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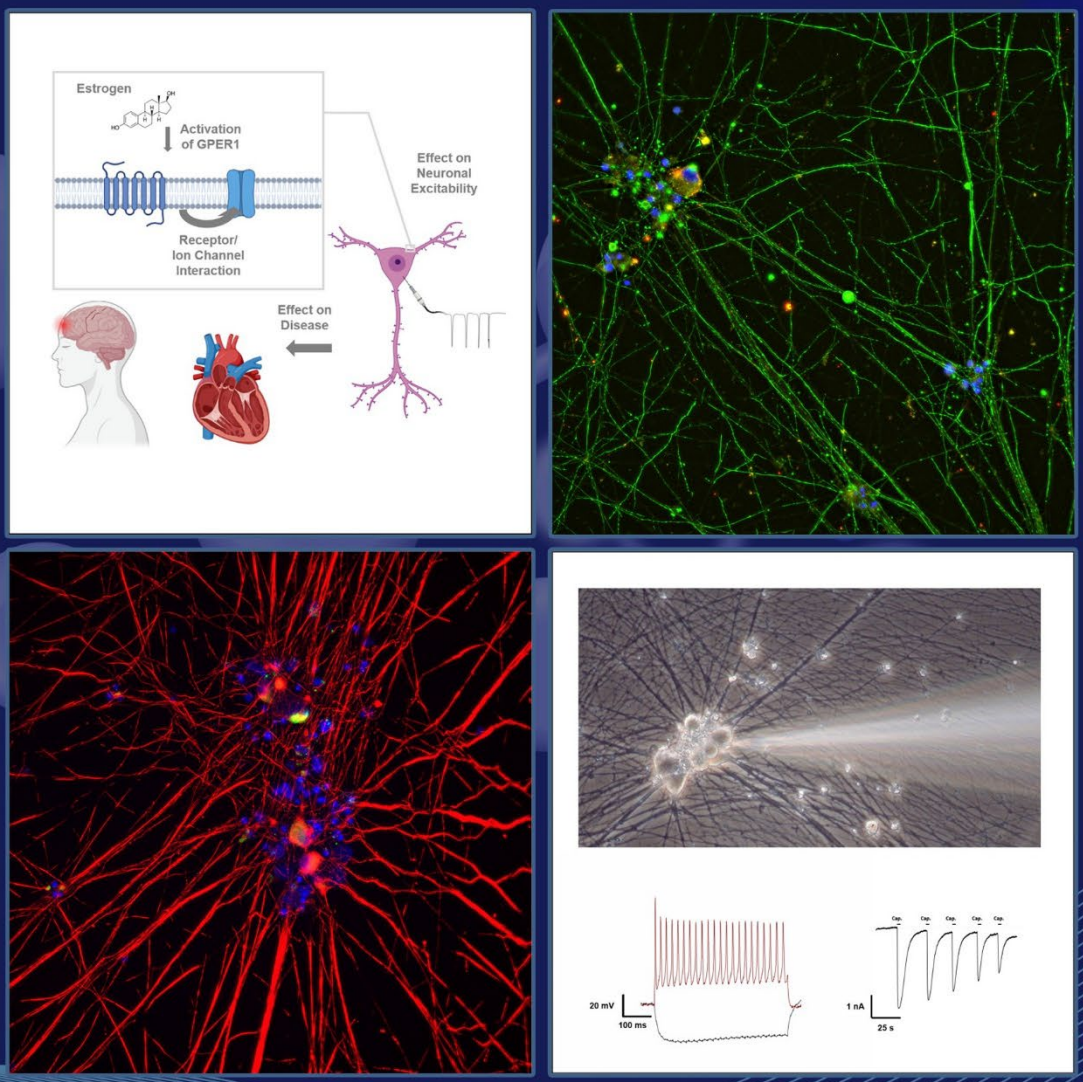

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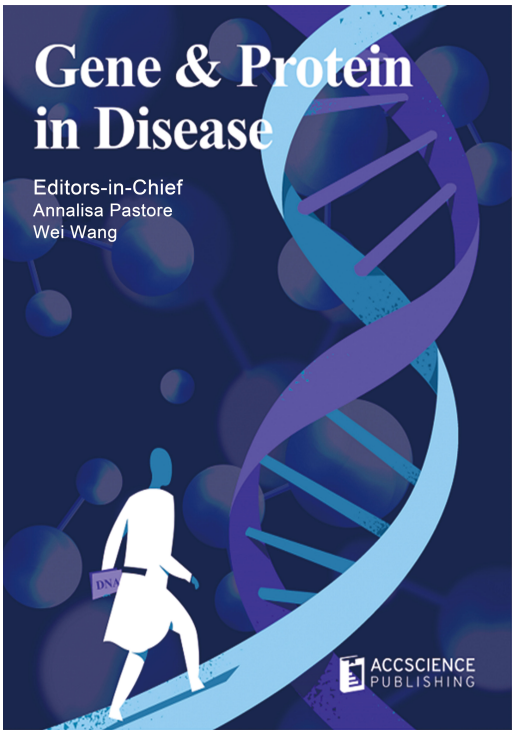

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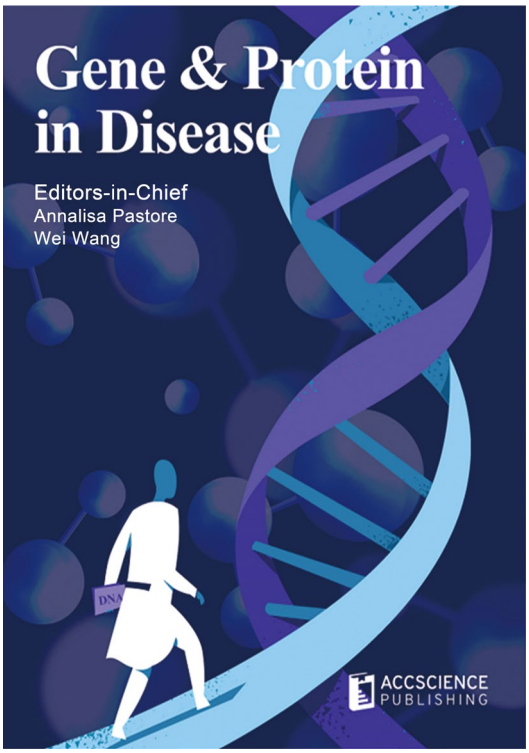

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A graphic illustration of double-stranded DNA

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Gene & Protein in Disease

EDITORIAL

Unveiling hidden genes: New drivers of cancer

Amancio Carnero[1,2]*

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Identifying new genes involved in cancer is crucial for cancer research. This process helps in discovering new oncogenes/tumor suppressors and establishing gene alterations in tumors whose drivers have not yet been identified.[1,2] It also helps identify genes that modify tumor behavior and drive tumor evolution, leading to tumor resistance to therapy, metastasis, or complications within the microenvironment.[3,4] Some common approaches used by researchers are as follows:

1. Genome-wide association studies: These studies analyze the genomes of individuals with and without cancer to identify genetic variations associated with cancer risk.[5]
2. Sequencing technologies: Next-generation sequencing allows researchers to sequence entire genomes or specific parts of the genome to identify mutations, alterations, or variations contributing to cancer development.[6-8]
3. Methylome analysis: This analysis involves studying and mapping DNA methylation patterns across the genome to identify regions of DNA methylation and understand the relevant impact on gene activity.[9]
4. Functional genomics: This involves studying gene functions and their interactions within cells. Techniques such as short hairpin RNA or short interfering RNA screening and clustered regularly interspaced short palindromic repeats/cas9 protein system (CRISPR/Cas9) gene editing can help researchers manipulate genes to understand their roles in cancer.[10,11]
5. Expression profiling: Comparing gene expression patterns between cancer cells and healthy cells can help identify genes upregulated or downregulated in cancer. This is achieved through techniques such as microarray analysis or RNA sequencing.[12-14]
6. Bioinformatics and data analysis: Computational approaches are crucial for analyzing large genomic datasets to identify potential candidate genes involved in cancer pathways or mechanisms.[15]
7. Animal models: Using genetically engineered animals that mimic human cancers can help identify genes involved in cancer development and progression.[16] This is accomplished through whole genome CRISPR/Cas9 gene knockout and functional phenotype analysis.[17]
8. Clinical studies and patient data analysis: Analyzing data from patients with cancer, including genetic profiles, treatment responses, and outcomes, provides insights into genetic factors influencing cancer susceptibility and progression.[18,19]

The integration of clinical data with genomic information and patient outcomes offers a comprehensive understanding of the genetic landscape of cancer. This integrative approach facilitates the identification of novel genes, pathways, and molecular mechanisms involved in cancer development. Furthermore, it supports the advancement of targeted therapies and personalized medicine for patients with cancer.

The use of artificial intelligence (AI) and complex algorithms to analyze and extract conclusions from various databases has become a pivotal aspect of cancer research. AI facilitates the analysis of vast datasets, accelerating the identification of new genes,

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biomarkers, and therapeutic targets. The involvement of AI in cancer research is multifaceted. It integrates clinical records as well as genomics, proteomics, and imaging data. AI algorithms efficiently process and integrate large volumes of heterogeneous data. AI techniques are used for data cleaning, normalization, and preprocessing to address issues such as missing data and variations in data formats. Machine learning and deep learning models can analyze data patterns to identify potential biomarkers associated with different cancer types. These biomarkers could be indicative of specific genetic mutations or other factors contributing to cancer development. They can predict tumor responses to therapy treatments, aiding in the development of better therapeutic options. AI is used to analyze genomic sequencing data to identify novel cancer-associated genes. Deep learning models can detect subtle patterns and mutations that might be missed by traditional methods. AI algorithms can annotate genetic variants, aiding researchers in understanding the functional significance of specific mutations. They assist in identifying potential drug targets, accelerating drug discovery by pinpointing genes or proteins that play a crucial role in cancer development. These tools can analyze complex biological pathways, revealing interconnected networks of genes and proteins involved in cancer. This holistic view aids in understanding underlying mechanisms and potential therapeutic therapy treatment. AI can also suggest existing drugs for repurposing based on their interaction with the identified genes or pathways.

Despite their relevance, AI-based findings should be experimentally validated before clinical translation. Collaboration among researchers, access to comprehensive databases, advanced technologies, and integration of multiple approaches can lead to the identification of new genes involved in cancer. These findings pave the way for a better understanding of the disease and the development of targeted therapies.

Conflict of interest

Amancio Carnero is an Associate Editor of this journal.

Further disclosure

There are tens of thousands of works describing techniques to identify new genes involved in cancer. It would have been impossible to cite all of them, even citations of only some landmark works using every technique. Therefore, we only suggest further reading on the same topic and the references listed in this editorial for a better understanding.

References

1. Colaprico A, Olsen C, Bailey MH, *et al.* Interpreting pathways to discover cancer driver genes with Moonlight. *Nat Commun.* 2020;11:69.

   doi: 10.1038/s41467-019-13803-0

2. Sinkala M. Mutational landscape of cancer-driver genes across human cancers. *Sci Rep.* 2023;13:12742.

   doi: 10.1038/s41598-023-39608-2

3. Santarius T, Shipley J, Brewer D, Stratton MR, Cooper CS. A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer*. 2010;10:59-64.

   doi: 10.1038/nrc2771

4. Martínez-Jiménez F, Muiños F, Sentís I, *et al.* A compendium of mutational cancer driver genes. *Nat Rev Cancer*. 2020;20:555-572.

   doi: 10.1038/s41568-020-0290-x

5. Nielsen F, van Overeem Hansen T, Sørensen C. Hereditary breast and ovarian cancer: New genes in confined pathways. *Nat Rev Cancer*. 2016;16:599-612.

   doi: 10.1038/nrc.2016.72

6. Sosinsky A, Ambrose J, Cross W, *et al.* Insights for precision oncology from the integration of genomic and clinical data of 13,880 tumors from the 100,000 Genomes Cancer Programme. *Nat Med.* 2024;30:279-289.

   doi: 10.1038/s41591-023-02682-0

7. Koh G, Degasperi A, Zou X, Momen S, Nik-Zainal S. Mutational signatures: Emerging concepts, caveats and clinical applications. *Nat Rev Cancer*. 2021;21:619-637.

   doi: 10.1038/s41568-021-00377-7

8. Saito Y, Koya J, Araki M, *et al.* Landscape and function of multiple mutations within individual oncogenes. *Nature*. 2020;582:95-99.

   doi: 10.1038/s41586-020-2175-2

9. Terekhanova NV, Karpova A, Liang WW, *et al.* Epigenetic regulation during cancer transitions across 11 tumour types. *Nature*. 2023;623:432-441.

   doi: 10.1038/s41586-023-06682-5

10. Eifert C, Powers RS. From cancer genomes to oncogenic drivers, tumour dependencies and therapeutic targets. *Nat Rev Cancer*. 2012;12:572-578.

    doi: 10.1038/nrc3299

11. Katti A, Diaz BJ, Caragine CM, Sanjana NE, Dow LE. CRISPR in cancer biology and therapy. *Nat Rev Cancer*. 2022;22:259-279.

    doi: 10.1038/s41568-022-00441-w

12. Paczkowska M, Barenboim J, Sintupisut N, *et al.* Integrative pathway enrichment analysis of multivariate omics data. *Nat Commun*. 2020;11:735.

    doi: 10.1038/s41467-019-13983-9

13. Chen Q, Liu Y, Gao Y, *et al.* A comprehensive genomic and transcriptomic dataset of triple-negative breast cancers. *Sci Data*. 2022;9:587.

doi: 10.1038/s41597-022-01681-z

14. Nagasawa S, Kuze Y, Maeda I, *et al.* Genomic profiling reveals heterogeneous populations of ductal carcinoma *in situ* of the breast. *Commun Biol*. 2021;4:438.

doi: 10.1038/s42003-021-01959-9

15. Grassmann F, Pawitan Y, Czene K. A systems genomics approach to uncover the molecular properties of cancer genes. *Sci Rep.* 2020;10:18392.

doi: 10.1038/s41598-020-75400-2

16. White R, Rose K, Zon L. Zebrafish cancer: The state of the art and the path forward. *Nat Rev Cancer*. 2013;13: 624-636.

doi: 10.1038/nrc3589

17. Parvez S, Brandt ZJ, Peterson RT. Large-scale F0 CRISPR screens *in vivo* using MIC-Drop. *Nat Protoc*. 2023;18:1841-1865.

doi: 10.1038/s41596-023-00821-y

18. Li A, Chapuy B, Varelas X, Sebastiani P, Monti S. Identification of candidate cancer drivers by integrative Epi-DNA and Gene Expression (iEDGE) data analysis. *Sci Rep.* 2019;9:16904.

doi: 10.1038/s41598-019-52886-z

19. López-Cortés A, Paz-y-Miño C, Guerrero S, *et al.* OncoOmics approaches to reveal essential genes in breast cancer: A panoramic view from pathogenesis to precision medicine. *Sci Rep.* 2020;10:5285.

doi: 10.1038/s41598-020-62279-2

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Gene & Protein in Disease

REVIEW ARTICLE

Beyond chromosomal rearrangements: The expanding landscape of gene fusions and chimeric RNAs

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Abstract

BCR–ABL, the pioneering gene fusion resulting from chromosomal translocation, has marked a major milestone in understanding genetic alterations in cancer. Initially, gene fusions were linked solely to chromosomal rearrangements, serving as diagnostic markers and cancer drivers. However, advancements in high-throughput sequencing and bioinformatics have revealed additional mechanisms underlying gene fusion. The term "gene fusion" primarily refers to fusion events at the DNA level, whereas "chimeric RNA" encompasses a wide range of transcripts containing exons from different parental genes, including gene fusion transcripts. Recent developments have identified numerous chimeric RNAs in various cancer types, extending even to non-cancerous tissues. Chimeric RNAs, originating from events such as trans-splicing, read-through, and intergenic splicing, form a complex landscape with varied functions. While some chimeric RNAs have defined roles and therapeutic potential, a comprehensive understanding of their diverse functions remains a priority. Exploring the full spectrum of chimeric RNA activities is crucial for revealing their clinical and therapeutic implications. In addition, chimeric RNAs are key players in tumorigenesis, affecting cellular processes, and driving cancer progression. Understanding their intricate interactions with cellular pathways is essential for developing targeted therapies and precision medicine approaches. The dynamic nature of chimeric RNAs highlights the need for ongoing research to fully harness their diagnostic and therapeutic potential.

Keywords: Chimeric RNA; Gene fusion; Splicing; Cis-splicing; Trans-splicing; Cis-SAGe; Cancer

1. Introduction

Gene fusions and their products were initially believed to result solely from chromosomal rearrangements; thus, they were recognized as cancer drivers and used for diagnosis.[1,2] The fusion gene was first described in the 1960s by Peter Nowell.[3] The discovery of fusion genes opened new avenues for identifying fusions potentially related to or considered causative agents for cancer. *BCR–ABL*, originating from chromosomal translocation, was the first fusion gene identified in human cancer in 1973 by Rowley.[4] This fusion gene originated from a translocation involving the q arms of chromosomes 9 and 22, and it accounts for over 96% of chronic myelogenous leukemia cases.[5] Later, *BCR–ABL* was clinically used as a biomarker in chronic myeloid leukemia (CML).[6] The advent of new technologies and various bioinformatic tools revealed that interchromosomal translocation was not the sole mechanism for fusion gene formation. Additional mechanisms include intrachromosomal translocation, transcriptional read-through, chromosome deletion, inversion, insertion, chromothripsis, and cis-SAGe.[7,8]

The term "gene fusion" refers to fusion events at the DNA level, whereas "chimeric RNA" includes a wide range of transcripts containing exons from different parental genes, including gene fusion transcripts. Notably, chimeric RNA generation is not solely dependent on gene fusion transcription. Chimeric RNAs can also be formed through trans-splicing of two distinct precursor mRNAs and alternative splicing of a read-through transcript, known as cis-splicing of adjacent genes (cis-SAGe).[9] Increasing evidence has indicated that chimeric RNAs may also arise through various splicing mechanisms. Splicing, a well-studied process, involves the exclusion of introns from pre-mRNA and aids in the formation of mature mRNA. The splicing machinery utilizes spliceosomes, which are composed of small nuclear RNA molecules and a wide array of proteins, forming an RNA–protein complex. Trans-esterification reactions catalyzed by spliceosomes remove introns and connect exons.[8] Increasing research has led to the continuous identification of chimeric RNAs in both tumor cells and non-cancerous tissues. These data are stored in various databases, such as FusionGDB, ChimerDB, and ChiTaRS. Different numbers of fusions in various cancer types are deposited in the TCGA fusion gene database, as illustrated in Figure 1.

2. Mechanisms of chimeric RNA formation

Chromosome rearrangement, which alters chromosome structure, was believed to be the sole mechanism underlying chimeric RNA formation. However, various mechanisms lead to gene fusion formation, as shown in Figure 2.

(i) Chromosomal translocation is a well-known mechanism for generating gene fusions, with *BCR–ABL* fusion, resulting from translocation between chromosomes 9 and 22, being a common example.[3] Chimeric RNAs are also linked to chromosomal inversions, such as *EML4–ALK* fusion, which is associated with non-small cell lung cancer (NSCLC).[9,10] Another mechanism for chimeric RNA production involves interstitial deletion; *TMPRSS2–ERG*, frequently found in prostate cancer, is an example of this mechanism.[11,12] Tandem duplications can also produce fusion RNAs, such as *C2orf44–ALK* in colorectal cancer and *FGFR3–TACC3*.[13,14] Chromothripsis can also result in fusions, where a chromosome or its segments shatter into fragments and are incorrectly reassembled. Examples include *PVT1–NDRG1* and *PVT1–MYC* in medulloblastoma and *NDUFAF2–MAST4* in prostate cancer cell lines.[15,16]

(ii) Exposure to various physical, chemical, and biological factors can cause genetic mutations and trigger gene fusion, as shown in Figure 3. After the Chernobyl nuclear disaster, numerous studies reported a strong link between radiation exposure and genetic mutations as well as gene fusions in thyroid cancer cases. In radiation-induced papillary thyroid carcinoma (PTC), the occurrence rate of RET fusions is significantly increased, ranging from 35% to 80%.[17] A detailed analysis of the prevalence of *NCOA4–RET*, involving 2395 cases of radioactive and sporadic PTC, found that radiation exposure increased the likelihood of RET/PTC, specifically in the western population for the *NCOA4–RET* subtype.[18]

ETV6–NTRK3 fusion was identified in 14.5% of PTC cases caused by the Chernobyl disaster, with a strong link to radiation exposure.[19] In a study examining ionizing radiation and RET fusion in lung adenocarcinoma, human 201T lung cells exposed to 1 Gy of gamma rays showed RET fusions, and RET rearrangement was detected in two out of 37 patients exposed to radiation.[20] Other studies on genetic mutations in lung cancer revealed a significantly higher incidence of gene fusions in patients exposed to tobacco and coal, with ALK fusion and genetic rearrangement strongly associated with such exposure.[21] Another study investigating insecticides and cancer-related mutations found *ETV6–RUNX1* fusion in peripheral blood mononuclear cells significantly exposed to permethrin. Permethrin exposure also led to *ETV6–RUNX1* and *IGH–BCL2* fusions in K562 cells, whereas malathion triggered *KMT2A–AFF1* and *ETV6–RUNX1* fusions.[22] Similarly, *EGFR–PPARGC1A* fusion in squamous cell carcinoma of the head was linked to prolonged sunlight exposure.[23] Research by Holly *et al.* in 2017 showed that

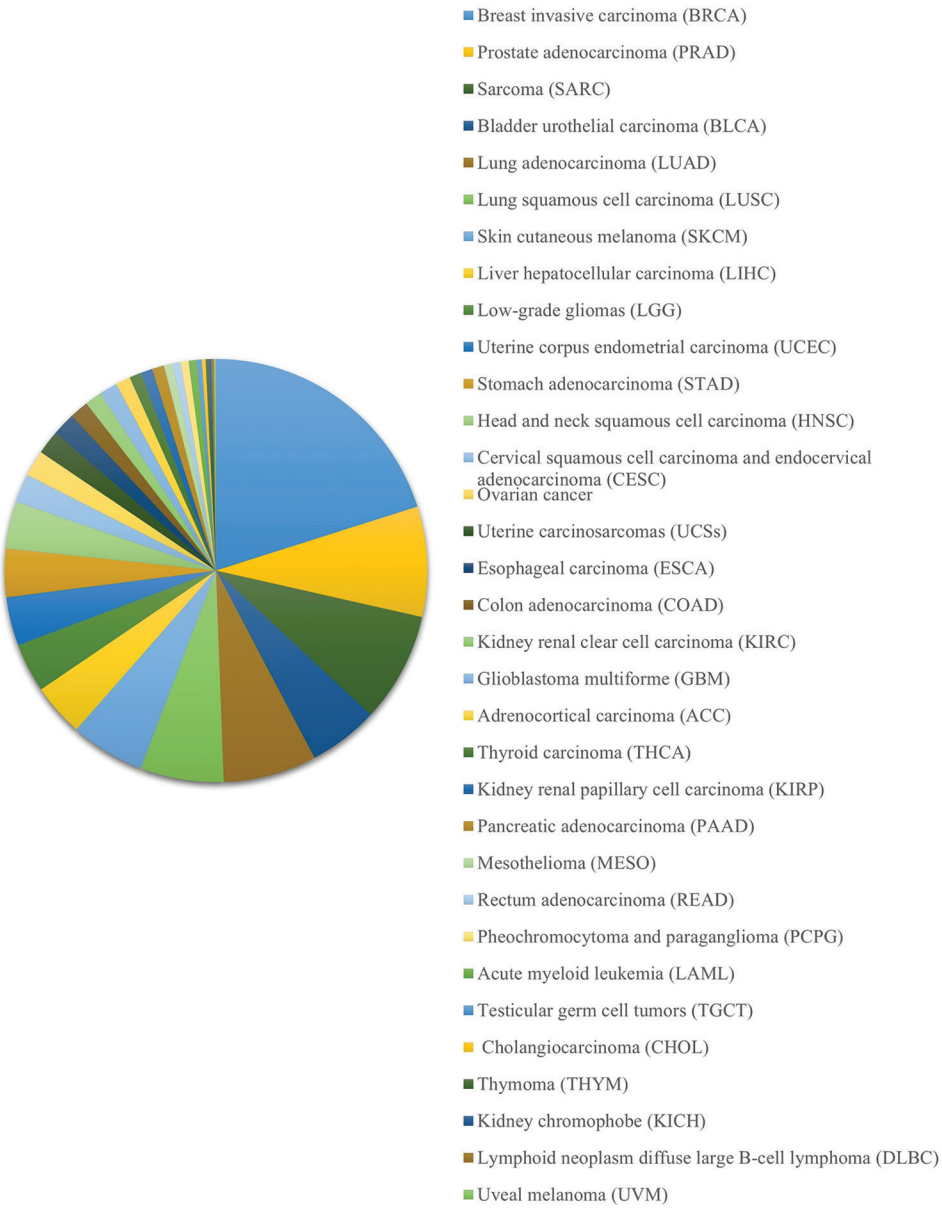

Figure 1. Number of gene fusions in various cancer types as deposited in the TCGA fusion gene database

elevated blood sugar stimulates *TMPRSS2–ERG* fusion in prostate cancer cells by increasing the expression of insulin-like growth factor binding protein-2 (IGFBP2). Elevated blood sugar also triggers *IGFBP2* production, increasing gene fusion events and lowering protein kinase C levels, indicating an effect on double-strand break repair rates.[24] Several studies have demonstrated that apoptotic signals, such as serum starvation, salicylic acid, and etoposide, can disrupt the TEL (ETV6) gene, resulting in fusion in developing B lymphocytes. *TEL–AML1* fusion is a common genetic alteration in pediatric acute lymphoblastic leukemia.[25] Prostate cancer has long been linked to androgen levels, and further research shows that androgen signaling activation triggers *TMPRSS2* and *ERG* expression. Gamma-ray exposure in cells also induces DNA double-strand breaks, promoting *TMPRSS2–ERG* fusion.[26] In conclusion, physical, chemical, and biological stimuli can lead to genomic mutations and gene fusions. A deeper understanding of the mechanisms by which these factors influence the creation of fusion transcripts will assist us in elucidating the role of fusion genes in tumorigenesis and will facilitate the discovery of novel approaches for the prevention and treatment of various types of cancers.

