introduces additional analytical complexities, necessitating advanced computational tools and specialized expertise. Overcoming these limitations is vital for translating microbiome research into clinically actionable interventions. Recent investigations highlight the necessity for standardized and reproducible methodologies to facilitate robust applications in personalized medicine. (59, 60).

Interpreting microbiome data for clinical application remains complex due to biological variability and incomplete understanding of host–microbe interactions. Many associations are correlative rather than causative, complicating decision-making in personalized interventions. Reproducibility across cohorts and populations is a major concern, necessitating rigorous study design, validation, and replication. Beyond technical issues, microbiome-based medicine raises privacy and ethical challenges. Microbial profiles can reveal sensitive health information, requiring secure data storage and controlled access. Informed consent, data sharing policies, and considerations of equity in personalized treatments are essential. Ethical frameworks must evolve alongside technological advances to ensure responsible use of microbiome data in clinical practice. Recent literature highlights these dual challenges, emphasizing both methodological rigor and ethical governance as cornerstones of safe and effective microbiome-based personalized medicine (61, 62). Table 3 presents recent studies highlighting future perspectives in gut microbiome research (2020–2025).

Future Perspectives and Research Directions

Artificial intelligence (AI) and machine learning (ML) are revolutionizing gut microbiome research by facilitating the analysis of intricate, high-dimensional datasets. Predictive models can identify microbial patterns associated with disease risk, therapeutic response, and host metabolic profiles. ML algorithms facilitate feature selection, biomarker discovery, and functional predictions from metagenomic, metatranscriptomic, and metabolomic data. Integrating AI with longitudinal microbiome studies enhances the ability to track temporal dynamics and predict individualized outcomes. Such approaches also allow simulation of personalized interventions, optimizing diet, probiotics, and other therapeutics based on predicted microbiome responses. Recent studies demonstrate that AI-driven models outperform traditional statistical methods in predicting disease and treatment outcomes, paving the way for precision medicine applications. However, model transparency, validation, and interpretability remain critical to clinical adoption (63, 64).

Upcoming microbiome research is shifting toward the integration of multi-omics datasets spanning metagenomics, metatranscriptomics, metabolomics, and proteomics within systems biology frameworks. This strategy offers a holistic perspective on microbial community architecture, functionality, and host interactions, facilitating precise mechanistic understanding. By combining multi-omics data with AI-driven predictive models, it is possible to direct the creation of fully personalized gut microbiome-based therapies, such as customized diets, probiotics, prebiotics, and fecal microbiota transplantation. Preliminary clinical trials incorporating these strategies have demonstrated enhanced therapeutic efficacy and fewer adverse effects, underscoring the potential of precision interventions. Ethical concerns, data privacy, and standardization will continue to be pivotal as these methodologies move into routine clinical practice. The

Table 3. Recent Studies on Future Perspectives in Gut Microbiome Research (2020–2025).

No	Authors (Year)	Technology / Approach	Focus / Application	Key Findings	Reference
1	Ghosh et al., 2021	Machine learning	Predictive microbiome modeling	ML models accurately predicted microbial signatures associated with disease risk	61
2	Reiman et al., 2022	Artificial intelligence	Personalized treatment simulation	AI-driven approaches improved prediction of individual responses to diet and therapy	62
3	Zhang et al., 2023	Multi-omics integration	Personalized therapeutics	Integration of metagenomics, metabolomics, and proteomics enabled precise microbiome-targeted interventions	63
4	Vatanen & Kostic, 2024	Multi-omics + computational modeling	Fully personalized therapies	Systems biology and modeling guided development of individualized microbiome-based treatments	64
5	Zmora et al., 2021	AI + diet personalization	Metabolic and immune health	AI-assisted diet interventions improved host metabolic outcomes based on individual microbiomes	61
6	Li et al., 2022	Multi-omics + ML	Therapeutic biomarker discovery	Identified predictive microbial and metabolite biomarkers for treatment response	62
7	Gonzalez et al., 2024	Multi-omics	Immunotherapy personalization	Microbiome and metabolomics integration predicted individual response to checkpoint inhibitors	63

synthesis of sophisticated computational techniques, high-resolution multi-omics, and individualized therapeutic design marks the next major advancement in microbiome-guided personalized medicine (61, 62).

DISCUSSION

The findings summarized in this review highlight the gut microbiome as a highly complex and individualized biological system with a central role in advancing personalized medicine. The considerable inter-individual variation in microbial composition and function driven by host genetics, environmental factors, dietary habits, lifestyle choices, and medical treatments offers a convincing rationale for the diverse disease susceptibility and differing therapeutic outcomes observed across patients. This variability positions the gut microbiome as a critical complementary layer to host genomic and clinical data within precision medicine frameworks.

Recent advances in high-throughput sequencing technologies, particularly shotgun metagenomics and integrated multi-omics approaches, have enabled strain-level resolution and functional characterization of microbial communities. These advancements have demonstrated that disease associations are frequently driven not just by the presence or absence of specific taxa, but by microbial functional capacity, metabolic activity, and gene expression patterns. Consequently, relying exclusively on taxonomic profiling may miss critical mechanistic insights, highlighting the significance of functional omics, such as metatranscriptomics and metabolomics, in microbiome research.

Notwithstanding these technological advancements, several challenges constrain the clinical translation of microbiome-based findings. A primary obstacle is the absence of complete standardization across sample collection, storage, DNA extraction, sequencing platforms, and bioinformatic pipelines, which hinders cross-study comparisons and meta-analyses. Furthermore, microbiome datasets are inherently compositional, sparse, and high-dimensional, presenting substantial statistical and computational hurdles. These factors can compromise reproducibility and impede the identification of robust, clinically actionable microbial biomarkers.

From a clinical perspective, many reported associations between microbiome features and disease states remain correlative rather than causative. Most existing studies are observational, emphasizing the need for well-designed longitudinal studies and randomized controlled trials to establish causal relationships and validate therapeutic interventions. Furthermore, population-specific factors such as ethnicity, geography, and dietary habits may limit the generalizability of identified microbial signatures, highlighting the importance of diverse and representative cohorts in microbiome research.

Ethical and regulatory frameworks further complicate the integration of microbiome profiling into routine clinical practice. Microbiome data can disclose sensitive information regarding health status, disease risk, and lifestyle, raising concerns regarding data confidentiality, patient autonomy, and fair distribution of resources personalized interventions. Addressing these ethical challenges is essential to ensure the responsible and trustworthy implementation of microbiome-based personalized medicine.

CONCLUSION

To conclude, the gut microbiome constitutes a highly personalized and dynamic ecosystem that significantly impacts host metabolism, immune regulation, disease susceptibility, and therapeutic results. Progress in metagenomics, high-resolution sequencing, and multi-omics integration has allowed for the detailed

characterization of individual-specific microbial signatures, thereby reinforcing the expanding role of the microbiome within personalized medicine.