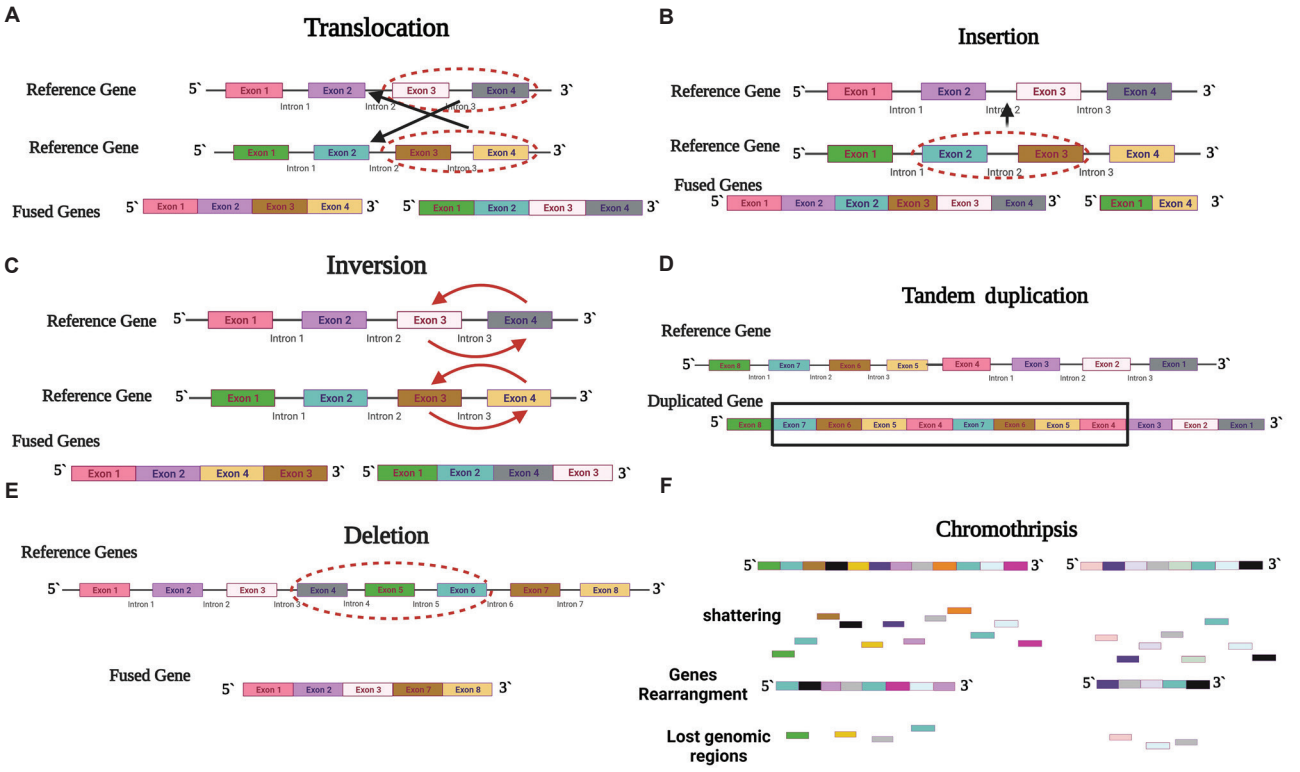

Figure 2. Mechanisms that facilitate gene fusion generation: (A) translocation, (B) insertion, (C) inversion, (D) tandem duplication, (E) deletion, and (F) chromothripsis

(iii) In addition to the aforementioned mechanisms, RNA processing events, such as cis- and trans-splicing, can generate chimeric RNAs (Figure 4). Cis-splicing between adjacent genes, or cis-SAGe is an RNA processing mechanism that occurs within a single pre-mRNA. In this process, the transcription machinery reads through the intergenic regions of two neighboring genes and performs alternative splicing between their exons.[27] Numerous chimeric RNAs have been identified through computational studies and paired-end RNA sequencing. These chimeric RNAs result from the fusion of adjacent genes, possibly arising from transcriptional read-through processes.[28-34] It is estimated that 4 – 5% of adjacent gene pairs in the human genome can participate in the mechanism, leading to chimeric RNA formation.[29,35] Cis-SAGe fusions typically occur when adjacent genes are within a 30-kb range, often linking the second-to-last exon of the 5' gene to the second exon of the 3' gene, following the 2 – 2 rule.[36] An example of cis-SAGe fusion is the chimeric RNA *SLC45A3–ELK4*.[36-38]

Trans-splicing is another mechanism that generates chimeric RNAs by joining exons from two distinct primary RNA transcripts. Based on the origin of the primary transcript, trans-splicing is categorized into two types: Intragenic and intergenic. Intragenic trans-splicing occurs when two RNA copies from the same genomic locus undergo a process leading to exon duplication and sense–antisense fusions.[39-41] Intergenic trans-splicing, on the other hand, occurs between two pre-mRNAs transcribed from different gene loci.[42,43] Trans-splicing is common in simpler organisms such as unicellular organisms, nematodes, and trypanosomes, where up to 70% of genes are involved in this process.[44-46]

3. Chimeric RNAs in cancer

Gene fusions significantly impact cancer progression, acting as exclusive oncogenic drivers in 1% of cancer cases and contributing to cancer development in approximately 16% of cases.[47] Recent advances in sequencing technologies and comprehensive profiling have provided novel insights into the prevalence of gene fusions. A study conducted in 2023 found that approximately one-third of metastatic breast cancers contained at least one highly expressed, high-confidence, cancer-specific fusion RNA, suggesting that gene fusions may be more common than previously thought.[49] Similarly, a 2024 study on cancer-associated transcript fusions in adult brain tumors revealed a significant presence of these fusions in cerebral gliomas and brain metastases, further emphasizing the role of gene fusions in cancer

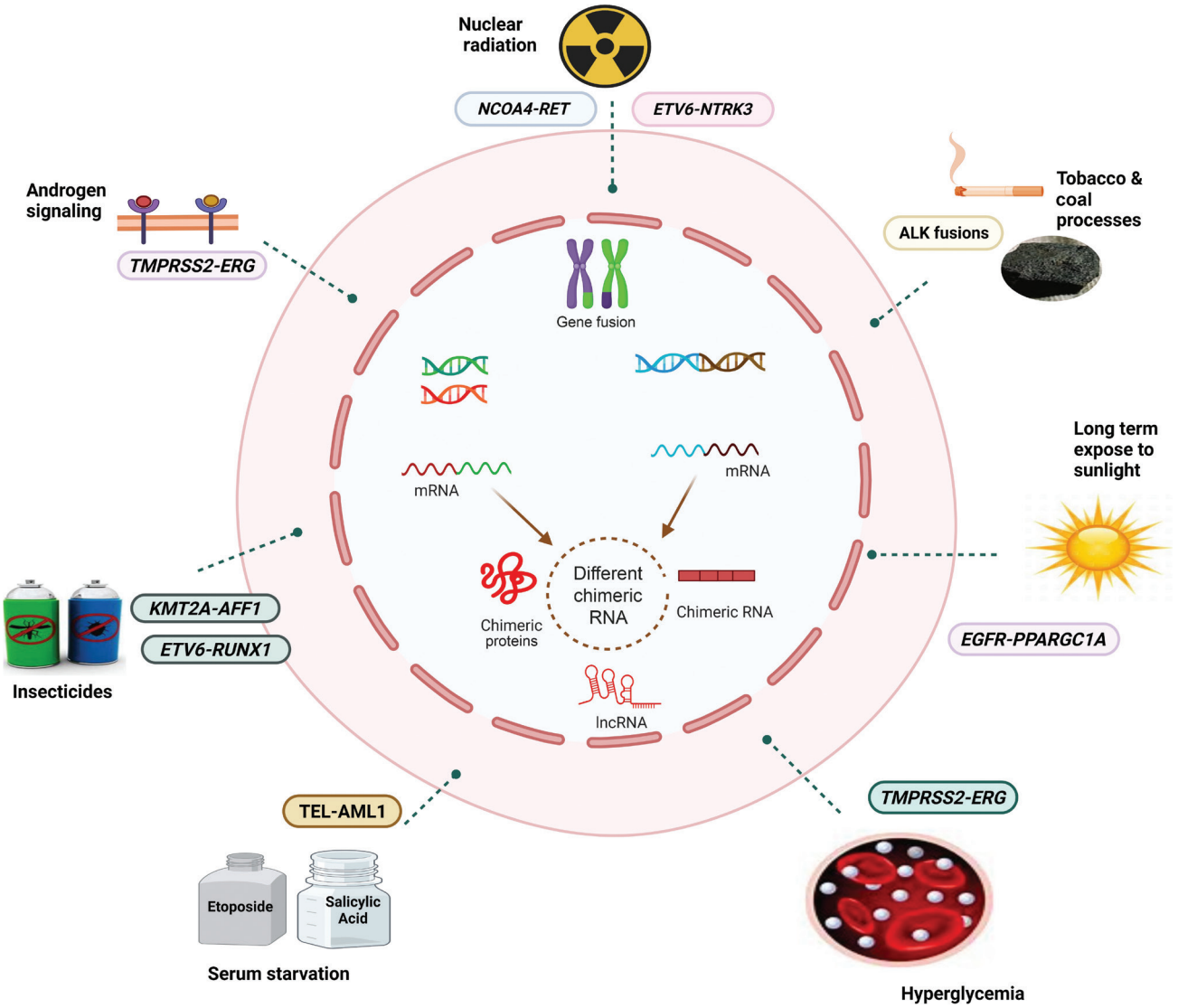

Figure 3. Various physical, chemical, and biological factors that can lead to genome mutations and induce gene fusions

progression and their potential as therapeutic targets.[50] Recent transcriptome-wide profiling has also identified numerous ribonuclease-targeting chimeras, expanding our understanding of chimeric RNAs in cancer.[51] Genomic instability, a hallmark of cancer cells, can lead to chimeric gene formation through genomic events, such as deletions, insertions, inversions, and translocations. While the structural changes resulting in the Philadelphia chromosome in CML were discovered in the 1960s, it was not until the 1980s that the first fusion gene, *BCR–ABL1*, was identified as a significant biomarker in CML.[6,48] Since the discovery of *BCR–ABL1* in CML, gene fusions producing chimeric oncoproteins have been recognized as common genomic alterations in cancer. Gene fusions drive oncogenesis by modifying the expression of oncogenes or tumor suppressors either through activation of a novel promoter or functional changes that disrupt key molecular pathways. In prostate cancer, chimeric RNAs such as *SLC45A3–ELK4* and *TMPRSS2–ERG* are associated with disease progression. Esophageal cancer, particularly esophageal squamous cell carcinoma (ESCC), has been linked to chimeric RNAs such as *GOLM1–NAA35* and *ASTN2–PAPPAas*. In NSCLC, gene fusions such as *KIF5B–MET* and *SOS1–ALK* play crucial roles in tumor development. Thyroid cancer, particularly PTC, has shown various fusions, including *STRN–ALK* and *TFG–RET*. Chimeric RNAs have also been found in other cancers, such as *COL7A1–UCN2* in laryngeal cancer, *SEPT7P2–PSPH* in nasopharyngeal carcinoma, and *LHX6–NDUFA8* in cervical cancer.[51] These concepts are further elaborated below.

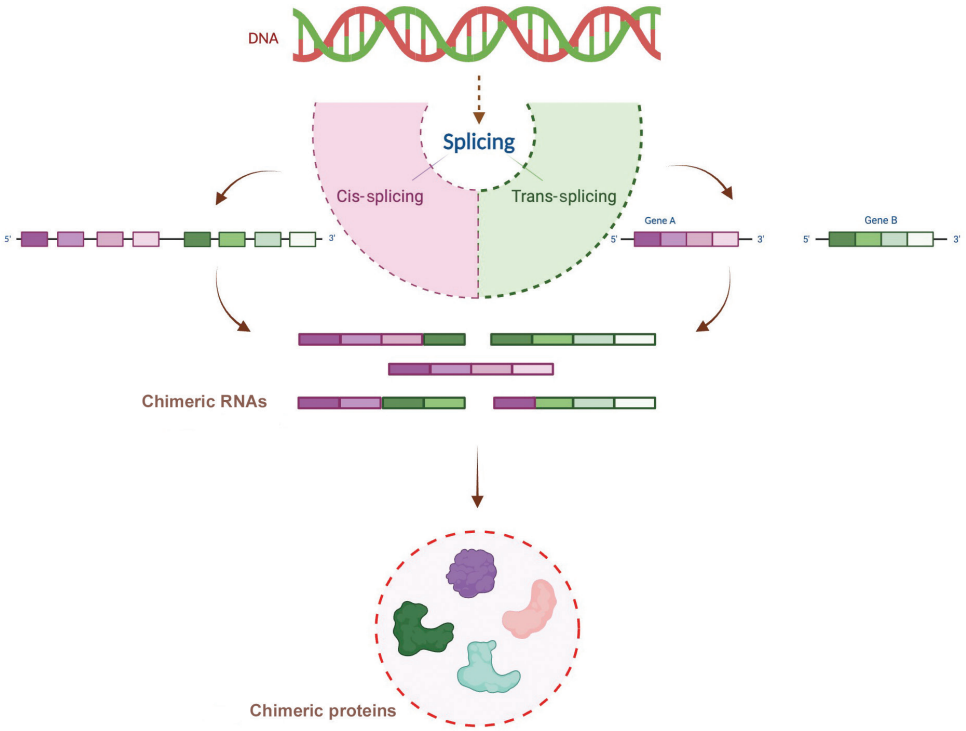

Figure 4. Mechanisms of chimeric RNA formation through cis- and trans-splicing

3.1. Chimeric RNAs in prostate cancer

Prostate cancer is the second most frequently detected cancer in western society, following skin cancer. A study conducted in 2018 reported that approximately 1.3 million people are newly diagnosed with prostate cancer annually.[49] Advances in technology and data mining have led to the discovery of numerous chimeric RNAs in prostate cancer. SLC45A3, a prostate-specific gene regulated by androgens, belongs to the solute carrier family 45, whereas ELK4, a part of the ETS transcription factor group, is an ETS transcription factor. *SLC45A3–ELK4* fusion arises from cis-splicing between neighboring genes rather than RNA trans-splicing. This fusion is correlated with prostate cancer progression, peaking in metastatic cases, and its introduction can regulate cellular proliferation. Moreover, *SLC45A3–ELK4* acts as long non-coding chimeric RNAs (lnccRNAs).[50,51] A study conducted by Zhou *et al.* in 2019 discovered that *TMPRSS2–ERG* leads to abnormal activation of the *ERG* oncogenic pathway, driving prostate cancer progression. They also discovered that the α1 and β1 subunits of guanylyl cyclase are regulated by ERG both *in vivo* and *in vitro*, specifically linked to *TMPRSS2–ERG*.[52] Another study conducted by Chakravarthi *et al.* in 2019 reported that approximately 30% of early prostate cancer cases had a fusion. This fusion involved the androgen receptor target gene *KLK4* and the non-coding pseudogene *KLKP1*, both part of the kallikrein family of serine proteases and located on chromosome 19 (q13.33 – q13.41). The fusion occurs through trans-splicing or in-frame fusion due to microdeletion, joining the first two exons of *KLK4* with exons 4 and 5 of *KLKP1*, affecting cell proliferation, invasion, intravasation, and tumor development.[53] Biomarkers have revolutionized the screening, detection, and prognosis of prostate cancer. A recent study identified a novel fusion transcript, *UNC5D-NRG1*, in prostate carcinoma, which is predicted to encode a protein and preserve the EGF-like domain.[9]

3.2. Chimeric RNAs in esophageal cancer

Esophageal cancer is a common cancer with high mortality and morbidity rates. It is the sixth leading cause of cancer-related deaths worldwide, with a 5-year survival rate below 25%.[55] Advances in cancer research technologies have led to the discovery of chimeric RNAs linked to esophageal cancer. The chimeric RNA formed by *GOLM1–NAA35* is highly expressed in ESCC compared with adjacent non-malignant tissue and is also present in the normal esophagus of individuals without ESCC. Research suggests that *GOLMI–NAA35* is not mutation-driven but formed through transcriptional read-through and splicing or trans-splicing, resulting in a chimeric protein.[56] Another study reported that abnormal splicing-induced *GOLM1–NAA35* expression in ESCC is associated with malignancy

progression and reduced overall survival. Inhibiting *GOLMI–NAA35* expression significantly slowed tumor growth *in vivo*, highlighting its role in ESCC progression.[57] A study conducted by Wang *et al*. in 2021 identified the chimeric RNA *ASTN2–PAPPA*as, which was likely generated through transcriptional read-through followed by splicing. This chimeric RNA originated from the splicing of exons and antisense introns of two neighboring genes and is predominantly found in cancer cells within tumors but absent in normal esophageal tissues. Inhibition of *ASTN2–PAPPAas* reduced cell migration and invasion while promoting ESCC cell spread to lymph nodes and enhancing stem cell characteristics via OCT4 regulation both *in vitro* and *in vivo*.[58] Luo *et al*. (2022) developed a tamoxifen-inducible knock-in mouse model expressing *ASTN2–PAPPAas* by integrating the human sequence into the mouse genome. Mice with the *ASTN2–PAPPAas* knock-in gene exhibited no abnormalities in growth, fertility, and histological and biochemical characteristics. The authors noted that this model could be used to study the role of chimeric RNAs in disease development and potential targeted treatments.[59]

3.3. Chimeric RNAs in lung cancer

In 2015, cancer accounted for 8.8 million deaths, with lung cancer responsible for approximately one-fifth (1.69 million) of these deaths, making it the leading cause of cancer-related deaths worldwide. Approximately 14% of newly diagnosed cancers are attributed to lung cancer, confirming it as the primary contributor to cancer-related deaths. Chimeric RNAs formed by gene fusions (RNA fusions) and splicing errors play a crucial role and represent viable treatment targets in NSCLC.[60] In 2018, Gow *et al*. identified two cases of *KIF5B–MET* fusion. One was found in a patient with adenocarcinoma and sarcomatoid tumor, whereas the other was found in a patient with pulmonary sarcomatoid carcinoma. In both cases, a fusion of exons 1 – 24 from *KIF5B* with exons 15 – 21 from MET was detected. The fusion protein includes the full kinesin motor and coil-coiled regions from *KIF5B* along with the C-terminal tyrosine kinase domain from *MET*. The authors confirmed that the *KIF5B–MET* fusion protein exhibits oncogenic properties and enhances tumor cell proliferation *in vitro* and *in vivo*.[61] Chen *et al*. (2020) used next-generation sequencing on a patient with metastatic lung adenocarcinoma, identifying a novel oncogenic fusion of *SOS1–ALK*. This fusion protein comprised amino acids from the N-terminal of *SOS1* and the C-terminal of *ALK*, resulting in the formation of the *SOS1–ALK* fusion with an allele frequency of 4.6%. The authors noted that the patient, treated with crizotinib for 6 months, showed a notable and sustained positive response without any significant adverse effects.[62] Izumi *et al*. (2021) discovered a novel fusion transcript, *CLIP1–LTK*, in NSCLC. *CLIP1* is part of the microtubule plus-end tracking protein family 7, whereas *LTK* belongs to the receptor tyrosine kinase 6 subfamily. The *CLIP1–LTK* fusion transcript combines exon 16 from *CLIP1* with exon 11 from LTK. Using *in vitro* and *in vivo* procedures, the authors found that *CLIP1–LTK* functions as an oncogenic driver in NSCLC and revealed that targeting the fusion protein with lorlatinib is a promising therapeutic strategy.[63,64]

3.4. Chimeric RNAs in thyroid cancer

Thyroid cancer is a common endocrine cancer, accounting for approximately 2.1% of all newly diagnosed cancer cases globally.[65] Over the last 30 years, thyroid cancer cases have tripled, with an increase in annual mortality rate of 1.1%.[66] It was previously believed that gene fusions in thyroid cancer were caused by exposure to radioactive rays. However, advancements in technology and research have led to the identification of numerous novel and known gene fusions in thyroid cancer. In a recent study conducted by Jurkiewicz *et al*. in 2021, a genetic fusion event involving an exon of the striatin gene (*STRN*) and the common anaplastic lymphoma kinase (*ALK*) gene breakpoint at exon 20 was observed.[67] The *STRN–ALK* fusion transcript includes the N-terminal caveolin-binding and coiled-coil domains of *STRN* connected to the juxta-membrane region of *ALK*, which contains the tyrosine kinase domain. Both genes are located on chromosome 2, at positions 2p22.2 and 2p23. The *STRN–ALK* fusion activates ALK kinase through ligand-independent dimerization facilitated by the STRN coiled-coil domain, leading to the activation of the MAPK signaling pathway.[68] Approximately 80% of thyroid cancer cases are PTC, with most gene fusions involving rearranged during transfection (RET) fusions.[69] Research by Staubitz *et al*. (2019) revealed *ANKRD26–RET* gene fusion in PTC. Both genes are located on chromosome 10, with the fusion composed of exon 29 of *ANKRD* and exon 12 of *RET*. The fusion of RET's tyrosine kinase region with protein–protein interaction motifs in *NKRD26–RET* may lead to the continuous activation of tyrosine kinase.[70] Similarly, Krishnan *et al*. (2020) revealed a new gene fusion, *TFG–RET*, in PTC. This fusion includes exons 1 – 4 from the 5' end of Trk fused gene (*TFG*) linked to the 3' end of *RET* tyrosine kinase. The *TFG–RET* fusion transforms human thyroid cells through a kinase-dependent mechanism. Moreover, the *TFG–RET* fusion forms oligomers based on the *PB1* domain, and this oligomerization process is essential for the oncogenic transformation induced by *TFG–RET*. Moreover, the authors reported that the *TFG–RET* fusion expression enhanced cell viability and proliferation, leading to tumor formation *in vivo*.[71]

Advancements in technologies such as high-throughput data analysis, deep sequencing, and accurate bioinformatic tools will further aid in the identification of new chimeric RNAs in thyroid cancer.