Combining gut microbiome data with host genomics, metabolomics, and clinical parameters improves predictive accuracy and facilitates the creation of customized interventions, such as personalized dietary plans, prebiotics, probiotics and fecal microbiota transplantation. These strategies hold the potential to enhance treatment efficacy, minimize adverse effects, and optimize patient outcomes across a broad spectrum of diseases.

Nevertheless, significant challenges remain before microbiome-informed personalized medicine can be fully implemented in clinical settings. Standardization of methodologies, improved reproducibility, large-scale longitudinal studies, and rigorous clinical validation are critical for translating research findings into reliable diagnostic and therapeutic tools. In parallel, ethical frameworks addressing data privacy, consent, and equitable access must evolve alongside technological advancements.

In the future, the incorporation of longitudinal multi-omics datasets with artificial intelligence and machine learning methodologies is anticipated to further enhance predictive models and facilitate the development of truly personalized therapeutic strategies. Such developments have the potential to transform healthcare from a population-based paradigm to a fully personalized approach, establishing the gut microbiome as a foundational pillar of future precision medicine.

Authors's Contribution

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Investigation of Gene Expression and DNA Methylation of IGF2, PPARγ, LEP, and CDKN1C in Gestational Diabetes Mellitus

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ABSTRACT

Gestational diabetes mellitus (GDM) is a prevalent complication of pregnancy associated with adverse outcomes for both mother and fetus. Epigenetic modifications, particularly DNA methylation, may play a significant role in its pathogenesis. This study aimed to evaluate the expression and methylation status of IGF2, PPARγ, LEP, and CDKN1C in women with GDM. In this case control study, 50 women with GDM and 50 healthy pregnant women were included. Gene expression levels and DNA methylation patterns were analyzed, and clinical risk factors were assessed. Significant differences were identified in both expression and methylation profiles of the studied genes between GDM patients and controls. Pre-pregnancy BMI, high-fat diet, and family history of diabetes were significantly associated with GDM. These results indicate that GDM is influenced by metabolic, environmental, and epigenetic factors, and that altered expression and methylation of IGF2, PPARγ, LEP, and CDKN1C may contribute to its development.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as hyperglycemia first detected during pregnancy based on IADPSG/WHO criteria and represents one of the most common pregnancy complications, affecting about 14% of pregnancies worldwide (1–3). Poorly controlled GDM is associated with adverse maternal outcomes such as gestational hypertension, pre-eclampsia, and Caesarean delivery, as well as long-term risks including cardiovascular disease, obesity, and progression to Type 2 diabetes in both mother and offspring (4–7).

The pathogenesis of GDM remains incompletely understood and appears to involve complex interactions among genetic, metabolic, and environmental factors (8). Established risk factors include advanced maternal age, pre-pregnancy obesity, family history of diabetes, prior GDM, infections such as HIV, smoking, and socioeconomic conditions (9- 14). Mechanistically, GDM is linked to β-cell dysfunction, insulin resistance, adipose tissue dysfunction, gluconeogenesis, gut microbiota imbalance, and oxidative stress (15), with growing evidence highlighting the contribution of epigenetic regulation (16). Epigenetic mechanisms—including DNA methylation, histone modifications, and miRNA-mediated regulation—can alter gene expression without changing DNA sequence (17). DNA methylation, particularly at CpG sites, plays a major regulatory role, influencing gene expression depending on its genomic location (18), although its direct relationship with expression remains inconsistently reported (19).

Several studies have explored methylation changes in GDM, including genome-wide alterations prior to diagnosis and gene-specific promoter methylation in metabolic pathways (20–22). Among key genes, LEP regulates energy homeostasis and insulin secretion (23, 24), PPARγ is involved in adipogenesis and glucose metabolism and is linked to diabetes and pregnancy metabolic adaptation (25–27), IGF2 influences insulin sensitivity and fetal growth (28–30), and CDKN1C controls cell proliferation, including pancreatic β-cell expansion (31–33). Despite these findings, most studies have examined methylation or gene expression separately, with limited integration of clinical and lifestyle factors. Therefore, this study aimed to simultaneously assess methylation and expression of these genes alongside relevant clinical and lifestyle variables in pregnant women with GDM.

This study aimed to investigate the expression levels and DNA methylation status of key metabolic genes, including IGF2, PPARγ, LEP, and CDKN1C, in women with GDM compared with healthy pregnant controls. In addition, the study sought to evaluate the association between these molecular alterations and clinical as well as lifestyle-related risk factors in order to provide a more comprehensive understanding of the mechanisms underlying GDM pathogenesis.

MATERIALS AND METHODS

Study design

This study included 50 pregnant women with GDM and 50 healthy pregnant women with normal blood glucose levels based on the IADPSG diagnostic criteria, and the two groups were matched for age and body mass index (BMI). People with metabolic diseases other than gestational diabetes and those taking certain medications, such as steroids, were excluded from the trial due to their potential impact on gene expression and methylation. All required information was collected with the consent of the participants in this study through a questionnaire and medical records.

Sampling and RNA extraction

5 ml of peripheral blood was collected from each participant in EDTA tubes and stored at -80°C until use. RNA was extracted from peripheral blood using the Blood RNA Isolation Kit (DENAzist co). The purified RNA was then converted to cDNA using the Reverse Transcription Kit (DENAzist co).

Real-Time Polymerase Chain Reaction

Real-time PCR analysis of adiponectin, IGF2, PPARγ, LEP, CDKN1C, and GAPDH (as the reference gene) was performed using the SYBR Green PCR Master Mix assay. Each reaction was carried out under specified conditions in a total volume of 10.0 µL, containing 5.0 µL of SYBR Green PCR Master Mix, 0.5 µL of forward primer and 0.5 µL of reverse primer for each target gene (Table 1) at a final concentration of 10 pmoL, 1.0 µL of cDNA template, and 3.0 µL of RNase-free water. The thermal cycling protocol consisted of denaturation at 95°C for 15 seconds, primer annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Data were analyzed using the ΔΔCt method.

DNA methylation screening

DNA extraction

DNA was extracted using the Blood DNA isolation kit (Denazist co) according to the protocol.

Bisulfite treatment

In this study, the Qiagen bisulfite treatment kit (EpiTect Bisulfite Kits) was used for DNA sequence treatment.

Methylation-specific PCR reaction

Methylation-specific polymerase chain reaction (MSP) is a technique that has facilitated the detection of promoter hypermethylation at CpG islands in cell lines and clinical samples (34). Specific primers were designed for methylated and unmethylated sequences of each gene (Table 1). Moreover, uni DNA and water were used as positive and negative controls. PCR reactions were performed in a thermocycler, and then the products were electrophoresed on a 2% agarose gel.