3.5. Chimeric RNAs in different cancers

With advancements in technology, various experimental techniques, including RNA sequencing, whole-genome sequencing, and bioinformatic software tools, have significantly contributed to the identification and confirmation of gene fusions across numerous cancer types. These findings have been deposited in various databases. A study conducted by Tao *et al.* (2018) identified a recurring chimeric RNA, *COL7A1–UCN2*, in human laryngeal cancer, which was formed through alternative splicing. Both genes are located on chromosome 3, with the COL7A1 fusion occurring at exons 113 – 117 and the junction with UCN2 at exon 2. The authors reported that laryngeal cancer patients positive for the *COL7A1–UCN2* fusion had significantly poorer overall survival than those negative for the fusion. In addition, *COL7A1* mRNA expression was reduced in malignant tissues, whereas the formation of *COL7A1–UCN2* chimeric RNA bypassed the tumor-suppressing effects of TGF-β1, facilitating tumor invasion and growth.[72]

Similarly, Wang *et al.* (2019) identified a *SEPT7P2–PSPH* chimeric transcript in nasopharyngeal carcinoma, which was formed through trans-splicing between adjacent genes. Both genes are located on chromosome 7, with the chimeric transcript resulting from the fusion of exon 1 from *SEPT7P2* and exon 4 from *PSPH*, separated by 10.2 Mb. Septin 7 pseudogene 2 (*SEPT7P2*) is a pseudogene similar to *SEPT7*, expressed across various tissues and involved in multiple biological processes. Phosphoserine phosphatase (*PSPH*) is a protein-coding gene, and its abnormal expression has been linked to several carcinomas. Further investigation revealed that suppressing the *SEPT7P2–PSPH* fusion transcript could enhance cell growth and cancer cell spread, likely due to the increased expression of *PSPH*.[73]

Furthermore, Wu *et al.* (2018) reported the presence of a chimeric RNA, *LHX6–NDUFA8*, in both cervical cancer tissue and pap smear specimens. This chimeric RNA has two isoforms: *LHX6–NDUFA8-e8e2* and *LHX6–NDUFA8-e8e3*, both showing significant prevalence in cervical cancer. To evaluate the potential of fusion RNAs for inclusion in pap smear screenings for cervical cancer and cervical intraepithelial neoplasia, the researchers analyzed the expression of both isoforms in pap smear samples. The findings showed a favorable detection rate for both isoforms, correlating with the presence of cervical cancer. Both isoforms of *LHX6–NDUFA8* result from cis-splicing events between neighboring genes, a process known as cis-SAGe.[74] Gene fusions detected in various cancer types are listed in Table 1.

4. Chimeric RNAs in healthy tissues and cells

Previously, chimeric RNAs were thought to be associated solely with tumorigenesis. However, advancements in

Table 1. Gene fusions in various cancer types

Cancer type	Gene fusion	Role in cancer	References
Prostate cancer	*SLC45A3–ELK4*	Regulates cell proliferation	77, 78
Prostate cancer	*TMPRSS2–ERG*	Oncogenic pathway activation	79
Prostate cancer	*KLK4–KLKP1*	Impacts cell proliferation	80
Prostate cancer	*UNC5D–NRG1*	Predicted protein-coding fusion	81
Esophageal cancer	*GOLM1–NAA35*	Tumor growth and progression	82
Esophageal cancer	*ASTN2–PAPPAas*	Enhances cell migration	83
Lung cancer	*KIF5B–MET*	Oncogenic properties	84
Lung cancer	*SOS1–ALK*	Metastatic lung adenocarcinoma	85
Lung cancer	*CLIP1–LTK*	Oncogenic driver in NSCLC	86
Thyroid cancer	*STRN–ALK*	Activates MAPK signaling pathway	87
Thyroid cancer	*ANKRD26–RET*	Continuous activation of tyrosine kinase	8
Thyroid cancer	*TFG–RET*	Oncogenic transformation	89
Laryngeal cancer	*COL7A1–UCN2*	Facilitates tumor invasion and growth	90
Nasopharyngeal cancer	*SEPT7P2–PSPH*	Promotes cell proliferation	91
cervical Cancer	*LHX6–NDUFA8*	Prevalence in cervical cancer	92

research, technology, and bioinformatics tools have led to the discovery of chimeric RNAs in various healthy tissues and cells. The fusion transcript *DUS4L–BCAP29* was initially linked to gastric cancer, but a study by Tang *et al.* (2019) revealed that *DUS4L–BCAP29* is also present in normal tissues, with the junction sequence identical to that reported in gastric cancer. Overexpression of this fusion transcript promoted cell growth and motility in non-cancerous cell lines.[75] Babiceanu *et al.* (2016) also reported the presence of chimeric RNAs in normal tissues and cells, identifying *CTNNBIP1–CLSTN1* and *CTBS–GNG5* fusion transcripts in both normal tissues and cell lines. Knocking down the *CTBS–GNG5* chimeric RNA suppressed cell growth and reduced cell motility, and inhibiting *CTNNBIP1–CLSTN1* also led to reduced growth and motility.[9] Li *et al.* (2008) reported the presence of the *JAZF1–JJAZ1* fusion transcript in normal endometrial stromal cells, which was formed as a result of RNA trans-splicing rather than chromosomal rearrangement.[42] The *PAX3–FOXO1* chimeric RNA, linked to rhabdomyosarcoma and used as a biomarker, was also detected in normal muscle cell lines and muscle biopsies.[43] Moreover, Chen *et al.* (2021) revealed the presence of chimeric RNAs in various non-malignant cell lines, including HEK-293T, HUVEC, and LO2 cells.[76]

When examining chimeric RNAs in healthy tissues, there is potential to uncover novel aspects of typical cellular function or regulation. These discoveries may provide a deeper understanding of how regular cellular mechanisms are co-opted in oncological contexts.[96] Analysis of RNA-seq data from 16 distinct healthy human tissues revealed that numerous sense–antisense chimeric transcripts were expressed across multiple tissues. This suggests a potential link between sense–antisense chimeras and normal physiological processes. Furthermore, an evolutionary analysis of sense–antisense fusions across different species identified several shared genes capable of producing these chimeric transcripts in humans, mice, fruit flies, and pigs. This finding implies that the emergence of sense–antisense chimeric transcripts from specific genes may offer evolutionary advantages, as these genes have been evolutionarily selected to enhance functional diversity in response to various cellular conditions.[97]

4.1. Potential effects of chimeric RNAs on subsequent gene expression

- *Gene regulation:* Chimeric RNAs can influence gene expression by acting as competitive endogenous RNAs (ceRNAs). These molecules bind to microRNAs, preventing them from degrading target mRNAs leading to increased expression of genes that would otherwise be suppressed by microRNAs[98]
- *RNA stability:* Chimeric RNAs have the potential to alter transcript stability. Depending on the combination of exons, these chimeric RNAs may either be more stable or more prone to degradation than their non-chimeric counterparts
- *Protein translation:* Some chimeric RNAs may be translated to produce novel fusion proteins. These fusion proteins might have combined or altered functions compared with the original proteins, potentially affecting cellular activities and pathways[99]
- *Epigenetic regulation:* Chimeric RNAs could also impact epigenetic modifications by influencing the recruitment of chromatin-modifying enzymes to specific genomic regions, thereby altering the expression of neighboring genes.[100]

5. Chimeric RNAs as a biomarker and molecular target

Since the discovery of the *BCR–ABL* fusion transcript, numerous fusion transcripts have been identified across various cancer types, emerging as promising biomarkers for cancer diagnosis and treatment. A simple example is shown in Figure 5. The *EML4–ALK* fusion serves as a biomarker in NSCLC, guiding the use of ALK inhibitors to improve patient outcomes.[77] Similarly, the *TMPRSS2–ERG* gene fusion is detectable in the early stages of prostate cancer and is valuable for prognostic assessments.[78] In a study by Lin *et al.* (2019), the chimeric RNA *seGOLM1–NAA35* was proposed as a potential biomarker and early indicator for human ESCC, potentially predicting early recurrence or disease progression more effectively than standard radiological methods.[57] Wu *et al.* (2018) also reported the frequent presence of *LHX6–NDUFA8* in cervical cancer and pap smear specimens, suggesting it could be used as a biomarker for cervical cancer detection.[73] Furthermore, Izumi *et al.* (2019) identified *CLIP1–LTK* fusion in patients with NSCLC. This fusion has been recognized as an oncogenic driver in NSCLC, with individuals harboring this fusion showing a strong, favorable response to lorlatinib, a tyrosine kinase inhibitor that targets receptor tyrosine kinases linked to cancer development. The fusion protein can be targeted using lorlatinib. A list of gene fusions with diagnostic and therapeutic potential is provided in Table 2.[102-117]

Similarly, intergenically spliced chimeric RNAs offer a unique set of biomarkers and potential therapeutic targets. In NEPC, several chimeric RNAs, including *TMPRSS2–ERG* (e2e4), *EEF2–SLC25A42*, *SNX13–ATP2C1*, and *FXYD2–DSCAML1*, have been identified and validated, all exhibiting specific expression patterns in NEPC cells. Notably, *TMPRSS2–ERG* (e2e4) showed elevated

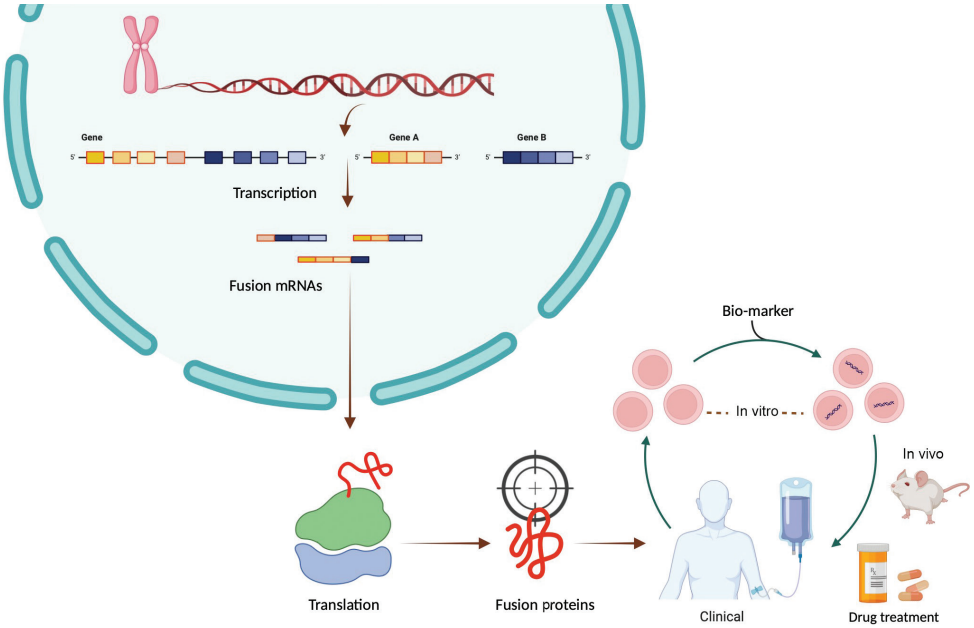

Figure 5. Graphical representation of how a fusion protein can be used as a molecular target or biomarker for tumors

expression levels in tumors, correlating with poor prognosis in the TCGA PCa study. Interestingly, the parental genes of this chimeric RNA did not show a similar correlation, highlighting its potential as a novel biomarker for NEPC. Furthermore, an in-depth analysis of chimeric RNAs extracted from the Cancer Cell Line Encyclopedia prostate RNA-seq dataset has outlined the chimeric RNA landscape across various PCa subtypes, including hormone-sensitive and castration-resistant PCa. In summary, chimeric RNAs expand the functional genome, offering valuable insights into the emerging mechanisms of tumorigenesis and serving as a promising source for discovering biomarkers and therapeutic targets.[118]

6. Limitations of using chimeric RNAs in therapy and as biomarkers

Chimeric RNAs exhibit significant heterogeneity, implying that diverse cancer cells, even within the same tumor, may express distinct chimeric RNAs. This diversity makes it challenging to develop a unified therapeutic strategy that can effectively target all relevant chimeric RNAs.[119] When developing RNA-based therapies, such as siRNA or antisense oligonucleotides, there is a concern regarding off-target effects. These effects can occur when the therapeutic intervention unintentionally affects normal RNA transcripts that share sequence or structural similarities with the chimeric RNA, potentially leading to unintended side effects.[120] In addition, chimeric RNAs are inherently unstable, which can lead to rapid degradation and complicate their reliable detection in biological samples. This instability not only hinders the development of targeted therapies but also limits their usefulness as reliable biomarkers.[121] Chimeric RNAs are also found in healthy tissues, which complicates their use as precise indicators of lingering disease or treatment effectiveness, as their presence may not always indicate disease progression. At present, no universally accepted, standardized approach exists for identifying and measuring chimeric RNAs in clinical settings. The lack of standardization can lead to inconsistent outcomes, further complicating their use as indicators of disease or treatment efficacy.

7. Databases for chimeric RNAs

The rate at which chimeric transcripts are being identified has surged exponentially.[79] Numerous efforts have been made to organize these identified transcripts using various bioinformatics tools and text-mining methodologies.[80] As previously mentioned, fusion transcripts have been discovered in various carcinomas as well as in non-diseased tissues and cells and have been deposited in multiple databases, including Mitelman,[81] FusionGDB,[82] ChimerDB 4.0,[83] ChiTarRs 5.0,[84] TumorFusions,[85] dbCRID,[86] TICdb,[87] ConjoinG[88] and COSMIC.[89] A list of databases for gene fusions is provided in Table 3.

8. Future prospectives

In the coming years, as sequencing technologies advance, particularly in full-length sequencing and the development of new software, we will be better equipped to explore

Table 2. Gene fusions with diagnostic and therapeutic potential

Tumor type	Gene fusion	Relevance	Diagnostic use	Therapeutic use
Chronic myeloid leukemia (CML)	*BCR–ABL*	Drives proliferation and survival of leukemic cells	Diagnostic marker for CML	Targeted by tyrosine kinase inhibitors (e.g., imatinib)
Acute promyelocytic leukemia (APL)	*PML–RARA*	Blocks differentiation of promyelocytes	Diagnostic marker for APL	Targeted by all-trans retinoic acid and arsenic trioxide
Ewing sarcoma	*EWSR1–FLI1*	Oncogenic driver fusion that alters transcription	Diagnostic marker for Ewing sarcoma	Investigational targeted therapies
Synovial sarcoma	*SS18–SSX1/SSX2*	Alters chromatin remodeling, driving oncogenesis	Diagnostic marker for synovial sarcoma	No specific targeted therapies currently
Non-small cell lung cancer (NSCLC)	*EML4–ALK*	Constitutive ALK signaling drives tumor growth	Diagnostic marker for ALK-positive anaplastic large-cell lymphoma	Targeted by ALK inhibitors
Prostate cancer	*TMPRSS2–ERG*	ERG overexpression leads to tumorigenesis	Used as a biomarker in prostate cancer	Potential target in ongoing research
Acute lymphoblastic leukemia (ALL)	*ETV6–RUNX1*	Promotes leukemogenesis through altered transcription	Diagnostic marker in pediatric ALL	No specific targeted therapies currently
Glioblastoma	*FGFR3–TACC3*	Constitutively active FGFR3 signaling	Investigational diagnostic marker	Targeted by FGFR inhibitors (e.g., erdafitinib)
Adenoid cystic carcinoma	*MYB–NFIB*	Leads to overexpression of oncogene MYB	Investigational diagnostic marker	No specific targeted therapies currently
Colorectal cancer	*ETV6–NTRK3*	Constitutive NTRK signaling drives oncogenesis	Diagnostic marker NTRK fusion-positive cancers	Targeted by TRK inhibitors (e.g., larotrectinib)
Papillary thyroid carcinoma	*RET–PTC*	Activates RET kinase, driving tumorigenesis	Diagnostic marker for RET fusion-positive thyroid cancer	Targeted by RET inhibitors (e.g., selpercatinib)
Glioma	*KIAA1549–BRAF*	Activates MAPK pathway, driving tumor growth	Diagnostic marker for pediatric low-grade gliomas	Targeted by BRAF inhibitors (e.g., vemurafenib)
Soft tissue sarcomas	*COL1A1–PDGFB*	Activates PDGF signaling, promoting tumor growth	Diagnostic marker for dermatofibrosarcoma protuberans	Targeted by PDGF inhibitors (e.g., imatinib)
Mantle cell lymphoma	*IGH–CCND1*	Cyclin D1 overexpression drives cell cycle progression	Diagnostic marker for mantle cell lymphoma	No specific targeted therapies currently
Thyroid cancer	*PAX8–PPARy*	Alters transcription and differentiation	Diagnostic marker for follicular thyroid carcinoma	Investigational targeted therapies
Cholangiocarcinoma	*FGFR2–BICC1*	Activates FGFR signaling, promoting tumorigenesis	Diagnostic marker for FGFR fusion-positive cholangiocarcinoma	Targeted by FGFR inhibitors (e.g., infigratinib)

Abbreviations: ALL: Acute lymphoblastic leukemia; ALCL: Anaplastic large-cell lymphoma; APL: Acute promyelocytic leukemia; ATRA: all-trans retinoic acid; CML: Chronic myeloid leukemia; Non-small cell lung cancer (NSCLC).

chimeric RNAs. Gene fusion events and the resulting chimeric RNAs have played a pivotal role in transforming cancer diagnosis and therapy. The identification of newer tumor driver genes has provided deeper insights into the onset and progression of tumors. Unconventional chimeric RNAs, produced through mechanisms other than trans-splicing and cis-SAGe, are expected to contribute significantly to the discovery of new pharmaceuticals and biomarkers for disease treatment. While some fusion proteins may not be ideal therapeutic targets, understanding their regulation, impact on downstream targets, and interference with cellular processes can provide crucial insights for discovering novel therapies for cancer. Despite advancements in RNA sequencing technology and molecular biology, several challenges remain. These include combating drug resistance, identifying mutant genes,

Table 3. Databases cataloging gene fusions

Database	Tumor	Non-tumor	Total entry	URL
Mitelman	Yes	No	51,184	https://mitelmandatabase.isb-cgc.org/
FusionGDB	Yes	No	43,895	https://ccsm.uth.edu/FusionGDB/index.htm
ChimerDB[4.0]	Yes	No	67,610	https://www.kobic.re.kr/chimerdb/
ChiTaRS 5.0	Yes	No	1,11,582	http://chitars.md.biu.ac.il/
LiGeA	Yes	No	3,77,540	http://hpc-bioinformatics.cineca.it/fusion/
Tumor Fusion Gene Data Portal	Yes	Yes	20,731	https://www.tumorfusions.org/
dbCRID	Yes	Yes	2643	http://c1.accurascience.com/dbCRID/
TICdb	Yes	No	1374	https://genetica.unav.edu/TICdb/
ConjoinGene	Yes	Yes	800	https://metasystems.riken.jp/conjoing/index
COSMIC	Yes	No	305	https://cancer.sanger.ac.uk/cosmic/fusion

uncovering new mutations, refining detection techniques, and understanding the mechanisms of chimeric RNA signaling pathways. Although many of the identified chimeric RNAs are believed to be unique to cancer, they have also been detected in healthy tissues and cells. Therefore, it is important to validate a sufficient number of non-tumor control samples before designating chimeric RNAs as cancer indicators. A major challenge in the study of chimeric RNAs is the lack of high-throughput methods for investigating their functionality. The formation of non-conventional chimeric RNAs and their relationship with gene fusions pose unresolved queries that require further investigation. However, by leveraging innovative high-throughput technologies such as deep sequencing, full-length sequencing, and precise bioinformatics tools, these challenges can be effectively addressed.