Statistical analysis

For comparative analysis of gene expression levels between groups, the relative expression ratio was used with the ΔΔCt method in Real-Time PCR. To compare quantitative variables between groups, an independent t-test was used, and to examine qualitative variables, chi-square test was used. Data were reported as Mean ± SD. To examine the association of risk factors with GDM, logistic regression model was used. Results were reported as Odds Ratio (OR) with 95% Confidence Interval (CI). A significance level of $p < 0.05$ was considered as the threshold of significance in all tests. All analyses were performed with SPSS version 26 software. GraphPad Prism software was also used to prepare visual graphs. In addition, the intensity of methylated and unmethylated bands was evaluated for semi-quantitative analysis by ImageJ software.

RESULTS

Patient characteristics

Participant characteristics are presented in Table 2. The age range of patients with Gestational diabetes mellitus (GDM) was 20 to 41 years (mean: 32.5

Table 1. Sequence of primers used and product size *M: methylated DNA, U: unmethylated DNA

q-PCR			
Gene	**Primer sequence**	**Tm ◦C**	**Product size (bp)**
IGF2	F: GTGGCATCGTTGAGGAGTG R; CACGTCCCTCTCGGACTTG	60	92
PPARY	F: ACCAAAGTGCAATCAAAGTGGA R: ATGAGGGAGTTGGAAGGCTCT	60	100
LEP	F: TGCCTTCCAGAAACGTGATCC R: CTCTGTGGAGTAGCCTGAAGC	61	164
CDKN1C	F: GTGAGCCAATTTAGAGCCCA R: CGGTTGCTGCTACATGAACG	59	104
GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	60	197
MSP			
IGF2-M	F: GACGTTAATTTTCGGGGACGTT R: AACGCGAAATAAAACGAACGTATACGA	55	363
IGF2-U	F: TTTTGGATGTTAATTTTTGGGGATGTT R: AAAAAAACACAAAATAAAACAAACATATACAA	64	373
PPARY-M	F: AAGACGGTTTGGTCGATC R: CGAAAAAAAATCCGAAATTTAA	52	124
PPARY-U	F: GGGAAGATGGTTTGGTTGATT R: TCCAAAAAAAAATCCAAAATTTAA	53	128
LEP-M	F: TTTGGAGGGATATTAAGGATTTTTC R: TACAACCGCTAACGCTACGAT	59	301
LEP-U	F: TTTGGAGGGATATTAAGGATTTTTT R: ACCTTACAACCACTAACACTACAAT	59	305
CDKN1C-M	F: GTTAGTTGGCGTAGGAGGTTTAC R: AAAACGCTACGAACGATAACG	55	257
CDKN1C-U	F: TTAGTTGGTGTAGGAGGTTTATGG R: TAAAAAACACTACAAACAATAACATA	55	259

years), while in the healthy control group it ranged from 22 to 43 years (mean: 33.4 years). The findings indicated significant associations between pre-pregnancy BMI, high-fat diet, and family history of diabetes with GDM (P=0.04, Odds ratio: 2.062, 95% CI: 1.009–4.213; P=0.000, Odds ratio: 0.193, 95% CI: 0.081–0.459; P=0.011, Odds ratio: 3.777, 95% CI: 1.342–10.628). In contrast, no significant associations were found between GDM and maternal age, systolic blood pressure, physical activity, gestational weight gain, or smoking.

Comparison of the expression status

Based on the results obtained, IGF2 gene expression was increased 1.6-fold in the GDM group (P=0.0.041). Regarding the PPARγ gene, a 0.44-fold decrease in expression was observed in the GDM group (P=0.039). The LEP gene showed a 1.8-fold increase in expression

Table 2. Association between clinical characteristics of control and GDM groups and risk factors with GDM

Clinical characteristics/risk factors	GDM (n=50)	Control (n=50)	p-value*
Maternal age (Mean ± SD)	32.5±3.2	33.4±2.8	0.923
Pre-pregnant BMI (kg/m2) (Mean ± SD)	30.2±3.1	16.6 ± 2.5	**0.04**
Systolic blood pressure (mmHg) (Mean ± SD)	120 ± 12	116 ± 10	0.887
Physical activity			
Yes	18(36%)	24(48%)	0.225
No	32(64%)	26(52%)	
Diet			
High-fat	31(62%)	12(24%)	**0.000**
Low-fat	19(38%)	38(76%)	
Weight gain during pregnancy (kg) (Mean ± SD)	18 ± 4	12 ± 3	0.337
Smoking	4(8%)	5(10%)	0.727
Non-smoking	46(92%)	45(90%)	
Family history of diabetes			
Yes	١٧(34%)	6(12%)	**0.011**
No	33(66%)	44(88%)	

in the GDM group compared to the control group (P=0.0.036). A 0.5-fold decrease in expression was observed in the GDM group for the CDKN1C gene (P=0.028) (fig 1).

Comparison of the DNA methylation status

Table 3 shows the methylation status of IGF2, PPARγ, LEP, and CDKN1C genes in the control and GDM groups.

IGF2 gene

The methylation level was significantly reduced in the GDM group. In the GDM group, 38% of the samples were methylated, while in the control group this value was 64% (P=0.01, Odds ratio: 0.344, 95 % CI: 0.153-0.776).

PPARγ gene

No significant difference was observed in the methylation level of the PPARγ gene between the two groups. The methylation percentage in the GDM group was 52%, and in the control group was 56%.

LEP gene

Reduced methylation levels were observed in the LEP gene promoter in the GDM group. 36% of patient samples were methylated, while in the control group this value was 68% (P=0.001, Odds ratio: 3.777, 95 % CI: 1.649-8.651).

CDKN1C gene

Increased methylation of the CDKN1C gene was observed in the GDM group. 60% of samples in the GDM group were methylated, while this value was 28% in the control group (P=0.001, Odds ratio: 0.259, 95 % CI: 0.112-0.599).

In figure 2, the results of the bands obtained from 2% gel electrophoresis related to the 4 genes IGF2, PPARγ, LEP, and CDKN1C using the MSP-PCR technique, along with positive and negative controls, can be seen.

DISCUSSION

GDM is a multifactorial disorder shaped by genetic, epigenetic, and environmental influences (35). In the present study, the expression and methylation status of four key genes IGF2, PPARγ, LEP, and CDKN1Cwere examined in patients with GDM and compared with those in the control group. The findings revealed significant alterations in both expression and methylation patterns of these genes in GDM, highlighting their potential involvement in the pathophysiology of the disease.