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References

1. Rabbitts TH. Chromosomal translocations in human cancer *Nature*. 1994;372:143-149.

   doi: 10.1038/372143a0

2. Heim S, Mitelman F. Molecular screening for new fusion genes in cancer. *Nat Genet*. 2008;40:685-686.

doi: 10.1038/ng0608-685

3. Nowell P. The minute chromosome (Phl) in chronic granulocytic leukemia. *Blut*. 1962;8:65-66.

doi: 10.1007/BF01630378

4. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243:290-293.

doi: 10.1038/243290a0

5. Rowley JD. The critical role of chromosome translocations in human leukemias. *Annu Rev Genet*. 1998;32:495-519.

doi: 10.1146/annurev.genet.32.1.495

6. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. *Nature*. 1985;315:550-554.

doi: 10.1038/315550a0

7. Tuna M, Amos CI, Mills GB. Molecular mechanisms and pathobiology of oncogenic fusion transcripts in epithelial tumors. *Oncotarget*. 2019;10:2095.

doi: 10.18632/oncotarget.26777

8. Chwalenia K, Facemire L, Li H. Chimeric RNAs in cancer and normal physiology. *Wiley Interdiscip Rev RNA*. 2017;8:e1427.

doi: 10.1002/wrna.1427

9. Zhang Y, Gong M, Yuan H, Park HG, Frierson HF, Li H. Chimeric transcript generated by cis-splicing of adjacent genes regulates prostate cancer cell proliferation. *Cancer Discov*. 2012;2(7):598-607.

doi: 10.1158/2159-8290.CD-12-0042

10. Soda M, Choi YL, Enomoto M, *et al*. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448:561-566.

doi: 10.1038/nature05945

11. Li Y, Li Y, Yang T. Clinical significance of EML4-ALK fusion gene and association with EGFR and KRAS gene mutations in 208 Chinese patients with non-small cell lung cancer. *PLoS One*. 2013;8:e52093.

doi: 10.1371/journal.pone.0052093

12. Hermans KG, Van Marion R, Van Dekken H, Jenster G, Van Weerden WM, Trapman J. TMPRSS2: ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer Res*. 2006;66:10658-10663.

doi: 10.1158/0008-5472.CAN-06-1871

13. Perner D, Vierkötter A, Sugiri D, *et al*. Association between sun-exposure, smoking behaviour and plasma antioxidant levels with the different manifestation of skin ageing signs between Japanese and German women--a pilot study. *J Dermatol Sci*. 2011;62:138-140.

doi: 10.1016/j.jdermsci.2011.02.010

14. Gisselsson D, Pettersson L, Höglund M, *et al*. Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity. *Proc Natl Acad Sci U S A*. 2000;97:5357-5362.

doi: 10.1073/pnas.090013497

15. Ally A, Balasundaram M, Carlsen R, *et al*. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. *Cell*. 2017;169:1327-1341.e1323.

doi: 10.1016/j.cell.2017.05.046

16. Northcott PA, Shih DJ, Peacock J, *et al*. Subgroup-specific structural variation across 1,000 medulloblastoma genomes. *Nature*. 2012;488:49-56.

doi: 10.1038/nature11327

17. Teles Alves I, Hiltemann S, Hartjes T, *et al*. Gene fusions by chromothripsis of chromosome 5q in the VCaP prostate cancer cell line. *Hum Genet*. 2013;132:709-713.

doi: 10.1007/s00439-013-1308-1

18. Efanov AA, Brenner AV, Bogdanova TI, *et al*. Investigation of the relationship between radiation dose and gene mutations and fusions in post-Chernobyl thyroid cancer. *J Natl Cancer Inst*. 2018;110:371-378.

doi: 10.1093/jnci/djx209

19. Su X, Li Z, He C, Chen W, Fu X, Yang A. Radiation exposure, young age, and female gender are associated with high prevalence of RET/PTC1 and RET/PTC3 in papillary thyroid cancer: A meta-analysis. *Oncotarget*. 2016;7:16716.

doi: 10.18632/oncotarget.7574

20. Leeman-Neill RJ, Kelly LM, Liu P, *et al*. ETV6-NTRK3 is a common chromosomal rearrangement in radiation-associated thyroid cancer. *Cancer*. 2014;120:799-807.

doi: 10.1002/cncr.28484

21. Dacic S, Luvison A, Evdokimova V, *et al*. RET rearrangements in lung adenocarcinoma and radiation. *J Thorac Oncol*. 2014;9:118-120.

doi: 10.1097/JTO.0000000000000015

22. Chen Y, Li G, Lei Y, *et al*. Lung cancer family history and exposure to occupational/domestic coal combustion contribute to variations in clinicopathologic features and gene fusion patterns in non-small cell lung cancer. *Thorac Cancer*. 2019;10:695-707.

doi: 10.1111/1759-7714.12987

23. Navarrete-Meneses M, Salas-Labadía C, Sanabrais-Jiménez M, *et al*. Exposure to the insecticides permethrin and malathion induces leukemia and lymphoma-associated gene aberrations *in vitro*. *Toxicol In Vitro*. 2017;44:17-26.

doi: 10.1016/j.tiv.2017.06.013

24. Egashira S, Jinnin M, Ajino M, *et al.* Chronic sun exposure-related fusion oncogenes EGFR-PPARGC1A in cutaneous squamous cell carcinoma. *Sci Rep*. 2017;7:12654.

    doi: 10.1038/s41598-017-12836-z

25. Holly JM, Broadhurst J, Mansor R, Bahl A, Perks CM. Hyperglycemia promotes TMPRSS2-ERG gene fusion in prostate cancer cells via upregulating insulin-like growth factor-binding protein-2. *Front Endocrinol* (*Lausanne*). 2017;8:305.

    doi: 10.3389/fendo.2017.00305

26. Eguchi-Ishimae M, Eguchi M, Ishii E, *et al*. Breakage and fusion of the TEL (ETV6) gene in immature B lymphocytes induced by apoptogenic signals. *Blood*. 2001;97:737-743.

    doi: 10.1182/blood.v97.3.737

27. Mani RS, Tomlins SA, Callahan K, *et al*. Induced chromosomal proximity and gene fusions in prostate cancer. *Science*. 2009;326:1230-1230.

    doi: 10.1126/science.1178124

28. Jividen K, Li H. Chimeric RNAs generated by intergenic splicing in normal and cancer cells. *Genes Chromosomes Cancer*. 2014;53:963-971.

    doi: 10.1002/gcc.22207

29. Wang K, Ubriaco G, Sutherland LC. RBM6-RBM5 transcription-induced chimeras are differentially expressed in tumours. *BMC Genomics*. 2007;8:348.

30. Parra G, Reymond A, Dabbouseh N, *et al*. Tandem chimerism as a means to increase protein complexity in the human genome. *Genome Res*. 2006;16:37-44.

    doi: 10.1101/gr.4145906

31. Denoeud F, Kapranov P, Ucla C, *et al.* Prominent use of distal 5′ transcription start sites and discovery of a large number of additional exons in ENCODE regions. *Genome Res*. 2007;17:746-759.

    doi: 10.1101/gr.5660607

32. Siepel A, Diekhans M, Brejová B, *et al*. Targeted discovery of novel human exons by comparative genomics. *Genome Res*. 2007;17:1763-1773.

    doi: 10.1101/gr.7128207

33. Kannan K, Wang L, Wang J, Ittmann MM, Li W, Yen L. Recurrent chimeric RNAs enriched in human prostate cancer identified by deep sequencing. *Proc Natl Acad Sci U S A*. 2011;108:9172-9177.

    doi: 10.1073/pnas.1100489108

34. Nacu S, Yuan W, Kan Z, *et al*. Deep RNA sequencing analysis of readthrough gene fusions in human prostate adenocarcinoma and reference samples. *BMC Med Genomics*. 2011;4:1-22.

    doi: 10.1186/1755-8794-4-11

35. Hernández-Torres F, Rastrojo A, Aguado B. Intron retention and transcript chimerism conserved across mammals: Ly6g5b and Csnk2b-Ly6g5b as examples. *BMC Genomics*. 2013;14:1-12.

    doi: 10.1186/1471-2164-14-199

36. Akiva P, Toporik A, Edelheit S, *et al*. Transcription-mediated gene fusion in the human genome. *Genome Res.* 2006;16:30-36.

    doi: 10.1101/gr.4137606

37. Qin F, Song Z, Babiceanu M, *et al*. Discovery of CTCF-sensitive Cis-spliced fusion RNAs between adjacent genes in human prostate cells. *PLoS Genet*. 2015;11:e1005001.

    doi: 10.1371/journal.pgen.1005001

38. Kumar-Sinha C, Kalyana-Sundaram S, Chinnaiyan AM. SLC45A3-ELK4 chimera in prostate cancer: Spotlight on cis-splicing. *Cancer Discov*. 2012;2:582-585.

    doi: 10.1158/2159-8290.CD-12-0212

39. Rickman DS, Pflueger D, Moss B, *et al*. SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. *Cancer Res*. 2009;69:2734-2738.

    doi: 10.1158/0008-5472.CAN-08-4926

40. Wu CS, Yu CY, Chuang CY, *et al*. Integrative transcriptome sequencing identifies trans-splicing events with important roles in human embryonic stem cell pluripotency. *Genome Res*. 2014;24:25-36.

    doi: 10.1101/gr.159483.113

41. Murphy L, Dotzlaw H, Hamerton J, Schwarz J. Investigation of the origin of variant, truncated estrogen receptor-like mRNAs identified in some human breast cancer biopsy samples. *Breast Cancer Res Treat*. 1993;26:149-161.

    doi: 10.1007/BF00689688

42. Pink J, Fritsch M, Bilimoria M, Assikis V, Jordan V. Cloning and characterization of a 77-kDa oestrogen receptor isolated from a human breast cancer cell line. *Br J Cancer*. 1997;75:17-27.

    doi: 10.1038/bjc.1997.4

43. Li H, Wang J, Mor G, Sklar J. A neoplastic gene fusion mimics trans-splicing of RNAs in normal human cells. *Science*. 2008;321:1357-1361.

    doi: 10.1126/science.1156725

44. Yuan H, Qin F, Movassagh H, *et al*. A chimeric RNA characteristic of rhabdomyosarcoma in normal myogenesis process. *Cancer Discov*. 2013;3:1394-1403.

    doi: 10.1158/2159-8290.CD-13-0186

45. Sutton RE, Boothroyd JC. Evidence for trans splicing in trypanosomes. *Cell*. 1986;47:527-535.

    doi: 10.1016/0092-8674(86)90617-3

46. Krause M, Hirsh D. A trans-spliced leader sequence on actin mRNA in *C. elegans*. *Cell*. 1987;49:753-761.

doi: 10.1016/0092-8674(87)90613-1

47. Nilsen TW. Evolutionary origin of SL-addition trans-splicing: Still an enigma. *Trends Genet*. 2001;17:678-680.

doi: 10.1016/s0168-9525(01)02499-4

48. Gao Q, Liang WW, Foltz SM, *et al*. Driver fusions and their implications in the development and treatment of human cancers. *Cell Rep.* 2018;23:227-238.e223.

doi: 10.1016/j.celrep.2018.03.050

49. Nowell PC, Hungerford DA. Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. *J Natl Cancer Inst*. 1961;27:1013-1035.

50. Priedigkeit N, Golub T. *Potentially Targetable Fusion RNAs May Be More Common in Metastatic Breast Cancer Than Previously Realized*. Massachusetts: Dana-Farber Cancer Institute; 2023.

51. Metellus P, Camilla C, Bialecki E, *et al*. The landscape of cancer-associated transcript fusions in adult brain tumors: A longitudinal assessment in 140 patients with cerebral gliomas and brain metastases. *Front Oncol*. 2024;14:1382394.

doi: 10.3389/fonc.2024.1382394

52. Tong Y, Su X, Rouse W, *et al*. Transcriptome-wide, unbiased profiling of ribonuclease targeting chimeras. *J Am Chem Soc*. 2024;146:21525-21534.

doi: 10.1021/jacs.4c04717

53. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.

doi: 10.3322/caac.21492

54. Qin F, Zhang Y, Liu J, Li H. SLC45A3-ELK4 functions as a long non-coding chimeric RNA. *Cancer Lett*. 2017;404:53-61.

doi: 10.1016/j.canlet.2017.07.007

55. Zhou F, Gao S, Han D, *et al*. TMPRSS2-ERG activates NO-cGMP signaling in prostate cancer cells. *Oncogene*. 2019;38:4397-4411.

doi: 10.1038/s41388-019-0730-9

56. Chakravarthi BV, Dedigama-Arachchige P, Carskadon S, *et al*. Pseudogene associated recurrent gene fusion in prostate cancer. *Neoplasia*. 2019;21:989-1002.

doi: 10.1016/j.neo.2019.07.010

57. Ptáková N, Martínek P, Holubec L, *et al*. Identification of tumors with NRG1 rearrangement, including a novel putative pathogenic UNC5D-NRG1 gene fusion in prostate cancer by data-drilling a de-identified tumor database. *Genes Chromosomes Cancer*. 2021;60:474-481.

doi: 10.1002/gcc.22942

58. Torre LA, Bray F, Siegel RL, Ferlay L, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;65:87-108.

doi: 10.3322/caac.21262

59. Zhang H, Lin W, Kannan K, *et al.* Aberrant chimeric RNA GOLM1-MAK10 encoding a secreted fusion protein as a molecular signature for human esophageal squamous cell carcinoma. *Oncotarget*. 2013;4:2135.

doi: 10.18632/oncotarget.1465

60. Lin Y, Dong H, Deng W, *et al*. Evaluation of salivary exosomal chimeric GOLM1-NAA35 RNA as a potential biomarker in esophageal carcinoma. *Clin Cancer Res.* 2019;25:3035-3045.

doi: 10.1158/1078-0432.CCR-18-3169

61. Wang L, Xiong X, Yao Z, *et al*. Chimeric RNA ASTN2-PAPPAas aggravates tumor progression and metastasis in human esophageal cancer. *Cancer Lett.* 2021;501:1-11.

doi: 10.1016/j.canlet.2020.10.052

62. Luo Y, Du L, Yao Z, *et al*. Generation and application of inducible chimeric RNA ASTN2-PAPPAas knockin mouse model. *Cells*. 2022;11:277.

doi: 10.3390/cells11020277

63. Blidner RA, Haynes BC, Hyter S, *et al*. Design, optimization, and multisite evaluation of a targeted next-generation sequencing assay system for chimeric RNAs from gene fusions and exon-skipping events in non-small cell lung cancer. *J Mol Diagn*. 2019;21:352-365.

doi: 10.1016/j.jmoldx.2018.10.003.

64. Gow CH, Liu YN, Li HY, *et al*. Oncogenic function of a KIF5B-MET fusion variant in non-small cell lung cancer. *Neoplasia*. 2018;20:838-847.

doi: 10.1016/j.neo.2018.06.007

65. Chen HF, Wang WX, Xu CW, *et al.* A novel SOS1-ALK fusion variant in a patient with metastatic lung adenocarcinoma and a remarkable response to crizotinib. *Lung Cancer*. 2020;142:59-62.

doi: 10.1016/j.lungcan.2020.02.012

66. Izumi H, Matsumoto S, Liu J, *et al*. The CLIP1-LTK fusion is an oncogenic driver in non-small-cell lung cancer. *Nature*. 2021;600:319-323.

doi: 10.1038/s41586-021-04135-5

67. Kitahara CM, Sosa JA. The changing incidence of thyroid cancer. *Nat Rev Endocrinol.* 2016;12:646-653.

doi: 10.1038/nrendo.2016.110

68. Seib CD, Sosa JS. Evolving understanding of the epidemiology of thyroid cancer. *Endocrinol Metab Clin*. 2019;48:23-35.

doi: 10.1016/j.ecl.2018.10.002

69. Jurkiewicz M, Cimic A, Murty VV, *et al*. Detection of STRN-ALK fusion in thyroid nodules with indeterminate cytopathology facilitates papillary thyroid cancer diagnosis. *Diagn Cytopathol*. 2021;49:E146-E151.

doi: 10.1002/dc.24647

70. Kelly LM, Barila G, Liu P, *et al*. Identification of the transforming STRN-ALK fusion as a potential therapeutic target in the aggressive forms of thyroid cancer. *Proc Natl Acad Sci U S A*. 2014;111:4233-4238.

doi: 10.1073/pnas.1321937111

71. Agrawal N, Akbani R, Arman Aksoy B, *et al*. Integrated genomic characterization of papillary thyroid carcinoma. *Cell*. 2014;159:676-690.

doi: 10.1016/j.cell.2014.09.050

72. Staubitz JI, Musholt TJ, Schad A, *et al*. ANKRD26-RET-a novel gene fusion involving RET in papillary thyroid carcinoma. *Cancer Genet*. 2019;238:10-17.

doi: 10.1016/j.cancergen.2019.07.002

73. Krishnan A, Berthelet J, Renaud E, *et al*. Proteogenomics analysis unveils a TFG-RET gene fusion and druggable targets in papillary thyroid carcinomas. *Nat Commun*. 2020;11:2056.

doi: 10.1038/s41467-020-15955-w

74. Tao Y, Gross N, Fan X, *et al*. Identification of novel enriched recurrent chimeric COL7A1-UCN2 in human laryngeal cancer samples using deep sequencing. *BMC Cancer*. 2018;18:248.

doi: 10.1186/s12885-018-4161-8

75. Wang J, Xie GF, He Y, *et al*. Interfering expression of chimeric transcript SEPT7P2-PSPH promotes cell proliferation in patients with nasopharyngeal carcinoma. *J Oncol*. 2019;2019:1654724.

doi: 10.1155/2019/1654724

76. Riegler J, Gill H, Ogasawara A, *et al*. VCAM-1 density and tumor perfusion predict T-cell infiltration and treatment response in preclinical models. *Neoplasia*. 2019;21(10):1036-1050.

doi: 10.1016/j.neo.2019.08.003

77. Ali RM, McIntosh SA, Savage KI. Homologous recombination deficiency in breast cancer: Implications for risk, cancer development, and therapy. *Genes Chromosomes Cancer*. 2021;60(5):358-372.

doi: 10.1002/gcc.22921

78. Hartman ML, Gajos-Michniewicz A, Talaj JA, *et al*. BH3 mimetics potentiate pro-apoptotic activity of encorafenib in BRAFV600E melanoma cells. *Cancer Lett*. 2021;499:122-136.

doi: 10.1016/j.canlet.2020.11.036

79. Mamouni K, Zhang S, Li X, *et al*. A novel flavonoid composition targets androgen receptor signaling and inhibits prostate cancer growth in preclinical models. *Neoplasia*. 2018;20(8):789-799.

doi: 10.1016/j.neo.2018.06.003

80. Sala L, Mancin M, Pastore A, Seminati D, Cortinovis D, Bidoli P, Alberti A. Aplastic anemia in a patient with advanced lung adenocarcinoma during first line osimertinib: A case report and literature review. *Lung Cancer*. 2020;142:120-122.

doi: 10.1016/j.lungcan.2020.02.019

81. Ford DJ, Dingwall AK. Corrigendum to "The cancer COMPASS: Navigating the functions of MLL complexes in cancer" [Cancer Genetics 208 (2015) pp. 178-191]. *Cancer Genet*. 2019;233:102.

doi: 10.1016/j.cancergen.2019.03.003

82. Moon JY, Zolnik CP, Wang Z, *et al*. Gut microbiota and plasma metabolites associated with diabetes in women with, or at high risk for, HIV infection. *EBioMedicine*. 2018;37:392-400.

doi: 10.1016/j.ebiom.2018.10.037

83. Singh S, Qin F, Kumar S, *et al*. The landscape of chimeric RNAs in non-diseased tissues and cells. *Nucleic Acids Res*. 2020;48(4):1764-1778.

doi: 10.1093/nar/gkz1223

84. Elfman J, Pham LP, Li H. The relationship between chimeric RNAs and gene fusions; potential implications of reciprocity in cancer. *J Genet Genomics, Yi Chuan Xue Bao*. 2020;47(7):341.

doi: 10.1016/j.jgg.2020.04.005

85. Sun Y, Li H. Chimeric RNAs discovered by RNA sequencing and their roles in cancer and rare genetic diseases. *Genes* (*Basel*). 2022;13(5):741.

doi: 10.3390/genes13050741

86. Wu P, Yang S, Singh S, *et al*. The landscape and implications of chimeric RNAs in cervical cancer. *EBioMedicine*. 2018;37:158-167.

doi: 10.1016/j.ebiom.2018.10.059

87. Tang Y, Guan F, Li H. Case study: The recurrent fusion RNA DUS4L-BCAP29 in noncancer human tissues and cells. *Methods Mol Biol*. 2020;2079:243-258.

doi: 10.1007/978-1-4939-9904-0_19

88. Babiceanu M, Qin F, Xie Z, Jia Y, *et al*. Recurrent chimeric fusion RNAs in non-cancer tissues and cells. *Nucleic Acids Res*. 2016;44:2859-2872.

doi: 10.1093/nar/gkw032

89. Chen C, Haddox S, Tang Y, Qin F, Li H. Landscape of chimeric RNAs in non-cancerous cells. *Genes* (*Basel*).

2021;12:466.

doi: 10.3390/genes12040466

90. Mukherjee S, Detroja R, Balamurali D, *et al*. Computational analysis of sense-antisense chimeric transcripts reveals their potential regulatory features and the landscape of expression in human cells. *NAR Genom Bioinform*. 2021;3(3):lqab074.

doi: 10.1093/nargab/lqab074

91. Shaw AT, Yeap BY, Solomon BJ, *et al*. Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: A retrospective analysis. *Lancet Oncol*. 2011;12:1004-1012.

doi: 10.1016/S1470-2045(11)70232-7

92. Adamo P, Ladomery M. The oncogene ERG: A key factor in prostate cancer. *Oncogene*. 2016;35:403-414.

doi: 10.1038/onc.2015.109

93. Hehlmann R. Chronic myeloid leukemia in 2020. *Hemasphere*. 2020;4(5):e468.

doi: 10.1097/HS9.0000000000000468

94. Liquori A, Ibañez M, Sargas C, Sanz MÁ, Barragán E, Cervera J. Acute promyelocytic leukemia: A constellation of molecular events around a single PML-RARA fusion gene. *Cancers* (*Basel*). 2020;12(3):624.

doi: 10.3390/cancers12030624

95. García-García L, Fernández-Tabanera E, Cervera ST, *et al*. The transcription factor FEZF1, a direct target of EWSR1-FLI1 in Ewing sarcoma cells, regulates the expression of neural-specific genes. *Cancers* (*Basel*). 2021;13(22):5668.

doi: 10.3390/cancers13225668

96. Zaborowski M, Vargas AC, Pulvers J, *et al*. When used together SS18-SSX fusion-specific and SSX C-terminus immunohistochemistry are highly specific and sensitive for the diagnosis of synovial sarcoma and can replace FISH or molecular testing in most cases. *Histopathology*. 2020;77(4):588-600.

doi: 10.1111/his.14190

97. Elliott J, Bai Z, Hsieh SC, *et al*. ALK inhibitors for non-small cell lung cancer: A systematic review and network meta-analysis. *PLoS One*. 2020;15(2):e0229179.

doi: 10.1371/journal.pone.0229179

98. Stopsack KH, Su XA, Vaselkiv JB, *et al*. Transcriptomes of prostate cancer with TMPRSS2: ERG and other ETS fusions. *Mol Can Res*. 2023;1(1):14-23.

doi: 10.1158/1541-7786.MCR-22-0446

99. Østergaard A, Fiocco M, De Groot-Kruseman H, *et al*. ETV6: RUNX1 Acute Lymphoblastic Leukemia: How much therapy is needed for cure? *Leukemia*. 2024;38:1-11.

doi: 10.1038/s41375-024-02287-7

100. Haura EB, Hicks JK, Boyle TA. Erdafitinib overcomes FGFR3-TACC3-mediated resistance to osimertinib. *J Thorac Oncol*. 2020;15(9):e154-e156.

doi: 10.1016/j.jtho.2019.12.132

101. Wagner VP, Bingle CD, Bingle L. MYB-NFIB fusion transcript in adenoid cystic carcinoma: Current state of knowledge and future directions. *Crit Rev Oncol Hematol*. 2022;176:103745.

doi: 10.1016/j.critrevonc.2022.103745

102. Bungaro M, Garbo E. NTRK1/2/3: Biology, detection and therapy. *Precision Cancer Med*. 2024;6.

103. Wirth LJ, Sherman E, Robinson B, *et al*. Efficacy of selpercatinib in RET-altered thyroid cancers. *N Engl J Med*. 2020;383(9):825-835.

doi: 10.1056/NEJMoa2005651

104. Houghton PJ. Advances in the treatment of BRAF-mutant low-grade glioma with MAPK inhibitors. *Transl Pediatr*. 2024;13(3):513.

doi: 10.21037/tp-23-541

105. Antar DA, Laabid K, Darfaoui M, El Omrani A, Khouchani, M. The role of Imatinib mesylate in the treatment of metastatic dermatofibrosarcoma protuberans: A case report and literature review. *Sch J Med Case Rep*. 2023;6:1219-1223.

106. Goktas E, Sanal S, Tekinalp A. Blastoid variant mantle cell lymphoma with amplified IGH/CCND1 fusion: A unique case and current literature review. *Med Bull Haseki*. 2024;62(2):124-127.

doi: 10.4274/haseki.galenos.2024.9567

107. George N, Agarwal A. 25 Genetic landscape and molecular mechanisms in thyroid cancer. In: *Textbook of Endocrine Surgery.* California: Saunders; 2022: p. 216.

108. King G, Javle M. FGFR inhibitors: clinical activity and development in the treatment of cholangiocarcinoma. *Curr Oncol Rep*. 2021;23:108.

doi: 10.1007/s11912-021-01100-3

109. Li H, Wang Q. Chimeric RNAs and their implication in prostate cancer. *Cancer Pathog Ther*. 2023;1(3):216-219.

doi: 10.1016/j.cpt.2023.04.003

110. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov*. 2010;9(1):57-67.

doi: 10.1038/nrd3010

111. Mahmoudian-Sani MR, Jalali A, Jamshidi M, *et al*. Long non-coding RNAs in thyroid cancer: Implications for pathogenesis, diagnosis, and therapy. *Oncol Res Treat*. 2019;42(3):136-142.

doi: 10.1159/000495151

112. Mertens F, Johansson B, Fioretos T, Mitelman F. The emerging complexity of gene fusions in cancer. *Nat Rev Cancer*. 2015;15:371-381.

doi: 10.1038/nrc3947

113. Tagore S, Gorohovski A, Jensen LJ, Frenkel-Morgenstern M. ProtFus: A comprehensive method characterizing protein-protein interactions of fusion proteins. *PLoS Comput Biol*. 2019;15:e1007239.

doi: 10.1371/journal.pcbi.1007239

114. Mitelman F. Recurrent chromosome aberrations in cancer. *Mutat Res*. 2000;462:247-253.

doi: 10.1016/s1383-5742(00)00006-5

115. Kim P, Zhou X. FusionGDB: Fusion gene annotation DataBase. *Nucleic Acids Res*. 2019;47:D994-D1004.

doi: 10.1093/nar/gky1067

116. Jang YE, Jang I, Kim S, *et al*. ChimerDB 4.0: An updated and expanded database of fusion genes. *Nucleic Acids Res*. 2020;48:D817-D824.

doi: 10.1093/nar/gkz1013

117. Balamurali D, Gorohovski A, Detroja R, Palande V, Raviv-Shay D, Frenkel-Morgenstern M. ChiTaRS 5.0: The comprehensive database of chimeric transcripts matched with druggable fusions and 3D chromatin maps. *Nucleic Acids Res*. 2020;48:D825-D834.

doi: 10.1093/nar/gkz1025

118. Hu X, Wang Q, Tang M, *et al*. TumorFusions: An integrative resource for cancer-associated transcript fusions. *Nucleic Acids Res*. 2018;46:D1144-D1149.

doi: 10.1093/nar/gkx1018

119. Kong F, Zhu J, Wu J, *et al*. dbCRID: A database of chromosomal rearrangements in human diseases. *Nucleic Acids Res*. 2010;39:D895-D900.

doi: 10.1093/nar/gkq1038

120. Novo FJ, De Mendíbil IO, Vizmanos JL. TICdb: A collection of gene-mapped translocation breakpoints in cancer. *BMC Genomics*. 2007;8:33.

doi: 10.1186/1471-2164-8-33

121. Prakash T, Sharma VK, Adati N, *et al*. Expression of conjoined genes: Another mechanism for gene regulation in eukaryotes. *PLoS One*. 2010;5:e13284.

doi: 10.1371/journal.pone.0013284

122. Tate JG, Bamford S, Jubb HC, *et al*. COSMIC: The catalogue of somatic mutations in cancer. *Nucleic Acids Res*. 2019;47:D941-D947.

doi: 10.1093/nar/gky1015

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

G protein-coupled estrogen receptor 1 and its role in sex-specific differences in neurological and cardiovascular diseases

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Abstract

Estrogen receptors (ERs) and their ligands play a crucial role in physiological and pathophysiological processes, particularly in the central nervous and cardiovascular systems. There is increasing evidence that besides the two cytosolic and nuclear ERs, namely, ERα and ERβ, the seven-transmembrane G protein-coupled ER 1 (GPER1) is of great importance in the molecular mechanisms underlying various neurological and cardiovascular diseases and is probably responsible for sex-specific differences. In contrast to ERα and ERβ, GPER1 mediates its effects through not only transcriptional regulation but also rapid nongenomic signaling. This emphasizes the role of GPER1 in the modulation of acute pathophysiological mechanisms involving changes in diverse signaling pathways related to neurological and cardiological aspects. In this review, we have summarized the role of GPER1 in disorders of excitable tissues, including neuroinflammation, learning and memory, Alzheimer's disease, Parkinson's disease, depression and mood disorders, schizophrenia, epilepsy, autism spectrum disorders and attention-deficit/hyperactivity disorder, migraine and pain, cardiovascular hypertension, cardiovascular function and fibrosis, hypertrophy, and atrial fibrillation, with a special focus on its involvement in sex-specific differences. We have assessed reports investigating the role of GPER1 in rodents and humans using *in vivo* and *in vitro* data. We have also reviewed the role of nutraceuticals, especially phytoestrogens, in this context. Furthermore, we have discussed the potential of GPER1 as a target for novel therapeutic interventions and prognostic indicator of neurological and cardiovascular diseases with a focus on sex-specific differences.

Keywords: G protein-coupled estrogen receptor 1; Sex-specific differences; Neurological diseases; Cardiovascular diseases; Estrogen; Estrogen receptors

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1. Introduction

Sex-specific differences in diseases of the central nervous system (CNS)[1] and cardiovascular system[2-6] are currently generally accepted, and the underlying molecular mechanisms have been widely investigated in the past few decades. As sex hormones play a key role in developmental and regulatory processes in physiological and pathophysiological conditions in both males and females, they are prominent candidates influencing the regulatory processes that result in sex differences. Together with the well-known intracellular estrogen receptor (ER) alpha (ERα) and ERβ, which regulate

cellular functions primarily at the transcriptional level,[7] the novel G protein-coupled ER 1 (GPER1, GPR30), a member of the seven-transmembrane G protein-coupled receptor family, is capable of transmitting rapid signaling transduction through G proteins. GPER1 binds to 17β-estradiol (E2) with high affinity, mediating rapid estrogen-dependent cellular signaling responses through second messengers such as cyclic adenosine monophosphate (cAMP) and calcium as well as harboring the additional ability to affect gene expression.[8-10] GPER1 is generally localized in the plasma membrane and intracellularly in the endoplasmic reticulum as well as early endosomes, but it is absent in the nucleus of human embryonic kidney 293 (HEK293) cells.[11] In general, the subcellular localization of GPER1 differs among various cell types, tissues, age, sex, and species.[12] In particular, GPER1 is reportedly located in the plasma membrane of breast cancer cells lacking ERα and ERβ (SKBR3)[10] and the inner membrane of the endoplasmic reticulum in monkey kidney fibroblasts.[9] In rat oxytocin neurons, GPER1 was expressed primarily in the Golgi but was absent in the plasma membrane.[13] In a study comprising male and female rats, GPER1 expression was detected in all neurons located in the trigeminal ganglion on the cell surface and in the cytoplasm.[14] However, in the cardiomyocytes of female rats, GPER1 expression was enhanced in the perinuclear region.[15] Live cell imaging of mouse myoblast C2C12 cells, Madin–Darby canine kidney epithelial cells, and human ductal breast epithelial tumor T47-D cells revealed that even after reaching the plasma membrane, GPER1 was detectable in distinct puncta, indicating that the receptor undergoes constitutive endocytosis, highlighting its dynamic processing.[16,17] The first step in GPER1-mediated rapid signaling is the activation of G proteins with subsequent activation of different downstream signaling pathways; GPER1 increases estrogen-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) activity *through* $G_{i/o}$-mediated activation[18] or G_s-mediated transactivation of adenylyl cyclase and epidermal growth factor receptor (EGFR).[19] Regarding the GPER1-dependent signaling cascades of non-genomic effects, the activation of GPER1 by E2 causes an increase in intracellular calcium levels, likely due to the release of stored calcium rather than the entry of calcium from the extracellular space.[20] The concentration of intracellular cAMP and phosphorylation of cAMP response element-binding protein (CREB) increased on the binding of the specific GPER1 agonist G-1 to GPER1. Consequently, the activation of GPER1 by G-1 facilitated cell proliferation *through* the cAMP/protein kinase A (PKA)/p-CREB pathway and subsequently upregulated the levels of cell cycle regulators such as cyclin D1/cyclin-dependent kinase (CDK) 6 and cyclin E1/CDK2.[21] Furthermore, protein kinases such as protein kinase C (PKC) and PKA can be activated by intracellular calcium, which acts as a second messenger to activate downstream effector proteins through phosphorylation. A previous study detected GPER1-mediated estrogen signaling in neuroblastoma SH-SY5Y cells, in which GPER1 activation is followed by rapid calcium mobilization and PKC activation; hence, these pathways play a key role in neurological signaling. Moreover, GPER1 mediates calcium signaling at the transcriptional level during several processes, such as regulation of opioid-dependent signaling in the brain.[20] Similarly, estrogen uncouples other $G_{i/o}$-GPCRs such as gamma-aminobutyric acid B and opioid receptor-like-1 by activating G protein-coupled inwardly rectifying potassium channels. This activation is also dependent on the activation of phospholipase C, PKA, and PKC.[22] Regarding the interactome of GPER, a study by Ahmadian Elmi *et al.* reported 73 potential GPER1 interactions, including a direct interaction of GPER1 with CLPTM1, regulator of GABA type A receptor forward trafficking, and an indirect interaction of GPER1 with regulatory beta and catalytic alpha subunits of glucosidase II, both of which associate with CLPTM1 regulator of GABA type A receptor forward trafficking. They showed that the overexpression of stromal interaction molecule 1 and additional depletion of calcium stores affected GPER1 maturation, trafficking, and calcium signaling, with GPER1 becoming more nuclear.[23] Furthermore, GPER1 signaling may be dependent on complex formation with other receptors such as the β1-adrenergic receptor, a membrane-associated guanylate kinase scaffold protein, and PKA-anchoring protein.[24] Moreover, GPER1 increases the expression of the hypoxia-inducible factor 1 subunit alpha-dependent vascular endothelial growth factor that enhances angiogenesis and progression in breast cancer.[25] At present, GPER1 is widely discussed as an important receptor mediating sex-specific differences in both health and disease,[26] including the CNS and cardiovascular system. Besides ERα and ERβ,[27] GPER1 is widely expressed in the excitable tissue of the brain, that is, in various regions of the nervous system, including the hypothalamus, hippocampus, cerebral cortex, dorsal horn of the spinal cord, and primary afferent neurons, emphasizing its emerging role in neurological diseases.[20,28-32] GPER1 is also expressed in the excitable tissue of the heart, that is, the arterial wall,[33] where its deletion causes left ventricular dysfunction and adverse remodeling, which was analyzed using sex-specific gene profiling.[34] Notably, E2 has previously been associated with sex-specific differences in hypertension,[35,36] hypertrophy,[37] heart failure,[38] and cardiac stress,[39,40] indicating that ERs play a crucial role in the development of cardiovascular diseases.

In addition, the impact of nutrition, particularly polyphenol metabolites, as well as environmental factors has been widely discussed in terms of neurodegenerative[41-43] and cardiovascular diseases.[44] Moreover, the binding of isoflavones to GPER1 is considered highly probable, as reported in several *in silico* molecular docking studies and *in vitro* studies.[45] As a central link between environmental factors and genetics,[46] epigenetic modifications of *GPER1* expression have recently been analyzed in the context of different diseases. Studies have shown that GPER1 expression profiles are driven by sex-specific epigenetics in a diabetic mouse model[47] or drive cell apoptosis in SKBR3 and MDA-MB-231 breast cancer cells through the epigenetic downregulation of GPER1 expression.[48] Furthermore, endocrine-disrupting chemicals, such as ethinylestradiol, alter the DNA methylation of several genes, with *GPER1* being a gene with the highest number of alterations in its methylation patterns across three generations of the model organism *Menidia beryllina*.[49] In a prenatal-exposure mouse model, benzophenone-3 induced apoptosis and neurotoxicity, accompanied with an altered methylation status of apoptosis-related and ER genes, including hypomethylation of *GPER1*. In addition, a decrease in the mRNA and protein expression levels of ERα and ERβ and additional upregulation of GPER1 expression were detected.[50] Recently, epigenetic modulation has been explored in Alzheimer's disease (AD) and Parkinson's disease (PD), wherein resveratrol (grapes), curcumin (turmeric), and epigallocatechin gallate (EGCG; green tea) exhibited their neuroprotective ability by modifying DNA methylation patterns, histone acetylation, and non-coding RNA expression.[51] Nevertheless, in the context of neurological or cardiovascular diseases, there is a lack of information on the direct relationship between epigenetic modifications that result in *GPER1* alterations. In this review, we have provided bundled information on GPER1 and its key role in sex-specific differences in cardiovascular and neurological diseases, based on state-of-the-art research that provided a deep insight into the role of GPER1 in several pathways and receptor interactions in both *in vitro* and *in vivo* models.

2. GPER1 and its role in neurological diseases

2.1. Neuroinflammation, learning, and memory

Neuroinflammation is not only an obvious link between several neurodegenerative diseases, such as AD, PD, and Huntington's disease but also a potentially crucial player in the pathophysiology of these diseases. In general, neuroinflammation acts as an inherent protective mechanism in the CNS, where glial cells react to injury and disease by recognizing specific patterns, which results in the secretion of inflammatory modulators, elimination of the burden, and the end of the inflammatory process. Nevertheless, in several cases, clearance of the inflammatory region is not sufficient and chronic neuroinflammation occurs.[52] Although several studies have already demonstrated a neuroprotective role by the activation of GPER1 in animal models,[53-55] Bai *et al.* showed that GPER1 is directly involved in the regulation of neuroinflammation by modulating microglial activation, thereby exerting cognitive-improving effects in rats. In particular, not only reduced microglial activation but also suppression of NOD-, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3)-inflammasome activation and interleukin (IL)-1β signaling, as well as upregulation of the endogenous anti-inflammatory factor IL-1 receptor antagonist, was demonstrated in rat neurons in the hippocampal CA1 region after global cerebral ischemia.[56] Similar results were demonstrated by Pan *et al.*, who reported reduced neuronal apoptosis and favored microglial polarization to the anti-inflammatory type M2 *through* the intravenous injection of G-1 into male rats suffering from traumatic brain injury.[57] Consistent with this, Zhang *et al.* revealed that G-1-mediated activation of GPER1 on microglia in ovariectomized (OVX) female mice after middle cerebral artery occlusion inhibited toll-like receptor 4 (TLR4)-mediated microglial inflammation and improved neurological deficits as well as alleviated neuronal injuries.[58] This GPER1-associated neuroprotective mechanism was also reported by Peng *et al.*, who depicted a neural stem cell-derived exosome-mediated transport of zinc-finger-enhancer-binding protein 1 to microglia promoting the expression of GPER1 and reducing the activation of the TLR4/nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway and neuroinflammation.[59] However, not only microglia but also astrocytes were shown to be regulated by GPER1 activation in the context of neuroprotection. For instance, Wang *et al.* demonstrated that G-1-mediated neuroprotection in mice after middle cerebral artery occlusion is completely blocked in *GPER1*-knockout (KO) mice (no statement on sex) and that GPER1 activation led to reduced inflammatory cytokine release and restoration of autophagy imbalance in glutamate-exposed cultured murine astrocytes.[60] This anti-inflammatory effect of GPER1 activation in astrocytes was also reported by Yuan *et al.*, who demonstrated that silencing GPER1 inhibited the protective effect of insulin-like growth factor 1 (IGF-1) on 1-methyl-4-phenylpyridinium-induced inflammatory responses both *in vitro* and *in vivo* in male mice.[61] Finally, in the context of the gut–brain axis, Yin *et al.* showed an ameliorative effect of dietary tryptophan on neurodegeneration and inflammation in aging male mice accompanied with an increase in the expression

of GPER1 in the colon and brain.[62] More recently, they confirmed their results and clarified a key role of GPER1/AMP-activated protein kinase/sirtuin-1 signaling in the prevention of aging-related neurodegeneration and inflammation induced by indoles from the gut microbiota in male mice.[63]

Besides its role in the regulation of neuroinflammation, there are a few studies on the involvement of GPER1 in neuronal apoptosis, learning, and memory. Researchers have shown that GPER1 activation after subarachnoid hemorrhage, bleeding in the space between the brain and the surrounding membrane, in rats resulted in less neuronal apoptosis through Src/EGFR/signal transducer and activator of transcription 3 signaling, improved neurobehavioral performance, and long-term neurofunction in male rats, but not in female rats.[64] It was also shown that GPER1 is crucial in the reduction of cognitive impairment and neuronal damage in a mouse model of vascular dementia. In this study, the ginsenoside Rg1 increased the GPER1 expression level and reduced neuronal apoptosis and hippocampal neuronal loss in male mice.[65] Kim *et al.* also showed the beneficial impact of the GPER1 agonist G-1 with respect to recognition and spatial memory consolidation in OVX female mice,[66] as well as increased CA1 dendritic spine density and hippocampal memory consolidation similarly in female mice, which was dependent on actin polymerization and c-Jun N-terminal kinase signaling.[67] Similar results were reported by Wang *et al.*, who analyzed the effect of G-1 in OVX female rats after global cerebral ischemia. They demonstrated a G-1-induced upregulation of neurogenesis in the hippocampal dentate gyrus and CA1 region, less CA1 neuronal damage, and improved memory function after 28 days. Mechanistically, astrocyte-derived aromatase-brain-derived estrogen signaling was shown to be involved in long-term neuroprotection because the usage of an aromatase antagonist eliminated these effects.[68] It was also demonstrated that *GPER1* KO in astrocytes but not in the neurons of female mice caused significant impairment of learning and memory, which was accompanied with reactive astrocytes releasing proinflammatory cytokines that damage neurons. The cAMP/PKA/CREB signaling was involved in this effect at the molecular level, regulating the beneficial effects of GPER1 on Praja1 (PJA1), a RING ubiquitin ligase, which binds to Serpina3n, indicating astrocytic neuroinflammation. This mechanism was also confirmed for female human astrocytes, as GPER1 activation resulted in increased expression of PJA1 and activated CREB. Accordingly, the mRNA levels of *GPER1* and *PJA1* decreased in the plasma of postmenopausal women with an increase in *Serpina3n* expression.[69] In gonadectomized male mice, *in vivo* GPER1 activation through G-1 in the dorsal hippocampus was also shown to regulate memory consolidation, with CREB phosphorylation playing a role equivalent to that in OVX female mice.[70] Moreover, two recently published reviews focused on the effects of estrogen on age-dependent memory loss and AD.[71,72] Nonetheless, only a few studies have directly analyzed the GPER1 and AD interaction. AD generally exerts a massive impact on a large number of patients worldwide, and its pathology manifests in brain areas with increased amounts of amyloid beta (Aβ) protein, neuropil threads, and neurofibrillary tangles. The protective effect of GPER1 on rat neuronal cells against the Aβ-induced toxicity was mediated on the administration of the GPER1 agonist G-1 by changing the cell oxidative parameters.[73] Consistent with these findings, Deng *et al.* demonstrated an oxabicycloheptene sulfonate-induced protective effect on Aβ-promoted cytotoxicity through activation of the GPER1-phosphoinositide 3-kinase (PI3K)-/Akt and ERK pathway in an *in vitro* rat glial cell model of AD.[74] Furthermore, the improving effect of G-1 on recognition and memory was confirmed in an AD mouse model, where G-1 improved novel object recognition as well as long-term (24 h) recognition memory in female but not male mice.[75] Meng *et al.* revealed sex-specific differences in bisphenol A (BPA) sensitivity, as juvenile male rats exhibited elevated BPA-induced neurotoxicity as well as higher impairment of learning and memory compared with that in juvenile female rats. Interestingly, they demonstrated an involvement of GPER1 in this context, as low doses of BPA reduced GPER1 protein expression, especially in male rats, with only high doses affecting the expression of ERα and ERβ. In particular, the use of the GPER1 antagonist G-15 reduced neuronal morphogenic damage, emphasizing the role of GPER1 signaling in this process. Similar effects on reduced neuronal morphogenetic impairment were detected by the application of the phytoestrogen EGCG, a flavanol and the major component of green tea polyphenols. Moreover, in BPA-treated male rats, *in vivo* treatment with EGCG improved their spatial memory ability,[76] probably based on its antioxidant activity[77] and competitive binding to GPER1, resulting in depleted binding of E2 or BPA to GPER1.[78,79] This finding was also supported by Moreno-Ulloa *et al.*, who reported a comparable binding pattern between EGCG and GPER1 to that between GPER1 and its agonist G-1.[80] Another recent study demonstrated a role of GPER1 in the beneficial effects of the oral administration of the phytoestrogen secoisolariciresinol diglucoside (SDG) in an AD female mouse model. It was found that mice fed on the SDG diet exhibited accumulation of the gut microbial metabolites enterodiol and enterolactone, which are structurally similar to E2. Consequently, this led

to reduced neuroinflammation and cerebral Aβ deposition as well as alleviated cognitive impairment in female AD mice. Regarding GPER1, the study showed that the anti-inflammatory effect of SDG was mediated by GPER1 signaling and could be abolished by its antagonist G-15.[81] Equivalently, there is evidence that genistein (GEN), another phytoestrogen extracted from leguminous plants, inhibits lipopolysaccharide (LPS)-induced inflammation in microglia by reducing the production of inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF), IL-1β, and IL-6, which is dependent on GPER1 because G-15 treatment abolished the beneficial effects of GEN.[82] Another phytoestrogen, s-equol, a bacterial metabolite of the soy isoflavone daidzein, improved cerebellar development *in vitro* by influencing astrocytes and neurons while mediating GPER1 signaling.[83] Subsequently, it was confirmed that the isoflavone GEN, daidzein, and s-equol affect dendrite arborization and total number of Purkinje cells through GPER1 and ERα in murine primary cerebellar cultures. In particular, the effect of s-equol on neurite outgrowth was shown to be mediated by GPER1, as *GPER1* knockdown (KD) or G-15 treatment reduced the beneficial effects.[84] Consistent with this, s-equol diminished the LPS-induced production of NO and iNOS in astrocytes, which was partially induced by GPER1 because G-15 treatment attenuated the anti-inflammatory effect.[85] Regarding daidzein, a previous study on primary murine cerebellar cultures showed inhibition of the glutamate-mediated apoptotic pathway mediated by GPER1 as well as ERα as selective antagonists inhibited this effect.[86] Shen *et al.* also indicated that the ginsenoside Rg1, the primary active component of ginseng, prevents cognitive impairment and hippocampal neuronal apoptosis in a mouse model of vascular dementia, probably through GPER1.[65] In conclusion, GPER1 activation may play a key role in the regulation of neuroinflammation by acting on microglia, astrocytes, and cytokine release in both male and female individuals, which ultimately results in reduced neurodegeneration. Furthermore, it is also probably beneficial for learning and memory by acting on neuronal development, astrocytes, and actin polymerization as well as reducing neural apoptosis and Aβ-induced cytotoxicity (Figure 1).