Based on the results of comparing clinical characteristics and some risk factors between the GDM and control groups in this study, significant associations were observed between BMI, High-fat diet and family history of diabetes and GDM. These risk factors likely exacerbate the observed epigenetic and gene expression changes. This associations have

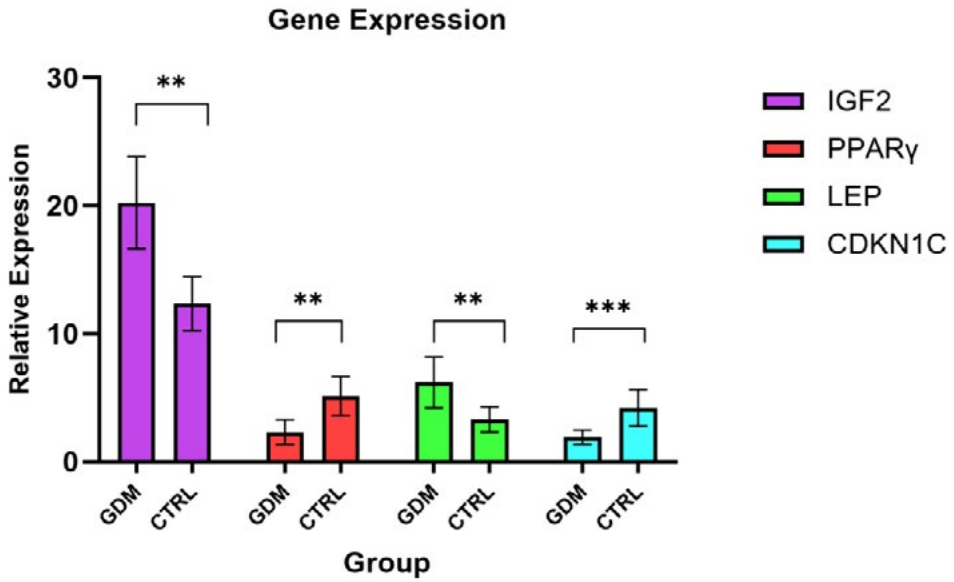

Fig 1. Schematic diagram comparing the expression levels of IGF2, PPARγ, LEP, and CDKN1C genes between the control and GDM groups using real-time PCR technique.

Table3. Comparison of methylation of IGF2, PPARγ, LEP and CDKN1C genes in control and GDM groups.

gene	methylation in the GDM group (n=50)	methylation in the control group (n=50)	odds ratio 95 % CI	P-value*
IGF2	19(38%)	32(64%)	0.344 0.153-0.776	**0.010**
PPARγ	26(52%)	28(56%)	0.851 0.3874-1.870	0.688
LEP	18(36%)	34(68%)	3.777 1.649-8.651	**0.001**
CDKN1C	30(60%)14(28%)	14(28%)	0.259 0.112-0.599	**0.001**

also been noted in other studies (36, 37, 38). In fact, it can be said that pre-pregnancy obesity and high BMI are among the most important risk factors for GDM and lead to increased insulin resistance and chronic inflammation (36). Changes in lipid profile (such as increased LDL cholesterol and triglycerides), which can be caused by obesity and an unhealthy, high-fat diet, are a characteristic of GDM and can affect the health of the mother and fetus (37). These types of diets lead to inflammation and insulin resistance (38). Also, advanced maternal age at the time of pregnancy and a family history of diabetes are strong risk factors for GDM (39).

Based on the findings, IGF2 gene expression was approximately 1.6-fold higher in the GDM group compared with the control group. In a study investigating alterations in the IGF axis during pregnancy, elevated serum IGF2 levels were reported in women with GDM, suggesting that increased IGF-2 may influence maternal and fetal metabolic processes (30). In contrast, another study examining IGF-I and IGF-II levels in mothers and fetuses and their association with fetal growth and gestational diabetes found no significant difference in maternal IGF-II levels between diabetic and non-diabetic groups (40). Additionally, Wei et al. reported increased IGF2 expression in placental tissues from patients with diabetes (including gestational diabetes and Type 2 diabetes) compared to controls, suggesting that this upregulation may represent an adaptive response to the intrauterine hyperglycemic environment (41). Elevated IGF2 expression may contribute to the development or exacerbation of GDM by promoting insulin resistance, impairing placental function, and enhancing nutrient

transfer to the fetus. Furthermore, studies in both murine and human samples demonstrated that increased IGF2 expression is associated with higher body weight, and that IGF2 levels in maternal peripheral blood and fetal cord blood are independently linked to macrosomia. These findings highlight the important role of IGF-2 in regulating growth and glucose–lipid metabolism during pregnancy and the postpartum period (42).

The expression of the PPARγ gene was significantly reduced in the GDM group, reaching 0.44 relative to the control group. This observation is in agreement with previous studies reporting an association between decreased PPARγ expression and disturbances in lipid and glucose metabolism (43). Given the key role of this gene in modulating insulin sensitivity, alterations in its expression may contribute to increased insulin resistance and the progression of GDM (44).

The expression level of the LEP gene was significantly increased in the GDM group compared to the control group (1.8-fold). The increased expression of this gene in GDM usually occurs due to insulin resistance and inflammatory changes. LEP is secreted by fat cells and in GDM, the level of this hormone is also increased due to hyperglycemia and increased adipose tissue (45). These results are consistent with previous studies investigating the role of leptin in insulin resistance and inflammation. For example, a study examined the association between maternal plasma LEP levels in early pregnancy and the risk of GDM. According to the study, early pregnancy LEP levels were significantly associated with an increased risk of GDM. It also introduced LEP as a predictive marker for GDM and emphasized the need for more extensive studies in this field (46). Another study has implicated LEP as a key adipokine in the development of GDM through insulin resistance and placental inflammatory processes. Alterations in the levels of this hormone disrupt fetal metabolic development, increase the risk of birth defects and intrauterine growth restriction, and increase the likelihood of developing metabolic diseases in the future (47).

Unlike IGF-2 and leptin, the expression of the CDKN1C gene in the present study was reduced by half in the GDM group compared to the control group. The change in the expression of this gene could be due to epigenetic effects and molecular pathways. Since the product of this gene is a cell cycle inhibitor, the change in its expression level could lead to an increase in uncontrolled cell division in some tissues and impaired placental development. It also indirectly increases the risk of metabolic complications in infants born to mothers with GDM (48). While the provided papers do not directly associate CDKN1C with gestational diabetes mellitus (GDM), they offer insights into its broader implications in metabolic regulation and growth disorders, which could indirectly relate to GDM. CDKN1C's role in regulating β-cell proliferation and its involvement in growth syndromes suggest potential pathways through which it might influence conditions like GDM (49, 50, 51).

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Pentagalloylglucose Suppresses Glioblastoma Progression via Wnt/β-Catenin Pathway Inhibition and EMT Reversal

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ABSTRACT

Background: Glioblastoma multiforme (GBM) is the most lethal primary brain tumor. GBM exhibits rapid growth and invasiveness along with a nadir prognosis. Epithelial–mesenchymal transition (EMT), combined with activated Wnt/β-catenin signaling, contributes to GBM progression and associated therapy resistance. *Pentagalloylglucose* (PGG), a polyphenolic compound from nature, has been shown to be anticancer in multiple cancers by inhibiting proliferation, migration, and EMT. The objectives are to demonstrate the effects of PGG on GBM cells and examine the modulation of EMT and the Wnt/β-catenin pathway.

Methods: U87-MG cells were treated with PGG (0.5–40 μM) for 24, 48, and 72 hours. Cell viability was assessed using the MTT assay. The expression levels of epithelial–mesenchymal transition (EMT) markers, including *E-cadherin*, *N-cadherin*, *Vimentin*, *Snail*, *Slug*, as well as *β-catenin*, were quantified by qRT-PCR. Cell migration was evaluated using a wound healing assay. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

Results: PGG demonstrated a marked reduction in cell viability in a dose- and duration-dependent manner with IC_{50} values at 18.4, 12.7, and 8.9 μM at 24, 48, and 72 hours, respectively. The upregulation of *E-cadherin* and downregulation of *N-cadherin*, *Vimentin*, and the *Snail* protein show that mesenchymal markers are being transcriptionally silenced. It was also a striking loss of *β-catenin* expression, which suggests Wnt/β-catenin suppression. Wound healing assay showed that PGG treatment resulted in a marked reduction of cell migration.

Conclusion: PGG significantly inhibits the progression of GBM by inhibiting EMT and downregulating the Wnt/β-catenin signaling pathways. Overall, PGG has potential as a natural, low-toxicity therapeutic or combinatory drug for glioblastoma, and future studies in vivo and in human trials will be needed to reaffirm this conclusion.

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INTRODUCTION

Glioblastoma multiforme (GBM) is a grade IV tumor by the World Health Organization and is the most aggressive of these cancer subtypes. GBM is characterized by high proliferation, high angiogenic ability, poor prognosis, and resistance to medication. The prevalence of glioblastoma multiforme (GBM) is approximately 2-3 per 100,000 population and is responsible for 15-20% of all primary brain tumours. GBM primarily presents in older patients

with a median age of diagnosis at 64 years. Despite developments in the field of neuro-oncology, the median survival in GBM patients remains around 12-15 months with a < 5% survival at 5 years (1, 2).

One of the key biological processes implicated in GBM development is epithelial–mesenchymal transition (EMT), a reversible transdifferentiation process wherein cells relinquish the epithelial characteristics (e.g., *E-cadherin*-mediated cell–cell attachment) and gain mesenchymal characteristics (e.g., heightened motility, invasiveness, and apoptosis resistance). Epithelial-mesenchymal transition (EMT) promotes the acquisition of cancer stem cell-like characteristics, which result in tumor recurrence and drug resistance (3, 4).

The Wnt/β-catenin signaling pathway is an essential and evolutionarily conserved mechanism that plays a key role in regulating epithelial–mesenchymal transition (EMT), embryogenesis, tissue homeostasis, and stem cell maintenance. In cancer, aberrant activation of this pathway leads to nuclear accumulation of *β-catenin*, which associates with TCF/LEF transcription factors to drive the expression of EMT-related genes, including Snail, Slug, ZEB1, and Twist. In Glioblastoma multiforme, dysregulation of Wnt/β-catenin signaling has been linked to enhanced tumor cell migration, invasion, angiogenesis, and therapeutic resistance. Therefore, targeting this pathway pharmacologically represents a promising strategy for reducing the aggressiveness of GBM (5-7).

1,2,3,4,6-*Pentagalloylglucose* (PGG), a natural polyphenolic molecule classified as a hydrolyzable tannin, is prevalent in medicinal plants such as Punica granatum, Rhus chinensis, and Terminalia chebula. The anticancer effects of PGG demonstrated inhibition of proliferation, induction of apoptosis and autophagy, inhibition of angiogenesis, and reduction of metastatic potential by regulating many oncogenic pathways populations (e.g., Wnt/β-catenin and GSK3β/β-catenin) (8, 9).

PGG has recently been shown to inhibit epithelial-mesenchymal transition (EMT) in various cancer models that displayed increased epithelial markers (e.g., *E-cadherin*) and reduced mesenchymal markers (e.g., *vimentin*, *N-cadherin*), as a result of inhibiting Wnt/β-catenin transcriptional activity (9, 10). Additionally, PGG has demonstrated advantageous effects in glioma cell lines by reducing fatty acid production, influencing energy metabolism, and lessening invasive properties (8). The results suggest that PGG may act as a significant inhibitor of GBM growth through various molecular pathways.

Given the significance of epithelial-mesenchymal transition (EMT) and Wnt/β-catenin signaling in the pathogenesis of glioblastoma (GBM), along with the increasing evidence supporting the anti-EMT properties of PGG- particularly in relation to the Wnt/β-catenin signaling pathway- this study aims to clarify the mechanistic connection between PGG treatment and the temozolomide (TMZ)-induced suppression of EMT in GBM cells. A deeper understanding of PGG may facilitate its potential application as either an adjunct or primary treatment for highly invasive brain tumors.

MATERIALS AND METHODS

Reagents and Chemicals

PGG was obtained from Sigma-Aldrich (St. Louis, MO, USA; CAS No.: 14937-32-7). A 10 mM stock solution was prepared by dissolving the compound in DMSO and stored at −20°C. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (Pen/Strep), and trypsin–EDTA were purchased from Gibco (Thermo Fisher Scientific, USA). The Add Prep Total RNA Extraction Kit, AddScript cDNA Synthesis Kit, and SYBR Green qPCR Master Mix were supplied by Addbio. Primers targeting epithelial–mesenchymal transition (EMT)-associated genes, including *E-cadherin, N-cadherin, Vimentin, Snail, Slug, and β-catenin*, as well as the housekeeping gene *GAPDH*, were synthesized by GenScript in China.

Cell Culture and Treatment

The U87-MG was obtained from IBRC, Iran (cell code: IBRC C10982), and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep) under standard culture conditions at 37°C in a humidified incubator with 5% CO_2. Cells were seeded into 6-well plates at a density of 2×10^5 cells per well and incubated for 24 hours to allow attachment. Subsequently, cells were treated with PGG at concentrations of 0.5, 1, 5, 10, 20, and 40 µM for 24, 48, and 72 hours. Control groups received equal volumes of dimethyl sulfoxide (DMSO) at concentrations below 0.1%.

Cell Viability Assay (MTT Assay)

Cell viability was assessed using the MTT assay. U87-MG cells were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to incubate overnight. Subsequently, the cells were treated with increasing concentrations of PGG (1–40 µM) for 24, 48, and 72 hours. Following treatment, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. The resulting formazan crystals were dissolved in 100 µL of DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using the Add Prep Total RNA Extraction Kit according to the manufacturer's protocol. RNA quantity and quality were assessed with a Nanodrop spectrophotometer. cDNA was synthesized from 1 µg of total RNA using the AddScript cDNA Synthesis Kit. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primers were designed based on sequences obtained from the NCBI database. Detailed primer specifications are provided in Table 1.

Relative gene expression was determined using the 2^-ΔΔct technique and normalized to *GAPDH*. All responses were conducted in triplicate.