2.2. PD

PD is a neurodegenerative movement disorder characterized by the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta.[87] Consequently, a reduced release of striatal dopamine (DA) results in the pathogenesis of PD with underlying genetic and functional causes.[88,89] It remains controversial whether there exists a sex difference in the risk of developing PD.[90,91] Nevertheless, the beneficial effect of estrogen has been well-described in both *in vitro* and *in vivo* models,[92-94] leading to the assumption of sex-specific differences in PD development and therapy. The major challenges in understanding sex-specific differences in PD development include the identification of new pathways and correlation of sex with heterogeneous clinical outcomes. Reekes *et al.* indicated that men are more cognitively impaired than women, revealing that sex is more strongly associated with cognitive performance than motor phenotype.[95] In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced animal model of parkinsonism, adult male C57BL/6 mice exhibited an increase in the number of tyrosine hydroxylase-immunoreactive cells, reduced activation of microglia, and decreased production of proinflammatory cytokines after 12 days of treatment with the GPER1 agonist G-1. The anti-inflammatory effect of G-1 was abolished by the treatment of wild-type (WT) mice with the GPER1 antagonist G-15. Further *in vitro* studies demonstrated that GPER1 activation reduced the release of proinflammatory cytokines from BV2 microglial cells after MPTP stimulation, indicating that GPER1 mediates the anti-neuroinflammatory effect of estrogen in experimental PD progression.[96] Treatment with G-1 exerted similar effects as those observed after treatment with E2, resulting in increased concentration and turnover of striatal DA in male mice. Consistent with these findings, the GPER1 antagonist G-15 blocked the effect of G-1 and partially prevented the effects of E2 treatment. In general, G-15–treated male mice were more susceptible to MPTP toxicity with a greater decrease in striatal DA concentration and DA transporter-specific binding.[97] Furthermore, G-1 and E2 have been shown to be equally potent in promoting a shift toward an anti-inflammatory immunophenotype that reduces MPTP-induced NF-κB and iNOS activation. G-15 is also capable of antagonizing the immunomodulatory effects of G-1, highlighting GPER1 as an immunomodulator and neuroprotector in PD.[98] Another study by Mendes-Oliveira *et al.* demonstrated that G-1 significantly decreased the phagocytic activity, expression of iNOS, and release of nitric oxide (NO) induced in N9 microglial cells after LPS treatment. However, G-1 treatment did not decrease the LPS-induced increase in the number of ionized calcium-binding adaptor molecule 1-positive cells; it significantly reduced the mRNA levels of *IL-1b*, cluster of differentiation 68 (*CD68*), and *iNOS*; completely inhibited DA cell loss; and consequently protected the motor function in male mice with PD. It was clearly demonstrated that G-1 can modulate microglial responses and protect DA neurons and motor functions against lesions induced by

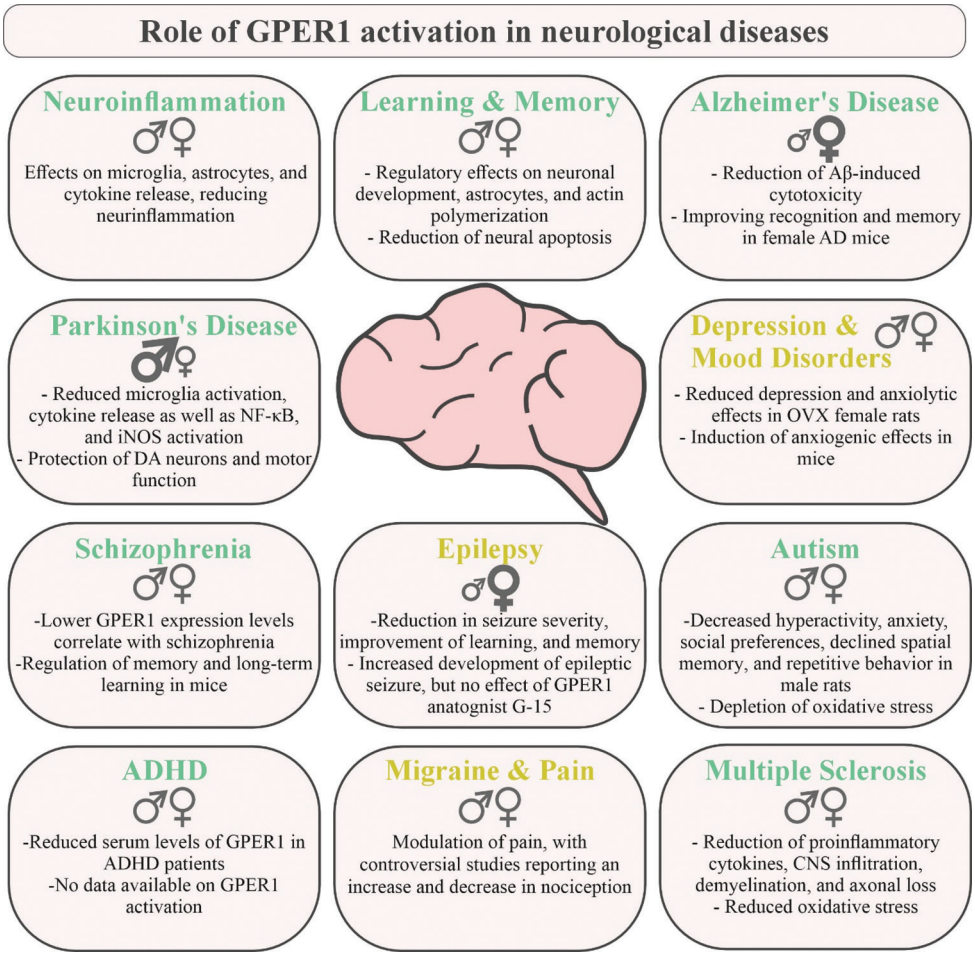

Figure 1. Role of the activation of GPER1 in neurological diseases. GPER1 is involved in various molecular mechanisms influencing the pathophysiology of several neurological diseases with differences between males and females. Here, the influence of GPER1 on disorders of the CNS is illustrated, with a positive effect of GPER1 activation (green headlines) and a controversially discussed effect (yellow headlines) on the outcome of the respective disease. Sex-specific differences in favoring the effects of GPER1 activation are illustrated by different sizes of the symbols of the biological sexes (same size: same effects in both sexes; outstanding female symbol: larger effect in females; and outstanding male symbol: larger effect in males). Image created by author
Abbreviations: ADHD: Attention-deficit/hyperactivity disorder; AD: Alzheimer's disease; CNS: Central nervous system; DA: Dopaminergic; GPER1: G protein-coupled estrogen receptor 1; G-15: GPER1 antagonist; iNOS: Inducible nitric oxide synthase; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; OVX: Ovariectomized.

inflammatory processes.[99] Poirier *et al.* also reported the protection of DA neurons against neuroinflammatory processes conferred by G-1 by showing a partial reduction of IL-1β levels in an MPTP-induced PD male mouse model.[100] Regarding nutraceuticals, the polyphenol EGCG plays a key role in the defense against neuronal inflammation (section 2.1) and PD. The neuroprotective effects of EGCG on cell survival, reduced reactive oxygen species production, and metal chelation have been clearly indicated in both *in vitro* and *in vivo* models.[101] Nevertheless, there exist no data on the direct interaction of EGCG with GPER1 in PD. Based on the findings of the effects of EGCG on GPER1 in neuroinflammation, the neuroprotective properties can probably be translated to PD pathology. Moreover, phytoestrogens can generally be used to investigate the disruption of endocrine signaling in various pathologies. To date, randomized studies in humans have demonstrated contradictory outcomes and do not clearly indicate a beneficial effect of phytoestrogens on PD pathology.[43] Du *et al.* reported a beneficial effect of GEN in PD through a partial interplay with GPER1 and IGF-1 receptor. In their study, LPS-treatment resulted in nigrostriatal injury in OVX female rats, with GEN application restoring LPS-decreased DA levels.[102] Epigenetic regulation of *GPER1* expression has been widely explored in various pathologies but not in PD-related models. To summarize, phytoestrogens can activate all three ERs, namely, ERα, ERβ, and GPER1, to regulate downstream signaling cascades with significant effects on neurite outgrowth and neuronal survival in

PD.[103] Furthermore, GPER1 activation results in the protection of DA cells through its anti-inflammatory effect in the context of PD (Figure 1).

2.3. Depression and mood disorders

Sex-specific differences with effects on onset, prevalence, and clinical phenotype have been reported in various studies on major depressive disorder (MDD).[104,105] Cross-national[106] and national[107] epidemiological studies have shown that women are affected twice as much as men by MDD, and symptoms during occurrence and progression vary between male and female patients. Despite the fact that women are more likely to develop depression, men commit suicide 3 times more frequently during depressive episodes.[108] Current research provides strong evidence for sex-specific molecular mechanisms in human depression, as a genome-wide association study (GWAS) on MDD reported that the underlying gene pathways of MDD are highly sex-dependent.[107] Ignoring critical, sex-specific molecular pathways, and targets for the development of new therapies is associated with the risk of missing new treatment strategies and mechanisms underlying MDD development. Therefore, research in the field of sex-specific differences in MDD is of special interest for developing novel therapeutic approaches. Patients with MDD show higher GPER1 serum levels than controls, indicating GPER1 as a potential biomarker for the presence of MDD. Accordingly, a positive correlation could be demonstrated between GPER1 serum levels and depression scores (Table 1).[109] Moreover, postmenopausal depression, caused by a decrease in female sex hormones, was correlated with MDD in OVX female rats. Intracerebral treatment with the GPER1 agonist G-1 in OVX female rats exerted antidepressant and anxiolytic effects by increasing translocator protein phosphorylation through PKA signaling.[110] Exposure to endocrine-disrupting chemicals such as dichlorodiphenyltrichloroethane (DDT; an insecticide) increases the risk of developing neuropsychiatric disorders, including depression, anxiety, and somatization.[111,112] p,p'-DDT, a DTT isomer, induced depressive-like behavior by decreasing the expression levels of serotonin 1A receptor and GPER1, as observed in the mouse brain tissue. p,p'-DDT also caused the attenuation of GPER1 expression, which was independent of sex and age. Consequently, the disruption of GPER1 signaling plays a key role in DDT-induced depressive-like symptoms.[113] A study demonstrated that extracts from the root bark of *Paeonia suffruticosa* (paeonol) were capable of restoring GPER1 expression levels in the prefrontal cortex and hippocampus, mimicking the effects of E2. Paeonol also increased the expression levels of the components of the PI3K/Akt/mammalian target of rapamycin signaling pathway in the prefrontal cortex and hippocampus and that of brain-derived neurotrophic factor in the hippocampus of OVX female mice. This study indicated that paeonol is beneficial for restoring postmenopausal cognitive impairment, anxiety, and depression.[114] Recent research has demonstrated the effect of E2 on ERβ and/or GPER1 in OVX female rats under chronic mild stress through shortened inhibitory avoidance latency tests using specific ERβ or GPER1 agonists. For instance, Tongta *et al.* showed that *GABA*- and neurosteroid-associated mRNAs related to anxiety were altered through ERβ, whereas GPER1 activation exerted no or opposing effects.[115] Consistent with these findings, Kastenberger *et al.* described GPER1 as an inductor of anxiogenic effects by estrogen in rodents. They revealed that a potential imbalance in the expression of ERβ (anxiolytic) and GPER1 (anxiogenic) may be responsible for the negative symptoms of estrogen replacement therapy in humans.[116] In general, estrogens have been used for the treatment of perimenopausal and postmenopausal depression in women, irrespective of antidepressant drug use, to attenuate uprising symptoms with fluctuating estrogen levels during the estrus cycle.[117]

Table 1. GPER1 serum levels in several neurological and cardiovascular diseases

Disease	Alteration in GPER1 serum level	Sex-specific differences	References
Depression and mood disorders	Elevated GPER1 serum levels in male and female patients diagnosed with MDD	No sex-specific differences detected	109
Schizophrenia	Elevated GPER1 serum levels in male patients diagnosed with schizophrenia	No difference in GPER1 serum levels between schizophrenia-affected women and healthy women	130
ASD	Reduced GPER1 serum levels in male and female patients with ASD	No sex-specific differences detected	156
ADHD	Reduced GPER1 serum levels in male and female patients with ADHD	No sex-specific differences detected	160
Hypertension	Reduced GPER1 serum levels in postmenopausal women with hypertension	No analysis of male patients	199

Abbreviations: ADHD: Attention-deficit/hyperactivity disorder; ASD: Autism spectrum disorder; GPER1: G protein-coupled estrogen receptor 1; MDD: Major depressive disorder.

Despite the impact of GPER1 on MDD itself, Lei *et al.* focused on the effects of low concentrations of fluoxetine (an antidepressant drug) resulting in the upregulation of ERα expression, which consequently mimics estrogen-like effects.[118] In contrast, *in vitro* studies have demonstrated that fluoxetine upregulates the expression of E2 and downregulates the expression of ERα and ERβ.[119,120] Koitmäe *et al.* investigated the modulation and sex-specific functionality of GPER1 in the hippocampal network in a *GPER1* KO mouse model using both male and female mice. GPER1 modulation resulted in reduced neuronal excitability, which may induce sex-specific cognitive deficits or sex-specific differences in mood disorders.[121] Over the past few decades, the relationship of depression, anxiety, and mood disorders with certain diets or supplementary nutrients has been widely discussed.[122-124] In particular, nutrients such as N-oleyl-ethanolamine and EGCG reportedly attenuate insulin resistance, depression, and eating disorders.[125] In this context, the N-methyl-d-aspartate receptor (NMDAR) pathway has been shown to be activated by the intake of EGCG-rich food, with beneficial effects on cognition[126] and hippocampal serotonin levels in a rat chronic mild stress model.[127] Besides the extensive literature on the impact of nutrients on mood disorders, there are no data on the direct impact of nutrients on GPER1. Based on GPER1 activation by EGCG in terms of neuroinflammation (section 2.1), a putative role in the development of mood disorders could be assumed. Current research in treating depression focuses on single pathways and/or single target receptors. Nonetheless, the pathogenesis of depression is a complex combination of various signaling molecules and targets. Alterations in estrogen levels can cause symptoms of depression, and stabilization of estrogen levels could alleviate the disease outcome involving GPER1 signaling (Figure 1).

2.4. Schizophrenia

Since the initial studies on sex-specific effects on the development of schizophrenia by Kraepelin in 1919, accumulated evidence has shown that sex-specific differences influence the epidemiology, symptomatic expression, life course, and response to antipsychotics in individuals with schizophrenia.[128,129] In a study involving 36 individuals with schizophrenia and 30 age- and sex-matched healthy control participants, male patients with schizophrenia showed higher serum GPER1 levels than healthy male controls, but there was no difference in GPER1 serum levels between schizophrenia-affected and healthy women (Table 1).[130] In 2020, a two-hit animal model of the uprising symptoms of schizophrenia combining exposure to perinatal infection (first-hit) with peripubertal unpredictable stress (second-hit) was investigated in rats that were exposed to polycytidylic acid (a viral mimetic) or saline. N-acetylcysteine (NAC) has been evaluated in schizophrenia due to the involvement of glutathione mechanisms in its neurobiology to prevent schizophrenic symptoms, which were assessed by behavioral and oxidative alterations in the prefrontal cortex and striatum as well as GPER1 expression. In particular, in female rats, there was an increase in the expression of GPER1, α7-nicotinic acetylcholine receptor, and parvalbumin in interneurons, which may contribute to a better response of female rats to NAC. These results emphasize the impact of sex-specific differences in the preventive effects of NAC in a rat two-hit schizophrenia model.[131] Peripheral blood mononuclear cells obtained from male and female patients with schizophrenia were converted into microglia-like cells to measure GPER1 expression levels. Furthermore, an elevated expression of GPER1 was detected in the hippocampal region of a two-hit female mouse model in the proestrus phase. In contrast, two-hit male mouse models with deficient prepulse inhibition of the startle reflex exhibited an overall lower hippocampal mRNA expression of *GPER1*.[132] A study comprising 90 patients with schizophrenia, including 64 patients with treatment-resistant schizophrenia (TRS; 45.3% males and 54.7% females) and 26 with partially responsive schizophrenia (PRS; 46.2% males and 53.8% females), investigated potential sex differences in the association between TRS and IL-6 levels.

The study by He *et al.*[133] indicated that patients with TRS had higher serum IL-6 levels than those with PRS. In female patients, IL-6 levels were significantly higher in the TRS cohort than in the PRS cohort, indicating a possible link between the inflammatory response system and severity of schizophrenia. This link may play a pivotal role in the pathogenesis of TRS in a sex-specific manner.[133] Another study by Zhang *et al.* investigated schizophrenia induced by the administration of dizocilpine (MK-801), a non-competitive NMDAR antagonist, in *GPER1* KO and WT mice. Subsequently, the effects on learning, memory, and social interaction as well as the protein expression levels of the NMDAR NR2B (NR2B)/calmodulin-dependent protein kinase II (CaMKII)/CREB signaling pathway were analyzed. Treatment of *GPER1* KO mice with MK-801 resulted in significant impairment of memory and long-term learning compared with that in the treated WT mice. Furthermore, in *GPER1* KO mice, the expression levels of proteins related to the NR2B/CaMKII/CREB signaling pathway were downregulated after MK-801 treatment, showing that GPER1 is an important player in cognitive, learning, and memory functions.[134] Recent studies have investigated the effect of certain nutrients and natural molecules through various research approaches.[135,136] The

direct impact of EGCG for schizophrenia treatment was disproved in a clinical trial conducted by Loftis *et al.*[137] In a ketamine-induced rat model of schizophrenia, the administration of GEN reduced schizophrenia-related symptoms in male rats in combination with standard clozapine treatment. However, the researchers did not indicate a direct relationship to the activation of GPER1 by GEN.[138] To summarize the results from recent studies, GPER1 plays a key role in the pathology of schizophrenia because its lower expression levels in certain brain regions were correlated with this disease. Hence, GPER1 expression is correlated with schizophrenia symptoms (Figure 1) and should be further investigated for novel therapeutic approaches. Moreover, the effect of GPER1 activation by estrogen or synthetic agonists has to be clarified in clinical studies.

2.5. Epilepsy

Sex-specific differences in epilepsy are well documented, although the alignment varies depending on the subtype of epilepsy.[139] A higher prevalence of localization-related symptomatic epilepsy has been reported in men; however, women are more often affected by idiopathic generalized epilepsy.[140] The molecular mechanisms behind this discrepancy remains to be clarified. In general, epilepsy is a chronic neurological disorder accompanied with repeated seizures involving hyperexcitability of the neuronal network based on impaired neuronal functions.[141] Although various mechanisms are discussed as molecular reasons for sex-specific differences in epilepsy, the involvement of steroids in the regulation of hyperexcitability is one of the most investigated connections.[142] The first studies of the mid-20th century implied a relationship between the sex hormone E2 and seizures in women with epilepsy;[143,144] however, currently, there is substantial evidence that GPER1 as a receptor might play a role in this axis. In a case report of a male child suffering from intellectual disability featuring autistic behavior and epilepsy, there was an unbalanced translocation involving a co-occurrent microdeletion in the chromosomal region 7p22.3 that also affects GPER1.[145] Two independent studies using rodent epilepsy models disclosed a protective role of GPER1 by reducing seizure severity[146] and improving learning and memory abilities as well as diminishing hippocampal neuron damage in epileptic rats.[147] In particular, Zuo *et al.* used lithium chloride- and pilocarpine-induced epileptic male rats, which were genetically modified by the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease technology to receive a *GPER1* KD for further experiments. Using this model, they demonstrated an increase in seizure severity in the epileptic rats with the *GPER1* KD, as well as intensified neuronal damage in the hippocampus after status epilepticus. Furthermore, a relationship between *GPER1* KD and increased neuronal inflammation was established based on increased microglial activation and elevated secretion of inflammatory factors in the hippocampus post status epilepticus[146] (section 2.1). Zhang *et al.* used a similar lithium chloride- and pilocarpine-based epilepsy model to explore temporal lobe epilepsy in male Sprague–Dawley rats, showing improvement in learning and memory abilities after GPER1 activation as well as assuming a role of GPER1 in synaptic plasticity.[147] Nevertheless, there are also contradictory results from Kurt *et al.* who reported increased development of pentylenetetrazole-induced epileptic seizures after injections with E2 or the GPER1 agonist G-1. However, the antagonist G-15 exerted no effect on seizures.[148] Consistent with the results of Zuo *et al.*[146] and Zhang *et al.*,[147] Wang *et al.* analyzed human patient-derived material and showed that a decrease in *GPER1* mRNA and GPER1 protein levels negatively correlated with seizure frequency in female but not in male patients with focal cortical dysplasia IIb (FCDIIb) and tuberous sclerosis complex (TSC), both of which are groups of malformations in cortical development and associated with epilepsy. Interestingly, the authors also confirmed a significant reduction in GPER1 protein levels in the microglia of female patients with FCDIIb and TSC, as well as elevated NF-κB-mediated inflammatory signaling. Remarkably, *in vitro* experiments revealed reduced NF-κB protein and neuronal excitability by the altered frequency of spontaneous excitatory postsynaptic currents under the application of the GPER1 agonist G-1. The authors, thus, concluded that GPER1 might exert an antiepileptogenic effect in female patients with FCDIIb and TSC.[149]

During the past few decades, the impact of nutrition, diet schemes, and certain secondary plant metabolites has emerged in terms of supportive therapy options for epilepsy.[150-153] Consistent with this, secondary plant metabolites such as EGCG and GEN, which exert a regulatory effect on GPER1 expression,[76,154] have also been discussed as promising candidates for additive nutrition in terms of epilepsy seizure prevention or attenuation. Nonetheless, there are no data on the direct interaction of these molecules with GPER1 or epigenetic modifications in epilepsy pathology or treatment. Altogether, these different data sets strongly suggest the involvement of GPER1 in the pathophysiology of epilepsy, particularly with regard to a potential role in the molecular mechanism underlying sex-specific differences. However, the exact underlying mechanism has to be clarified due to the controversial results regarding the role of GPER1 in epilepsy (Figure 1).