Cell Migration Assay

The wound healing assay approach solved for cell migration. In cell U87-MG, preparation in 6-well culture plates was continued until more than 90% confluency was attained. The cell suspension was scraped gently to form a wound, a cross-section. The cells were washed so that no debris was left, and subsequently, PGG was added; the cells were then moved to the plates microscopically. Captures were made during 0, 24, and 48 hours. Movement of the mouse and images captured were combined for data using the ImageJ software.

Statistical Analysis

All experiments were carried out in triplicate, and the results are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, CA, USA). Group comparisons were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A p-value less than 0.05 was considered indicative of statistical significance.

RESULTS

Cell Viability Assay (MTT Assay)

The MTT assay revealed that PGG reduced the viability of U87-MG glioblastoma cells in a clear dose- and time-dependent manner. At 24 h, low concentrations of PGG (0.5 and 1 µM) showed no statistically significant effect compared to the control group ($p > 0.05$). A mild but noticeable reduction in viability (~10–15%) was observed at 5 µM, whereas treatment with 10 µM resulted in approximately a 25% reduction in viability. Higher concentrations, i.e. 20 µM and 40 µM, significantly inhibited viability and reached ~50% and ~65% inhibition levels relative to the control ($p < 0.01$).

At 48 hours, the cytotoxic effects were more pronounced. At a concentration of 5 µM, PGG decreased cell viability by approximately 20%, whereas at 10 µM, the reduction was around 40%. At a concentration of 20 µM, cell viability declined to approximately 35% of the control level, while at 40 µM, viability fell below

Table 1. Primers used in qRT-PCR

Gene	Primer sequence	Pcr product (bp)
E-cadherin	F: GAGAACGCATTGCCACATACA R: ACCTTCCATGACAGACCCCTTAA	158
N-cadherin	F: TCTCTCACGCTGTGTCATCCAAC R: CACAGAGGTTCCTGGAAGAGCAC	152
Vimentin	F: AGTCCACTGAGTACCGGAGAC R: CATTTCACGCATCTGGCGTTC	98
Snail	F: TCCAGAGTTTACCCTTCCAGCA R: CTTTCCCACTGTCCTCATCTG	219
Slug	F: CTCACCTCGGGAGCATACAG R: GACTTACACGCCCCAAGGATG	121
β-catenin	F: CAATGACTCGAGCTCAGAGGGTAC R: TTTAGCAGTTTTGTCAGTTCAGGGA	223
GAPDH	F: AATCCCATCACCATCTTCCAG R: ATCAGCAGAGGGGGCAGAGA	168

25% ($p < 0.001$).

Significant inhibitory effects were noted at 72 hours. Treatment with 5 μM PGG resulted in a decrease in viability of approximately 30% and treatment with 10 μM resulted in a decrease of about 55%. Viability decreased to less than 30% of controls with treatment of 20 μM. The maximum concentration tested, 40 μM, decreased cell viability to under 15%, demonstrating significant cytotoxicity ($p < 0.001$).

The calculated IC_{50} values from the dose–response curves were approximately 18.4 μM at 24 hours, 12.7 μM at 48 hours, and 8.9 μM at 72 hours, indicating that extended exposure increases the anti-proliferative effects of PGG. The findings indicate that PGG significantly inhibits glioblastoma cell growth, with treatment concentration and duration being essential factors in its effectiveness.

Gene Expression Analysis (qRT-PCR)

Real-time PCR analysis showed that PGG treatment significantly affected the expression of genes associated with EMT and the Wnt/β-catenin pathway in U87-MG glioblastoma cells in a manner consistent with suppression of EMT characteristics. The epithelial marker *E-cadherin* showed significant upregulation with transcript levels 2.0–3.5-fold more than the control, indicating enhancement of cell-to-cell adhesion characteristics. Conversely, the two mesenchymal markers *N-cadherin* and *Vimentin* were significantly downregulated with decreases to 0.4–0.6-fold or 0.3–0.5-fold of the control ($p < 0.001$), respectively. Expression of the EMT transcription factor *Snail* also showed a marked decrease. However, although *slug* expression decreased by a slight amount in the PGG treatment group relative to the untreated group, the difference was not statistically significant ($p > 0.05$), suggesting that treatment with PGG did not significantly impact *Slug* gene expression in the current experimental conditions.

β-catenin, a central mediator of the Wnt signaling pathway, was downregulated to 0.4–0.7-fold, suggesting inhibition of Wnt/β-catenin–dependent transcriptional activity.

Cell migration (Wound healing assay)

The wound healing assay demonstrated that PGG treatment (10 μM) significantly diminished the migratory ability of U87-MG glioblastoma cells in comparison to the untreated control group. After 24 hours, the wound closure percentage in untreated cells was 62.66 ± 1.53%, while PGG-treated cells demonstrated a significantly reduced closure percentage of 40.67 ± 1.53% ($p < 0.01$). The inhibitory effect was significantly greater at 48 hours, with control cells demonstrating 89.67 ± 1.53% closure, whereas PGG-treated cells exhibited only 45.33 ± 1.53% closure ($p < 0.001$). The microscopy showed that at every time point, the PGG-treated group had a reduced number of cells invading the wound area, which was in accordance with the results of the MTT assay, which also supports the conclusion that PGG expresses time-dependent inhibition of glioblastoma cell migration.

DISCUSSION

Among the many primary brain tumors, GBM can have the most aggressive, lethal, and devastating effects; it is characterized by rapid growth and invasiveness and is often resistant to treatment. Despite some advances in surgery, radiotherapy, and chemotherapy, patients appear to have a median overall survival of under 15 months (1). One of the key molecular processes involved in the progression of GBM is the EMT. This process enables cancer cells to improve their migration and invasion capabilities, as well as to develop resistance to drugs (3). The Wnt/β-catenin pathway is imperative for activation of EMT in GBM, and researchers are attempting its targeting as a potential new way to inhibit tumor aggressiveness (5).

The findings of this study demonstrated that the natural compound PGG decreased the viability of GBM (U87-MG) cells in both a dose- and time-dependent manner. qRT-PCR analysis revealed a marked upregulation of the epithelial marker *E-cadherin*, alongside a reduction in mesenchymal markers, including *N-cadherin*, vimentin, and Snail. In addition, the expression level of *β-catenin*, a central element of the Wnt/β-catenin signaling pathway, was diminished. Furthermore, migration assay results showed that PGG significantly suppressed cell motility, suggesting a decrease in tumor cell invasive capacity.

The present study indicated that PGG inhibits the EMT process in GBM cells by decreasing the expression of mesenchymal genes and increasing *E-cadherin*. Furthermore, the reduction of the *β-catenin* level indicated the inhibition of the Wnt/β-catenin pathway. Inhibition of the Wnt/β-catenin pathway by PGG and reduction of EMT factors are findings that have been confirmed not only in the present study but also in other independent studies. Abnormal activation of the Wnt/β-catenin pathway is considered a key mechanism in invasion and drug resistance in GBM. In this pathway, nuclear accumulation of *β-catenin* leads to activation of EMT genes such as *Snail* and *Slug*, which is accompanied by a decrease in *E-cadherin* and an increase in *N-cadherin* and *vimentin* (11, 12).

A recent study indicated that PGG extracted from Bouea macrophylla significantly reduced the migration, invasion, and EMT abilities of MDA-MB231 cells. The mediation was driven by a decrease in *vimentin* and *β-catenin* expression and an increase in *E-cadherin*, as well as an inhibition of STAT3 phosphorylation. The compound also synergistically en-

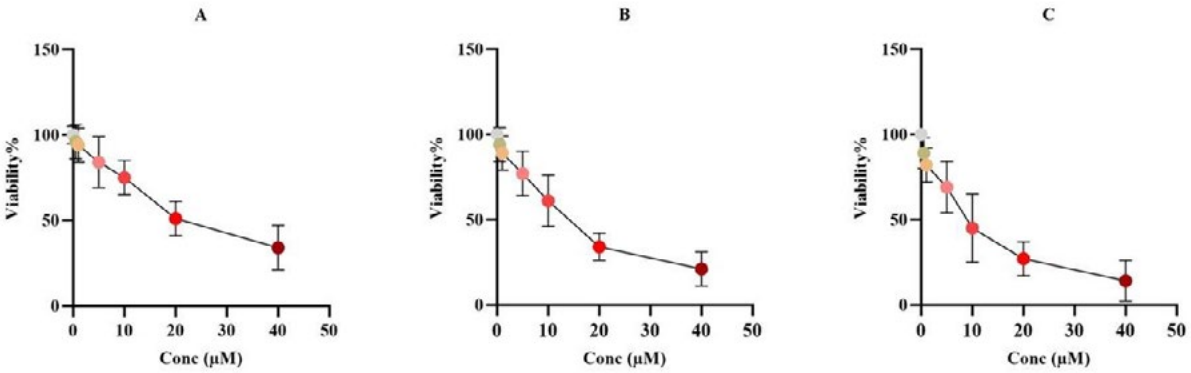

Fig 1. MTT assay to measure dose- and time-dependent inhibitory effects of PGG on U87-MG glioblastoma cell viability. Cells were treated with increasing concentrations of PGG (0.5–40 µM) for 24, 48, and 72 hours. Data are expressed as mean ± SD from three independent experiments. $p < 0.05$, $p < 0.01$, $p < 0.001$ versus control group.

hanced the effect of the drug doxorubicin (13). In a colon cancer model, PGG inhibited EMT and significantly reduced metastasis to the liver and lung by inhibiting the protease Cathepsin B and extracellular matrix-related pathways (FAK, cofilin). The expression of mesenchymal proteins was decreased, and epithelial markers were increased (14). PGG also inhibited cell migration and EMT in pancreatic cancer by suppressing stem cell factors (CD44v3, Nanog, and Sox-2) and inhibiting NF-κB and Foxo3 pathways (15).

The journal Biochemical and Biophysical Research Communications published research confirming the cytotoxic effects PGG has on glioblastoma cells, and as demonstrated with the data, PGG displayed the most significant level of inhibition on U251 glioma cells in comparison to other cell lines, including MDA-MB-231 and U87. The mechanism of this effect involves inhibition of the key enzyme of fatty acid synthesis (fatty acid synthase (FAS)), activation of the enzyme caspase-3, and induction of programmed cell death (apoptosis). Furthermore, at concentrations higher than 20 µM, PGG caused changes in the secondary structure of the FAS protein and its precipitation, indicating a strong inhibition of the function of this enzyme at the structural level. These findings indicate that PGG can act as an anti-glioblastoma agent not only through classical pathways such as inhibition of EMT and Wnt/β-catenin signaling but also through inhibition of lipid metabolism and induction of cell death (16).

Taken together, our data indicate that PGG may act as an anti-EMT and anti-proliferative agent to inhibit the growth of GBM cells. Since PGG was shown to enhance TMZ cytotoxicity through inhibition of EMT, it may offer a benefit when combined with TMZ in order to improve standard therapy effects. Additionally, due to the plant origin and distinct multi-mechanism activity of PGG, clinical investigation as a compound with low toxicity for use as an adjuvant treatment is also warranted.

The limitations of this study are as follows:

The study was performed only in an in vitro model with only the U87-MG cell line; therefore, results cannot necessarily be generalized to in vivo conditions or other cell lines, and further investigation is warranted. This study did not evaluate the impact of PGG on levels of the Wnt pathway or other associated signaling pathways. The decline of *Slug* gene expression, while a decline was observed, did not achieve statistical significance. This may be related to the duration of treatment and/or the sensitivity of the cell line.

CONCLUSION

The findings of this study clearly demonstrate that the natural compound PGG was able to significantly limit the growth, survival, and migration of GBM cells by inhibiting the EMT, reducing the expression of mesenchymal genes, and suppressing the Wnt/β-catenin pathway. Also, the dose- and time-dependent cytotoxic effects and the ability to induce apoptosis in tumor cells strengthen the position of PGG as a potential therapeutic candidate or a complementary combination with standard drugs such as temozolomide. Due to the natural origin, low toxicity, and multimodality of this compound, we advocate further testing with in vivo models and clinical trials to establish its efficacy and safety. The available information outlines new opportunities to develop more selective and less harmful therapeutic options in glioblastoma treatment.