2.6. Autism spectrum disorders (ASDs) and attention-deficit/hyperactivity disorder (ADHD)

Next to the previously described neurological disorders, a sexually dimorphic prevalence is also present in ASDs, as approximately four-fold more males are affected than females.[155] Regarding the involvement of GPER1, a previous study reported a significant negative correlation between GPER1 serum levels and ASD. They analyzed 45 children aged 3 – 12 years and 40 age- and sex-matched controls and found lower serum levels of GPER1 in patients with ASD than in healthy controls. However, the study showed no correlation between GPER1 serum level and E2 level nor between age in male or female young patients (Table 1).[156] Furthermore, a case report described a male patient suffering from neurodevelopmental abnormalities with autistic features and epilepsy, who harbored a translocation involving a co-occurrent microdeletion on chromosomal region 7p22.3 also affecting *GPER1*, further suggesting that GPER1 plays a role in ASD.[145] Another study explored the effect of GPER1 in a valproic acid-induced rat model of ASD, where the researchers analyzed the effect of the intraperitoneal application of the GPER1 agonist G-1 in male rats in two different doses for 21 days. They observed G-1-induced decrease in hyperactivity, anxiety, social preferences, declined spatial memory, and repetitive behavior in the male ASD rats. In terms of molecular biological changes, there was an increase in neurotransmission and depletion in oxidative stress in the hippocampus, as well as a reduced mRNA expression level of *IL-1b* but an increase in the expression of *GPER1*, retinoic acid-related orphan receptor-a (*RORa*), and aromatase in the hippocampus after G-1 treatment. This result strongly suggests a significant role of GPER1 in the sex-specific prevalence of ASD, and G-1 treatment appears to be a promising therapeutic approach.[157] Consistent with the results of Hameed *et al.*,[157] another study again emphasized the important role of GPER1 in ASD and developed a prediction model for ASD development based on *GPER1* expression among four other genes. The authors conducted a longitudinal follow-up study of 2623 newborns over 3 years and evaluated the transcript levels of superoxide dismutase-2 (*SOD2*), *RORa*, progesterone receptor, and *GPER1*, as well as two epigenetic markers at the *RORa* promoter and oxidative stress markers in umbilical cord blood mononuclear cell specimen. Their observations identified 41 children with ASD, who had significantly higher levels of oxidative stress as well as H3K9me3 histone modifications at the *RORa* promoter than healthy children. Moreover, children with ASD suffered from diminished gene expression levels of *SOD2*, *RORa*, and *GPER1*, whereas their progesterone receptor expression levels were higher. Using this specific gene expression pattern, the authors established a highly sensitive and specific ASD prediction model,[158] suggesting *GPER1* as a prognostic factor (Table 1) and underscoring its protective role in ASD (Figure 1). ASD and ADHD often co-occur and share characteristics and overlapping traits. Although ADHD represents one of the most frequent neuropsychiatric disorders in children, data from the ADHD Observational Research in Europe clearly revealed a higher prevalence of ADHD in boys than in girls, with the ratio varying by country, ranging from 3:1 to 16:1.[159] Based on the general known neuroprotective role of GPER1 and the association of its downregulation in other neurodevelopmental disorders such as ASD and schizophrenia, researchers also investigated the blood serum levels of E2 and GPER1 in children with ADHD and age- and sex-matched controls. Interestingly, children with ADHD showed significantly reduced GPER1 serum levels, but their E2 levels remained unaltered. Furthermore, there were no significant differences in GPER1 or E2 levels between male and female patients with ADHD or between ADHD subgroups (Table 1).[160] Similarly, a study from China investigated the role of a GPER1 polymorphism in the social function of 135 children with ADHD. No significant difference was detected in the genotypic frequencies of the c.-9T/C and c.789G/A loci between male and female children. However, higher school scores and improved learning were significantly associated with a TC genotype for the c.-9T to C locus compared with that in children with a TT genotype. This study further suggested an important role of GPER1 in the ADHD phenotype[161] (Figure 1). Nonetheless, neither the epigenetic regulation of *GPER1* nor the impact of nutraceuticals on GPER1 has been reported in the context of ASD or ADHD to date.

2.7. Migraine and pain

Based on the pioneering work of Berkley,[162] researchers have intensively examined sex-specific differences in pain-associated diseases over decades. At present, it is well-known that several pain conditions, such as migraine, fibromyalgia, and temporomandibular disorder, are more prevalent in women than in men. However, there are certain painful syndromes that affect men more than women, such as cluster headache.[163-165] The major origin for these sex-specific differences in pain are probably hormonal factors,[166,167] because the prevalence of migraine, for instance, significantly decreases at menopause,[168] thus suggesting a role of hormonal receptors (*e.g.*, GPER1) in the context of sex-specific differences in pain. GPER1 can mediate post-operative hyperalgesia in dorsal root ganglia neurons[169] in the rostral ventromedial medulla, which contributes to the chronification of post-operative pain;[170] orofacial inflammatory pain by microglia in the rostral ventral medulla;[171] and continuous neuronal sensitization

and neuroinflammation responses in neuropathic pain.[172] In a previous study, GPER1 was expressed in the anterior cingulate cortex where it mediated exacerbated neuropathic pain in an OVX female mouse model[173] and induced estrogen-mediated visceral hypersensitivity in a stressed rat model.[174] In another study, although estrogen levels were higher in females, GPER1 was expressed in the dorsal root ganglia and afferent terminals in the dorsal horn of male and female rats.[29] Araldi *et al.* investigated the side effects of triptans, which are widely used as therapeutics for migraine but are often negatively accompanied with injection-site pain in patients. They showed significant sex-specific differences in the complex dose dependence for sumatriptan-induced mechanical hyperalgesia in male and female rats. Interestingly, 1 ng of sumatriptan revealed hyperalgesia in male rats implanted with estrogen and in female rats but not in untreated male rats or OVX female rats, highlighting the role of estrogen. Investigations on the responsible ER revealed a significant involvement of GPER1 in sumatriptan-induced mechanical hyperalgesia in female rats. Moreover, the serotonin receptors 5-HT_{1D} and 5-HT_{1B} participate in sumatriptan-induced hyperalgesia, while 5-HT_{1D} is crucial in the induction of hyperalgesia in female and 5-HT_{1B} in male rats.[175] Another study revealed an important role of GPER1 in mechanical hyperalgesia, as high estrogen levels exacerbated postoperative allodynia in OVX female rats through GPER1 and matrix metallopeptidase 9. Mechanical hyperalgesia increased with the use of the GPER1 agonist G-1 and equivalently decreased with the use of the GPER1 antagonist G-15, emphasizing the role of GPER1 *in vivo*.[176] However, in a mouse model of arthritis, the opposite effect of GPER1 inhibition was observed, as biochanin treatment resulted in decreased production of proinflammatory cytokines and reduced hypernociception *via* GPER1. This is because G-15 treatment reversed this positive effect in mice with antigen-induced arthritis.[177] Accordingly, Liu and Duan investigated the role of progesterone in trigeminal neurons of female rats and showed that progesterone-mediated neuroexcitation is dependent on cAMP and GPER1, because the application of the GPER1 antagonist G-15 reversed the progesterone-induced enhancement in the excitability of Ah-type trigeminal neurons of female rats. Conversely, a single application of the GPER1 agonist G-1 resulted in an increase in the action potential frequency of Ah-type trigeminal neurons of female rats.[178] Investigations on two different mouse models of pain, reflecting chronic inflammatory and neuropathic pain, revealed anxiety-like behavior in OVX female mice suffering from both types of pain, which was accompanied with high GPER1 expression in the basolateral amygdala. Activation of GPER1 through G-1 significantly reduced the anxiety-like behavior but did not alter thermal hyperalgesia or mechanical allodynia. Mechanistically, this study revealed a role of GPER1 in maintaining the balance between excitatory and inhibitory transmissions in the basolateral amygdala synapses of OVX female mice suffering from chronic pain.[179] Liverman *et al.* showed that GPER1 expression is upregulated through inflammatory processes, suggesting that GPER1 is also upregulated during a migraine attack. Moreover, they revealed an increase in orofacial sensitivity in OVX female rats with induced trigeminal inflammatory pain using the GPER1 agonist G-1.[180] A study using a multibehavioral rat model of migraine revealed an equivalent enhancement of migraine-like behavior in female OVX rats through treatment with the xenoestrogen BPA. Moreover, they reported an increase in the mRNA levels of *ERa*, *GPER1*, *ERK1/2*, and $Na_V1.8$ (*SCN10A*) in the trigeminal neurons of BPA-treated rats, further suggesting a role of GPER1 in migraine and pain.[181] Analysis of GPER1 involvement in OVX female rats with pre-operative anxiety-induced post-operative hyperalgesia revealed a role of GPER1 in this process, because the application of the GPER1 antagonist G-15 reduced post-operative hyperalgesia.[169] Another study by Lu *et al.* demonstrated the opposite effect of GPER1 on pain induced by α, β-methylene ATP, activating the purinergic receptors P2X 1 and P2X 3 in OVX female rats. They showed that estrogen inhibited the pain induced by α, β-methylene ATP, whereas an ERα agonist and the GPER1 agonist G-1 mimicked the reduction in nociception, emphasizing a nocifensive role of GPER1 in this context. At the molecular signaling level, cAMP-PKA and ERK1/2 play crucial roles in mediating the E2-based reduction in pain threshold.[28] Regarding the epigenetic regulation of *GPER1* or the impact of nutraceuticals on GPER1 activity in the process of pain, no specific data are available. Conclusively, there are several studies confirming a relevant role of GPER1 in modulating migraine-associated pain; however, the results are controversial because some studies report an increase, whereas others describe a decrease in nociception by GPER1 activation (Figure 1). These discrepancies may be attributed to the different models used as well as the high number of downstream signaling cascades activated by GPER1.

2.8. Multiple sclerosis (MS)

MS is a chronic inflammatory autoimmune disease of the CNS, which is accompanied with inflammatory infiltration and subsequent impairment of oligodendrocytes, resulting in the destruction of myelin sheaths.[182] Similar to the sex-specific prevalence of the previously discussed

neurological diseases, MS exhibits an imbalance in sex distribution, as its prevalence is higher in women than in men, with a ratio of approximately 3:1 reported in Europe.[183] A prominent initial study on the influence of pregnancies on MS revealed a strong reduction in relapse rates during pregnancies,[184] emphasizing the vital role of sex hormones in the pathophysiology of neurological diseases. As already discussed in the neuroinflammation section (2.1), GPER1 activation may play a role in the regulation of neuroinflammation acting on microglia, astrocytes, and cytokine release, suggesting its importance in MS. This was confirmed by Blasko *et al.*, who reported an inhibition of LPS-induced cytokine production in human macrophages by the activation of GPER1 through its agonist G-1. Moreover, *in vivo* data have corroborated these results, wherein G-1 treatment of mice with MS (no data on sex reported in the publication) decreased the release of proinflammatory cytokines and thus the severity of the disease.[185] Similar results were reported by Bodhankar and Offner,[186] who analyzed the effect of the activation of ERs on the pathophysiology of MS in an animal model of experimental autoimmune encephalomyelitis (EAE). They reported a protective function of G-1 on CNS infiltration, demyelination, and axonal loss in the MS mouse model, highlighting the protective role of GPER1 in MS.[186] Consistent with these results, G-1 administration in demyelinated rats resulted in increased remyelination through enhanced oligodendrocyte maturation in the corpus callosum.[187] Analysis of the impact of *GPER1* KO in a female mouse model of EAE further confirmed that the E2-mediated reduction of EAE is only partially mediated by GPER1 activation.[188] However, Yates *et al.* exclusively reported an E2-induced reduction in EAE severity by GPER1 and not by ERα, because *GPER1* KO attenuated the beneficial effects of E2 on EAE in a female mouse model of EAE.[189] Similarly, analyses of the influence of Vitamin D3 on female EAE mice revealed preventive effects on clinical MS signs, demyelination, and CNS lesions induced by GPER1, as these effects were abolished in *GPER1* KO mice.[190] Besides its regulatory role in myelination, G-1-induced activation of GPER1 was shown to decrease oxidative stress through the upregulation of excitatory amino acid carrier 1 in rat glial cells. Mechanistically, the *in vitro* reduction of oxidative stress was mediated by GPER1-sphingosine kinase 1–fibroblast growth factor 2–ERK1/2 signaling.[191] Although there are no data on the impact of nutraceuticals, such as EGCG, on MS, their role in GPER1 activation and neuroinflammation (section 2.1) suggests that they are crucial in MS pathophysiology. Overall, GPER1 may mediate some beneficial effects on MS in rodent models through its action on neuroinflammation, oxidative stress, and myelination (Figure 1).

3. GPER1 and its role in cardiovascular diseases

3.1. Hypertension

Hypertension is a polyfactorial disease that can manifest as severe cardiovascular pathologies over time, such as heart failure and stroke.[192] Clinically, it is defined as a sustained resting systolic blood pressure level of ≥130 mmHg or a diastolic blood pressure level of ≥80 mmHg.[193] There is extensive evidence that estrogen confers protection against cardiovascular diseases, particularly coronary heart disease, heart failure, and arterial hypertension, in premenopausal women.[6] Interestingly, the blood pressure increases in postmenopausal women 5 – 10 years after the cessation of estrus cycling, such that the prevalence of hypertension becomes higher in women than in men.[194,195] Studies using postmenopausal hypertension rat models have shown that premenopausal females had lower blood pressure than age-matched males; however, with aging, the blood pressure increased significantly compared with that in their male counterparts.[196,197] This finding supports the assumption that sex hormones play a protective role in the vascular system.[198]

Recent studies have suggested that GPER1 has a major impact on vasodilatation and blood pressure regulation.[36] Compared with normotensive premenopausal women, low serum GPER1 levels are correlated with hypertension in postmenopausal women (Table 1).[199] GPER1 is widely expressed in the cardiovascular system, which has already been demonstrated in cardiomyocytes,[200] cardiac fibroblasts, coronary artery endothelial cells,[201] and coronary artery vascular smooth muscle cells.[202] Therefore, GPER1 is involved in a plethora of varying physiological functions, as previously described in the CNS[30] and cardiovascular system.[26,33] GPER1 activation affects diastolic function[203,204] and positively influences cardiac remodeling by preventing the proliferation of fibroblasts and mast cells through the inhibition of the cell cycle genes cyclin B1 and *CDK1*.[205,206] Besides these beneficial effects of GPER1, its genomic region on chromosome 7p22 has been linked to hypertension.[207] A further indication of the involvement of GPER1 in pathophysiological cardiovascular mechanisms was demonstrated by Liu *et al.* in 2018, who reported the association of GPER1 with hypertension as a protective factor in menopausal women, but not in premenopausal women.[199]

Studies using *GPER1* KO rodents have elucidated the GPER1-mediated physiological mechanisms underlying vasodilatation. For instance, Tropea *et al.* showed that GPER1 activation by G-1 induced NO-mediated vasodilation in the uterine arteries of pregnant and non-pregnant rats by modulating the NO/cyclic guanosine

monophosphate pathway.[208] Mice lacking *GPER1* exhibited increased endothelium-dependent contractility compared with normal mice, as G-1-induced GPER1 activation beneficially suppresses cyclooxygenase-derived endothelium-derived contractile factors such as endothelin1.[209] Other reports of GPER1 activation involved in the molecular pathways of vascular dilatation and relaxation have revealed the involvement of Rho-kinase-dependent[210] and cAMP-dependent[211] mechanisms. Activation of GPER1 through its highly selective agonist G-1[212] results in vasodilation of arteries and vasodilatation is maximal in postmenopausal woman compared with age-matched men.[213] Genetic linkage studies have corroborated the role of *GPER1* in regulating blood pressure, as GWAS have demonstrated that a genetic region associated with arterial hypertension is consistent with the location of *GPER1*.[207] Mechanistically, a single-nucleotide polymorphism (rs1154431) results in a substitution of leucine with proline at position 16 (GPER1 p.P16L), with a relative common allele frequency of ~20%.[214] This results in a hypofunctional receptor leading to an increased blood pressure in female rats but not in male rats, probably due to a lower activation level of ERK1/2 observed in a rat vascular smooth muscle cell line.[214] A study using patient-derived induced pluripotent stem cell models from three female patients with hypertension and heterozygous GPER1 p.P16L and CRISPR/Cas9 gene-edited isogenic normal controls indicated the specific involvement of *GPER1* in the development of hypertension. This study showed that the L16 variant reduced the anti-inflammatory effects of GPER1 in induced pluripotent stem cell-derived endothelial cells (iECs), and normal GPER1 plays a role in the regulation of cell adhesion molecules, as shown in a monocyte-to-iEC adhesion assay. Although the polymorphism in these patients did not significantly affect endothelial NO synthase and NO production compared with isogenic normal P16 or hypertension-associated L16 GPER1, these findings suggest that a modest elevation of inflammation can induce a mechanism contributing to elevated blood pressure and the risk of developing hypertension.[215] Another study reported that acute activation of GPER1 with the selective agonist G-1 resulted in vasodilatation in human endothelial cells through the liberation of NO.[216] At present, there are no data on whether the GPER1 p.P16L single-nucleotide polymorphisms play a role in cardiovascular function in men.

Aging and the consequent decreased estrogen level are some of the primary factors that reduce GPER1-dependent vasodilatation.[217] Studies have shown that E2 administration elicits blood pressure-lowering effects in different mammalian[218,219] and human models.[220] Moreover, the activation of GPER1 through G-1 results in similar vasodilation in both sexes of spontaneous hypertensive rats through different intracellular signaling pathways such as the H_2O_2 and NO pathways. In male rats, the relaxing response in the mesenteric arteries involves the NO pathway, whereas in female rats, the H_2O_2 pathway is predominant.[221] In conclusion, these data suggest a central regulatory role of GPER1 in hypertension and indicate a role of GPER1 agonists in the treatment of cardiovascular diseases in both biological sexes, placing a special focus on personalized approaches to treat these diseases in women (Figure 2).

3.2. Cardiovascular function

One of the most frequent causes of heart failure is the loss of contractile tissue after myocardial infarction, resulting in either heart failure with reduced ejection fraction (HFrEF) or heart failure with preserved ejection fraction (HFpEF). Remarkably, there is a marked sex-specific difference between men and women, as the prevalence of HFpEF in women is higher, with 1% in women and 0% in men in the age of 25 – 49 years and 8 – 10% in women aged >80 years compared to 4 – 6% in men of the same age. Men show an almost two-fold higher risk of HFrEF but not for HFpEF than women.[222] In a mouse model of induced heart failure through pressure overload, estrogen therapy could restore the ejection fraction (not further specified) of induced heart failure from 0.35 to a normal value of 0.53 by stimulating angiogenesis, suppressing fibrosis, and improving cardiac hemodynamics in both sexes, indicating a protective role of E2 through sarcoplasmic/endoplasmic reticulum calcium ATPase activation.[223] Furthermore, hypoxia or hypoxemia during myocardial infarction can trigger the upregulation of *GPER1* mRNA and GPER1 protein level in ERα- and ERβ-negative human breast adenocarcinoma cells (SKBR3) and HL-1 mouse cardiac muscle cells,[222,224] although there are yet no further data concerning *GPER1* epigenetics in human cardiomyocytes. GPER1 has been identified as a major player of cardioprotection in female and male Sprague–Dawley rats, based on the observation that activation of GPER1 with G-1 supported functional recovery after ischemia and reduced infarct size independently from biological sex.[38] Nevertheless, sex-specific differences were detected in cardiomyocyte-specific *GPER1* KO mice with pronounced impaired systolic and diastolic function in male compared with female KO mice. In addition, DNA microarray analysis revealed sex-specific differences in the gene expression profiles of the transcriptional networks in a cardiomyocyte-specific *GPER1* KO mouse model.[34] Interestingly, Wang *et al.* demonstrated that the gene set enrichment analysis of transcriptional networks revealed an enrichment of mitochondrial genes only in females

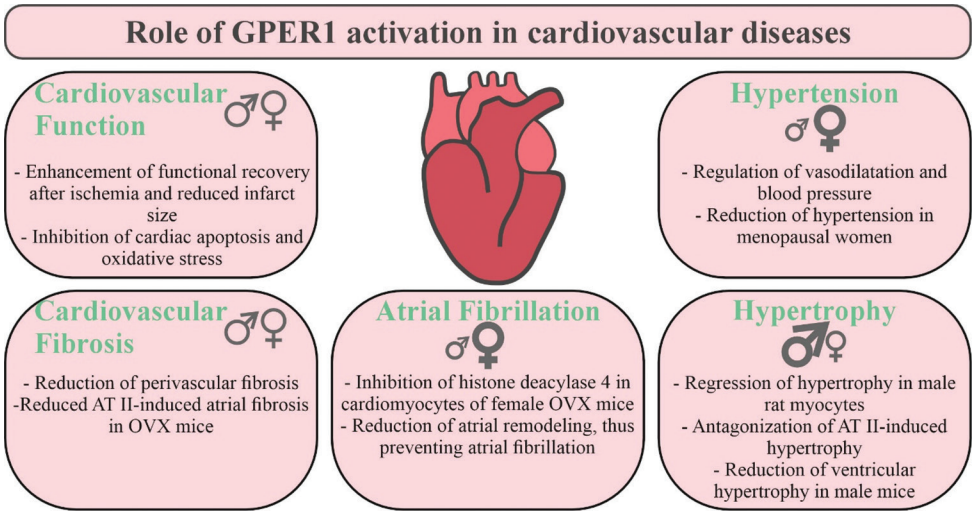

Figure 2. Role of GPER1 in cardiovascular diseases. Besides its involvement in neurological diseases, GPER1 plays a role in several molecular mechanisms influencing the pathophysiology of cardiological diseases with sex-specific differences. Here, the impact of GPER1 on disorders of the cardiovascular system is illustrated, with a favorable effect of GPER1 activation (green headlines) and a controversially discussed effect (yellow headlines) on the outcome of the respective disease. Sex-specific differences in the positive effects of GPER1 activation are illustrated by different sizes of the symbols of the biological sexes (same size: same effects in both sexes; outstanding female symbol: larger effect in females; and outstanding male symbol: larger effect in males) Abbreviations: AT II: Angiotensin II; GPER1: G protein-coupled estrogen receptor; OVX: Ovariectomized.

but only an upregulation of inflammatory response genes in male *GPER1* KO mice compared with that in WT and sex-matched animal groups.[34] Regarding both sexes, exposure to the xenoestrogen BPA during childhood and adulthood has been linked to a higher risk of developing cardiovascular diseases. Furthermore, children who have been exposed *in utero* to increased BPA levels, measured from maternal urinary concentrations at approximately 20 weeks of gestation, showed a significantly higher diastolic blood pressure at age 4 years.[225] BPA acts as an endocrine disruptor, and in high doses, it causes heart malformations, whereas EGCG could rescue these pathogenic effects during zebrafish heart development.[226] In an artificial aortic ring model and *in silico* studies, Moreno-Ulloa *et al.* demonstrated that (-)-epicatechin, another flavanol with cardiovascular beneficial effects, can mediate vasodilatation through GPER1 activation similar to G-1, through activation of the kinase c-Src and cross-talk with EGFR.[80] Phytoestrogens are considered promising therapeutics for postmenopausal atherosclerotic cardiovascular diseases.[227] *In vivo* as well as *in vitro* assays by Feng *et al.* demonstrated the anti-inflammatory and antiapoptotic effects of kaempferol, a naturally occurring flavonoid, by the activation of GPER1 in blood vessels, possibly attenuating atherosclerosis in postmenopausal women.[228] In addition, Zhao *et al.* demonstrated improvement of cardiac hypertrophy by GPER1 activation through the flavonoid phytoestrogen icariin in OVX female mice,[229] emphasizing the cardioprotective role of GPER1. Other phytoestrogens such as the non-flavonoid polyphenol resveratrol have also been shown to prevent hypertension induced by a high-fat diet in female rats.[230] In conclusion, different flavonoids as well as other phytoestrogens that activate GPER1 could be important for diverse clinical applications for cardiovascular diseases, although more specific research is required. Hypertrophy of the ventricle as a consequence of aortic hypertension is a risk factor for arrhythmias, ischemia, and sudden death, because long-term cardiac hypertrophy results in cardiac dilatation and heart failure.[231,232] Di Mattia *et al.* reported that activation of GPER1 with G-1 not only prevents hypertrophy but also regresses hypertrophy in neonatal cardiac male rat myocytes.[37] This GPER1-dependent effect reduces pERK1/2 and stimulates the activation of Akt signaling.[233,234] Furthermore, angiotensin II (AT II)-induced hypertrophy can be antagonized by the activation of GPER1 through the regulation of PI3K/Akt/mammalian target of rapamycin signaling.[235] Activation of the PI3K/Akt pathway through E2 receptors also reduces cardiomyocyte apoptosis in female mice.[236] Sex-specific protective effects have also been reported for the cAMP/L-type calcium channel pathway based on the observation of an increase in I_{CaL} density, contraction, and calcium transient amplitudes in isolated left ventricle apical myocytes in female and OVX E2-treated female mice compared with that in male and OVX female mice.[39] Interestingly, Machuki *et al.* also reported that these beneficial effects of E2 could be abolished by pretreatment of myocytes obtained from OVX female mice with the GPER1 antagonist G-15, whereas the ERα and ERβ antagonist ICI 182,780 did not cancel the positive effects of E2.[39] The receptor activity-modifying protein 3 (RAMP3),

a member of the RAMP family of single-transmembrane proteins, interacts with GPER1[237] and is transcriptionally upregulated by E2.[238] In HEK293 cells, cotransfection of *RAMP3* and *GPER1* could increase RAMP3 expression at the plasma membrane.[237] *In vivo* GPER1 activation by the administration of G-1 in *RAMP3* WT and *RAMP3* KO mice for 40 days led to a sex-dependent reduction in the left ventricular hypertrophy and perivascular fibrosis in *RAMP3* WT male mice but not in *RAMP3* KO male or female mice.[237] Furthermore, another study showed that RAMP3 plays a sex-dependent role in cardiac hypertrophy and heart failure by affecting only male *RAMP3* KO mice through increased activation of Akt signaling with no effect on female mice.[239] Modification of the RAMP3–GPER1 interaction and its associated intracellular pathways could help identify novel pharmacological targets for drugs against cardiac pathophysiological mechanisms, especially in male patients.