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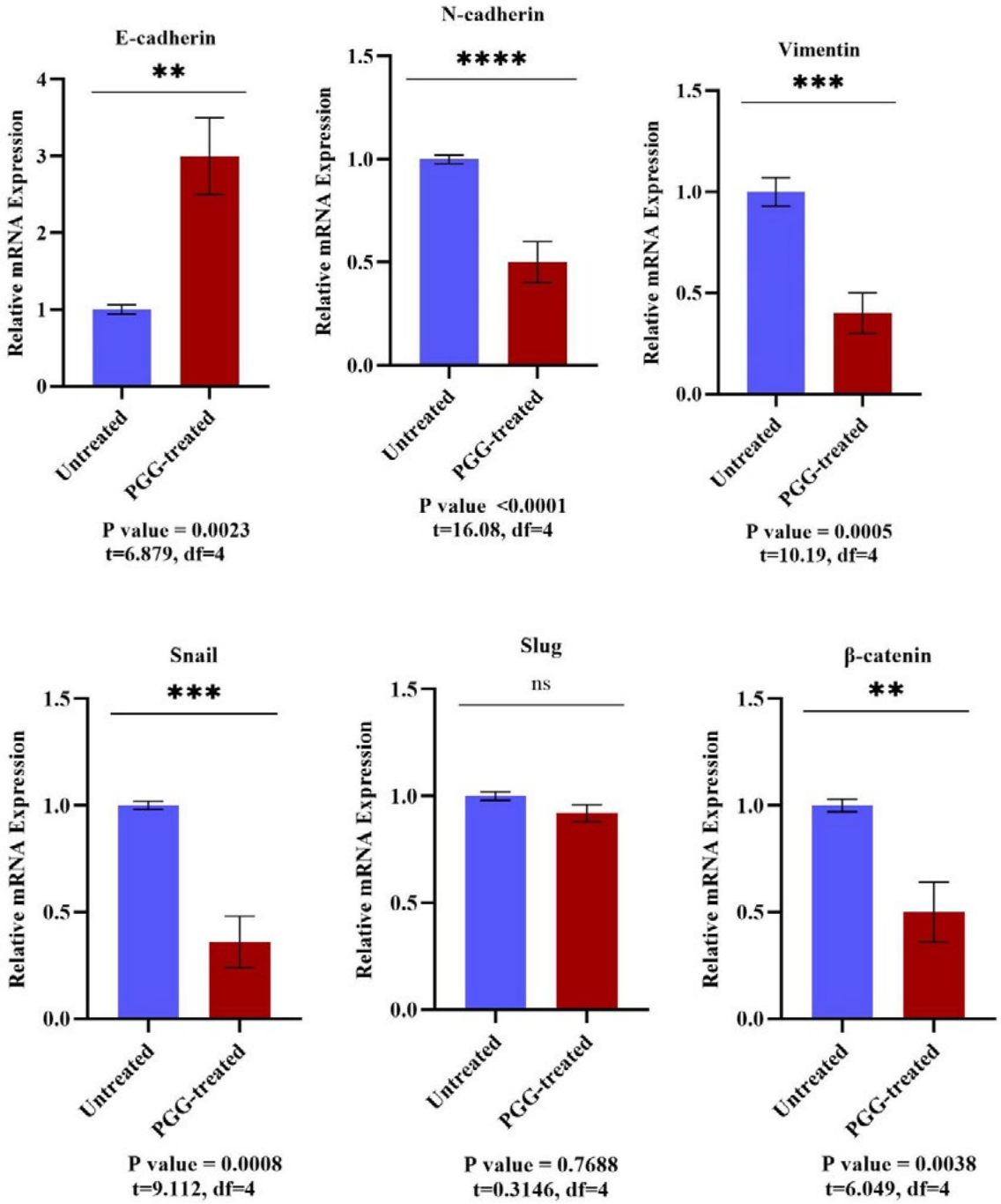

Fig2. The effect of PGG treatment on the expression of EMT-related genes and the level of β-catenin in U87-MG glioblastoma cells. The relative mRNA expression levels of E-cadherin, N-cadherin, Vimentin, Snail, Slug, and β-catenin were determined by qRT-PCR following 24 hours of treatment with PGG (10 μM). The relative expression levels are presented as fold change to the untreated control group and were normalised to GAPDH expression. PGG treatment significantly increased E-cadherin and decreased the mesenchymal markers (N-cadherin, Vimentin) and β-catenin. Snail expression was also significantly reduced, whereas the decrease in Slug was not statistically significant. Data represent mean ± SD of three independent experiments. $p < 0.05$, $p < 0.01$, $p < 0.001$ versus control group.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Data Availability Statement

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

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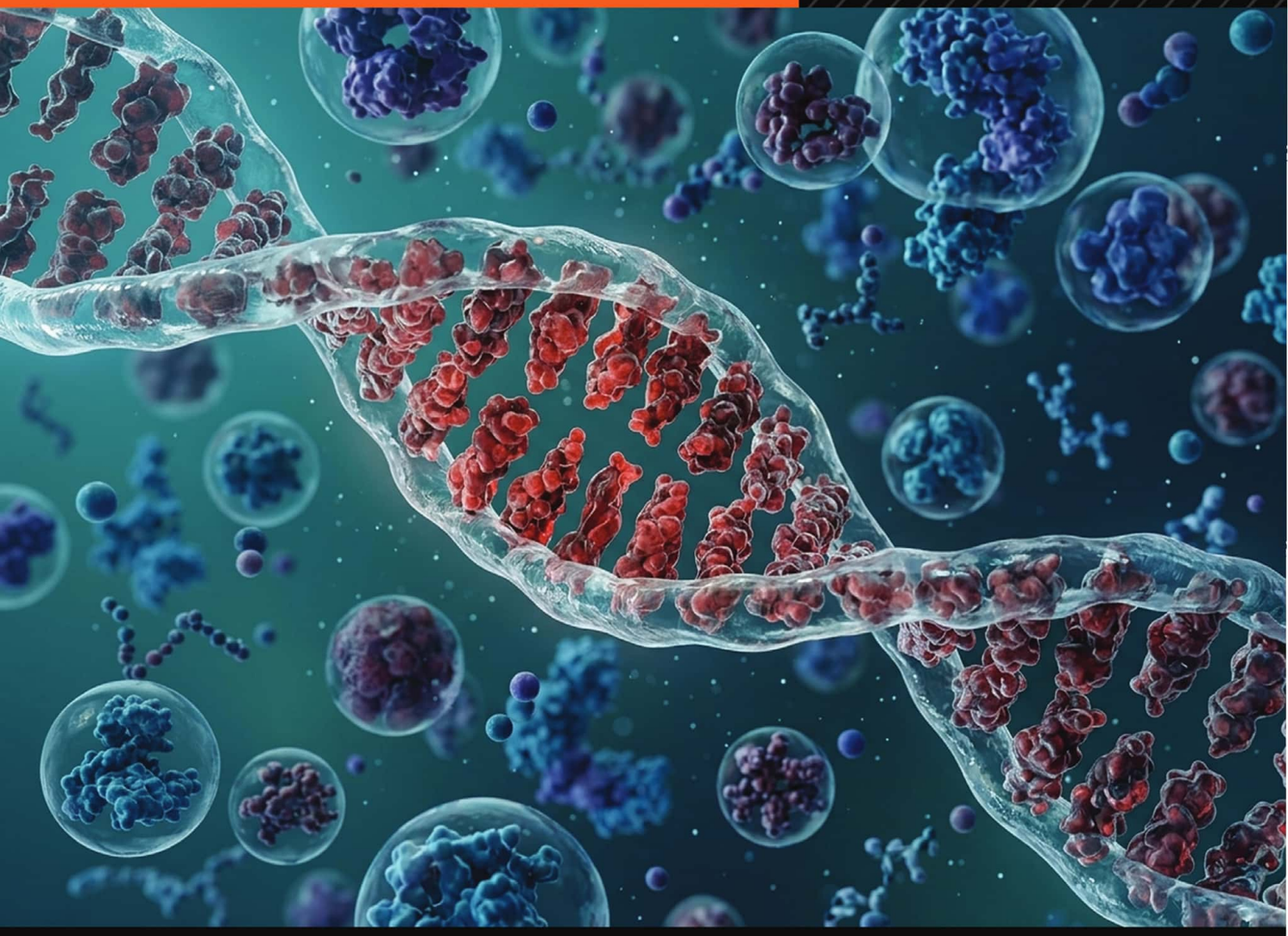

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