Another study investigated the cardioprotective effect of GPER1 in male rat hearts with regard to the translocation of PKC.[240] PKC activates the mitogen-activated protein kinase kinase 2 (MAPK2)/ERK1/2 signaling pathway and further phosphorylates glycogen synthase kinase 3 (GSK3) β in reperfusion injury.[241] The late onset of reperfusion after a period of ischemia causes apoptosis of the cardiac tissue.[242] Activation of GPER1 exerts a cardioprotective effect in H9C2 myocardial cells after ischemia-reperfusion injury by inhibiting cardiac apoptosis.[243] There is extensive evidence showing that inflammatory processes are critically involved, especially in reperfusion injury.[244] Using cardiomyocyte-specific *GPER1* KO mice, Wang *et al.* demonstrated GPER1-mediated downregulation of the NLRP3 inflammasome[245] (section 2.1), which contains, for instance, NLRP3, caspase 1, IL-1β, and IL-18 that represent inflammatory substances that could contribute to heart failure.[246,247] Doxorubicin, a drug used in cancer therapy, is often associated with the side effect of cardiotoxicity.[248] Activation of GPER1 could mitigate the adverse effects of doxorubicin therapy by reducing the levels of inflammatory mediators such as TNF.[249] Consistent with this, the cardiomyocyte-specific KO of *GPER1* in male mice resulted in a gene enrichment of inflammatory genes, whereas this effect was not observed in female *GPER1* KO mice.[34] KO of *GPER1* in female mice results in the left ventricular dysfunction as well as an upregulation of oxidative stress-related genes.[250] Based on animal[251] and human[252] studies, it is known that oxidative stress is elevated in males compared with that in females. A possible cellular mechanism is the opening of the mitochondrial permeability transition pore resulting in cell death that could be antagonized by the activation of GPER1.[253]

The peroxisome proliferator-activated receptor delta is a key player in the metabolic function of the heart. A recent study demonstrated that GPER1 can promote the nuclear translocation of peroxisome proliferator-activated receptor delta, thereby mitigating oxidative stress and apoptosis during sepsis.[254] Therefore, modulation of GPER1 through the action of interaction partners such as RAMP3 or inhibition or activation of kinases further downstream of GPER1-activated signaling pathways might represent promising targets for treating heart failure (Figure 2).[255]

3.3. Atrial fibrillation (AF)

AF is the most common tachycardic arrhythmia, which is associated with increased mortality as well as poor quality of life.[256] It causes a five-fold increase in the risk of developing stroke and heart failure[257] and a two-fold increase in the risk of developing myocardial infarction and coronary heart disease, especially in women.[258] In contrast, in a Taiwanese cohort study, Chao *et al.* reported a higher risk of developing myocardial infarction in men with AF than in women with AF.[259] An important interaction and dependency exist between several cardiac pathomechanisms, such as the association between hypertension and AF.[260] Atrial remodeling plays a key role in AF development, and histone deacetylases (HDACs) are associated with AF initiation.[261] Sawa *et al.* demonstrated in an *in vivo* mouse model that HDAC6 activation induced AF; in this case, HDAC6 was activated by chronic hypertension.[262] Moreover, Zhao *et al.* showed the involvement of GPER1 in the inhibition of HDAC4 in cardiomyocytes obtained from female OVX mice.[229] In particular, the risk of developing AF is higher in menopausal women than in premenopausal women.[263] Liu *et al.* demonstrated that GPER1 activation reduces atrial fibrosis induced by AT II in an OVX mouse model. They proposed a G-1-induced reduction of atrial remodeling, which prevented AF-induced mortality after receptor activation.[264] In addition, estrogen monotherapy considerably reduces AF and mortality rates in menopausal women, besides exerting additional cardioprotective effects.[265] In conclusion, GPER1 and its specific activation through its agonist G-1 provide a possible basis for antiarrhythmic therapy through the downregulation of HDACs (Figure 2), although there are currently no data on these possible beneficial effects and the involvement of HDACs in human cardiac tissue.

4. Conclusion

There has been an increase in the number of studies on GPER1 and its involvement in the regulation of various molecular mechanisms causing alterations at cellular and organism levels over the past few decades, and GPER1 has become an integral component of current

research. Based on the mediation of genomic and non-genomic rapid signaling and the ubiquitous expression of GPER1 in the excitable tissue, GPER1 plays a crucial role in several neurological and cardiovascular diseases (Figures 1 and 2). In particular, its exclusive protective role in multiple processes of cardiovascular diseases is obvious, including cardiovascular function, hypertension, hypertrophy, AF, and cardiovascular fibrosis. However, the previous studies have not consistently reported beneficial effects of GPER1 on both sexes. This is because in hypertrophy, males gained an advantage from GPER1 activation, whereas in hypertension, vasodilatation and AF data suggest marked positive effects in females (Figure 2). Equivalently, GPER1 activation may be highly favorable in almost all neurological diseases discussed in this review, including neuroinflammation, depression and mood disorders, schizophrenia, MS, ASD, AD, PD, and ADHD as well as learning and memory. Studies have emphasized a more favorable influence of GPER1 on females with AD, whereas males with PD have benefited more from GPER1. However, results regarding the impact of GPER1 activation on epilepsy, depression and mood disorders, and migraine and pain are controversial, and not only advantageous but also disadvantageous effects have been described (Figure 1). Nonetheless, GPER1 appears to be a promising candidate for further investigations, considering its use as a neuroprotective target in several disorders of the CNS. Furthermore, in cardiovascular and neurological diseases, particularly in depression and mood disorders, schizophrenia, ASD, ADHD, and hypertension, serum GPER1 level was analyzed as a predictor of the respective disorder (Table 1). In this regard, studies have reported elevated GPER1 serum levels in both sexes of patients with depression and mood disorders,[109] whereas elevated GPER1 levels were detected only in male patients with schizophrenia.[130] In patients with ASD[156] and ADHD,[160] reduced GPER1 serum levels were detected in both sexes. This is because in hypertrophy, reduced GPER1 levels were detected only in postmenopausal women with hypertension.[199] These results indicate the importance of GPER1 in these disorders as well as emphasize GPER1 as a possible diagnostic marker. GPER1 appears to be a promising target for developing novel therapeutics for diseases of the CNS and cardiovascular system, probably with sex-specific approaches. However, further clinical studies are warranted to confirm the promising results of *in vitro* and *in vivo* studies in rodents and humans to obtain a better knowledge of sex-specific pathology and future treatment options.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

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References

1. Ziemka-Nalecz M, Pawelec P, Ziabska K, Zalewska T. Sex differences in brain disorders. *Int J Mol Sci*. 2023;24:19.

   doi: 10.3390/ijms241914571

2. Singh R, Nasci VL, Guthrie G, *et al*. Emerging roles for G protein-coupled estrogen receptor 1 in cardio-renal health: Implications for aging. *Biomolecules*. 2022;12(3):412.

   doi: 10.3390/biom12030412

3. da Silva JS, Montagnoli TL, Rocha BS, *et al*. Estrogen receptors: Therapeutic perspectives for the treatment of cardiac dysfunction after myocardial infarction. *Int J Mol Sci*. 2021;22(2):525.

   doi: 10.3390/ijms22020525

4. Dinh QN, Vinh A, Arumugam TV, Drummond GR, Sobey CG. G protein-coupled estrogen receptor 1: A novel target to treat cardiovascular disease in a sex-specific manner? *Br J Pharmacol*. 2021;178(19):3849-3863.

   doi: 10.1111/bph.15521

5. Feldman RD. Sex-specific determinants of coronary artery disease and atherosclerotic risk factors: Estrogen and beyond. *Can J Cardiol*. 2020;36(5):706-711.

   doi: 10.1016/j.cjca.2020.03.002

6. Groban L, Tran QK, Ferrario CM, *et al*. Female heart health: Is GPER the missing link? *Front Endocrinol (Lausanne)*. 2019;10:919.

   doi: 10.3389/fendo.2019.00919

7. Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F. Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. *Steroids*. 2014;90:13-29.

   doi: 10.1016/j.steroids.2014.06.012

8. Filardo EJ, Quinn JA, Frackelton AR Jr., Bland KI. Estrogen action via the G protein-coupled receptor, GPR30: Stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol*. 2002;16(1):70-84.

   doi: 10.1210/mend.16.1.0758

9. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science*. 2005;307(5715):1625-1630.

   doi: 10.1126/science.1106943

10. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*. 2005;146(2):624-632.

    doi: 10.1210/en.2004-1064

11. Gonzalez de Valdivia E, Sandén C, Kahn R, Olde B, Leeb-Lundberg LMF. Human G protein-coupled receptor 30 is N-glycosylated and N-terminal domain asparagine 44 is required for receptor structure and activity. *Biosci Rep*. 2019;39(2):BSR20182436.

    doi: 10.1042/BSR20182436

12. Luo J, Liu D. Does GPER really function as a G protein-coupled estrogen receptor *in vivo*? *Front Endocrinol*. 2020;11:148.

    doi: 10.3389/fendo.2020.00148

13. Sakamoto H, Matsuda KI, Hosokawa K, *et al*. Expression of G protein-coupled receptor-30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology*. 2007;148(12):5842-5850.

    doi: 10.1210/en.2007-0436

14. Warfvinge K, Krause DN, Maddahi A, Edvinsson JCA, Edvinsson L, Haanes KA. Estrogen receptors α, β and GPER in the CNS and trigeminal system - molecular and functional aspects. *J Headache Pain*. 2020;21(1):131.

    doi: 10.1186/s10194-020-01197-0

15. Goncalves GK, Scalzo S, Alves AP, Agero U, Guatimosim S, Reis AM. Neonatal cardiomyocyte hypertrophy induced by endothelin-1 is blocked by estradiol acting on GPER. American journal of physiology. *Am J Physiol Cell Physiol*. 2018;314(3):C310-C322.

    doi: 10.1152/ajpcell.00060.2017

16. Sandén C, Broselid S, Cornmark L, *et al*. G protein-coupled estrogen receptor 1/G protein-coupled receptor 30 localizes in the plasma membrane and traffics intracellularly on cytokeratin intermediate filaments. *Mol Pharmacol*. 2011;79(3):400-410.

    doi: 10.1124/mol.110.069500.

17. Cheng SB, Quinn JA, Graeber CT, Filardo EJ. Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. *J Biol Chem*. 2011;286(25):22441-22455.

    doi: 10.1074/jbc.M111.224071

18. Gonzalez de Valdivia E, Broselid S, Kahn R, Olde B, Leeb-Lundberg LMF. G protein-coupled estrogen receptor 1 (GPER1)/GPR30 increases ERK1/2 activity through PDZ motif-dependent and-independent mechanisms. *J Biol Chem*. 2017;292(24):9932-9943.

    doi: 10.1074/jbc.M116.765875

19. Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: A novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol*. 2002;80(2):231-238.

    doi: 10.1016/S0960-0760(01)00190-X

20. Ding X, Gao T, Gao P, *et al*. Activation of the G protein-coupled estrogen receptor elicits store calcium release and phosphorylation of the Mu-opioid receptors in the human neuroblastoma SH-SY5Y cells. *Front Neurosci*. 2019;13:1351.

    doi: 10.3389/fnins.2019.01351

21. Chuang SC, Chen CH, Chou YS., Ho ML, Chang JK. G protein-coupled estrogen receptor mediates cell proliferation through the cAMP/PKA/CREB pathway in murine bone marrow mesenchymal stem cells. *Int J Mol Sci*. 2020;21(18):6490.

    doi: 10.3390/ijms21186490

22. Conde K, Meza C, Kelly MJ, Sinchak K, Wagner EJ. Estradiol rapidly attenuates ORL-1 receptor-mediated inhibition of proopiomelanocortin neurons via Gq-coupled, membrane-initiated signaling. *Neuroendocrinology*. 2016;103(6):787-805.

    doi: 10.1159/000443765

23. Ahmadian Elmi M, Motamed N, Picard D. Proteomic analyses of the G protein-coupled estrogen receptor GPER1 reveal constitutive links to endoplasmic reticulum, glycosylation, trafficking, and calcium signaling. *Cells*. 2023;12(21):2571.

    doi: 10.3390/cells12212571

24. Tutzauer J, Serafin DS, Schmidt T, Olde B, Caron KM, Leeb-Lundberg LMF. G protein-coupled estrogen receptor (GPER)/GPR30 forms a complex with the β1-adrenergic receptor, a membrane-associated guanylate kinase (MAGUK) scaffold protein, and protein kinase A anchoring protein (AKAP) 5 in MCF7 breast cancer cells. *Arch Biochem Biophys*. 2024;752:109882.

doi: 10.1016/j.abb.2024.109882

25. De Francesco EM, Pellegrino M, Santolla MF, *et al*. GPER mediates activation of HIF1α/VEGF signaling by estrogens. *Cancer Res*. 2014;74(15):4053-4064.

doi: 10.1158/0008-5472.CAN-13-3590

26. Prossnitz ER, Barton M. The G protein-coupled oestrogen receptor GPER in health and disease: An update. *Nat Rev Endocrinol*. 2023;19(7):407-424.

doi: 10.1038/s41574-023-00822-7

27. Weiser MJ, Foradori CD, Handa RJ. Estrogen receptor beta in the brain: From form to function. *Brain Res Rev*. 2008;57(2):309-320.

doi: 10.1016/j.brainresrev.2007.05.013

28. Lu Y, Jiang Q, Yu L, *et al*. 17β-estradiol rapidly attenuates P2X3 receptor-mediated peripheral pain signal transduction via ERα and GPR30. *Endocrinology*. 2013;154(7):2421-2433.

doi: 10.1210/en.2012-2119

29. Takanami K, Sakamoto H, Matsuda KI, *et al*. Expression of G protein-coupled receptor 30 in the spinal somatosensory system. *Brain Res*. 2010;1310:17-28.

doi: 10.1016/j.brainres.2009.11.004

30. Hazell GGJ, Yao ST, Roper JA, Prossnitz ER, O'Carroll AM, Lolait SJ. Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. *J Endocrinol*. 2009;202(2):223-236.

doi: 10.1677/JOE-09-0066

31. Kuhn J, Dina OA, Goswami C, Suckow V, Levine JD, Hucho T. GPR30 estrogen receptor agonists induce mechanical hyperalgesia in the rat. *Eur J Neurosci*. 2008;27(7):1700-1709.

doi: 10.1111/j.1460-9568.2008.06131.x

32. Brandt N, Rune GM. Sex-dependency of oestrogen-induced structural synaptic plasticity: Inhibition of aromatase versus application of estradiol in rodents. *Eur J Neurosci*. 2020;52(1):2548-2559.

doi: 10.1111/ejn.14541

33. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol*. 2011;7(12):715-726.

doi: 10.1038/nrendo.2011.122

34. Wang H, Sun X, Chou J, *et al*. Cardiomyocyte-specific deletion of the G protein-coupled estrogen receptor (GPER) leads to left ventricular dysfunction and adverse remodeling: A sex-specific gene profiling analysis. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863(8):1870-1882.

doi: 10.1016/j.bbadis.2016.10.003

35. Meyer MR, Haas E, Barton M. Gender differences of cardiovascular disease: New perspectives for estrogen receptor signaling. *Hypertension*. 2006;47(6):1019-1026.

doi: 10.1161/01.HYP.0000223064.62762.0b

36. Gohar EY. G protein-coupled estrogen receptor 1 as a novel regulator of blood pressure. *Am J Physiol Renal Physiol*. 2020;319(4):F612-F617.

doi: 10.1152/ajprenal.00045.2020

37. Di Mattia RA, Mariángelo JIE, Blanco PG, *et al*. The activation of the G protein-coupled estrogen receptor (GPER) prevents and regresses cardiac hypertrophy. *Life Sci*. 2020;242:117211.

doi: 10.1016/j.lfs.2019.117211

38. Deschamps AM, Murphy E. Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. *Am J Physiol Heart Circ Physiol*. 2009;297(5):H1806-H1813.

doi: 10.1152/ajpheart.00283.2009

39. Machuki JO, Zhang HY, Geng J, *et al*. Estrogen regulation of cardiac cAMP-L-type Ca2+ channel pathway modulates sex differences in basal contraction and responses to β2AR-mediated stress in left ventricular apical myocytes. *Cell Commun Signal*. 2019;17(1):34.

doi: 10.1186/s12964-019-0346-2

40. Ogola BO, Zimmerman MA, Sure VN, *et al*. G protein-coupled estrogen receptor protects from angiotensin II-induced increases in pulse pressure and oxidative stress. *Front Endocrinol (Lausanne)*. 2019;10:586.

doi: 10.3389/fendo.2019.00586

41. Carregosa D, Mota S, Ferreira S, *et al*. Overview of beneficial effects of (Poly)phenol metabolites in the context of neurodegenerative diseases on model organisms. *Nutrients*. 2021;13(9):2940.

doi: 10.3390/nu13092940

42. Brahmachari G. *Discovery and Development of Neuroprotective Agents from Natural Products*. Netherlands: Elsevier; 2018.

doi: 10.1016/C2014-0-04884-8

43. Goyal A, Verma A, Agrawal N. Dietary phytoestrogens: Neuroprotective role in Parkinson's disease. *Curr Neurovasc Res*. 2021;18(2):254-267.

doi: 10.2174/1567202618666210604121233

44. Cosselman KE, Navas-Acien A, Kaufman JD. Environmental factors in cardiovascular disease. *Nat Rev Cardiol*. 2015;12(11):627-642.

doi: 10.1038/nrcardio.2015.152

45. Żabińska M, Wiśniewska K, Węgrzyn G, Pierzynowska K. Exploring the physiological role of the G protein-coupled estrogen receptor (GPER) and its associations with human diseases. *Psychoneuroendocrinology*. 2024;166:107070.

doi: 10.1016/j.psyneuen.2024.107070

46. Hamilton JP. Epigenetics: Principles and practice. *Dig Dis*. 2011;29(2):130-135.

doi: 10.1159/000323874

47. Muhammad A, Hixon JC, Pharmacy Yusuf A, *et al*. Sex-specific epigenetics drive low GPER expression in gastrointestinal smooth muscles in type 2 diabetic mice. *Sci Rep*. 2024;14(1):5633.

doi: 10.1038/s41598-024-54213-7

48. Prestegui Martel B, Chávez-Blanco AD, Domínguez-Gómez G, *et al* N-(2-Hydroxyphenyl)-2-propylpentanamide (HO-AAVPA) induces apoptosis and cell cycle arrest in breast cancer cells, decreasing GPER expression. *Molecules*. 2024;29(15):3509.

doi: 10.3390/molecules29153509

49. Major KM, DeCourten BM, Li J, *et al*. Early life exposure to environmentally relevant levels of endocrine disruptors drive multigenerational and transgenerational epigenetic changes in a fish model. *Front Mar Sci*. 2020;7.

doi: 10.3389/fmars.2020.00471

50. Wnuk A, Rzemieniec J, Litwa E, Lasoń W, Kajta M. Prenatal exposure to benzophenone-3 (BP-3) induces apoptosis, disrupts estrogen receptor expression and alters the epigenetic status of mouse neurons. *J Steroid Biochem Mol Biol*. 2018;182:106-118.

doi: 10.1016/j.jsbmb.2018.04.016

51. Prasanth MI, Sivamaruthi BS, Cheong CSY, *et al*. Role of epigenetic modulation in neurodegenerative diseases: Implications of phytochemical interventions. *Antioxidants* (*Basel*). 2024;13(5):606.

doi: 10.3390/antiox13050606

52. Zhang W, Xiao D, Mao Q, Xia H. Role of neuroinflammation in neurodegeneration development. *Signal Transduct Target Ther*. 2023;8(1):267.

doi: 10.1038/s41392-023-01486-5

53. Lebesgue D, Traub M, de Butte-Smith M, *et al*. Acute administration of non-classical estrogen receptor agonists attenuates ischemia-induced hippocampal neuron loss in middle-aged female rats. *PLoS One*. 2010;5(1):e8642.

doi: 10.1371/journal.pone.0008642

54. Lebesgue D, Chevaleyre V, Zukin RS, Etgen AM. Estradiol rescues neurons from global ischemia-induced cell death: Multiple cellular pathways of neuroprotection. *Steroids*. 2009;74(7):555-561.

doi: 10.1016/j.steroids.2009.01.003

55. Tang H, Zhang Q, Yang L, *et al*. GPR30 mediates estrogen rapid signaling and neuroprotection. *Mol Cell Endocrinol*. 2014;387(1-2):52-58.

doi: 10.1016/j.mce.2014.01.024

56. Bai N, Zhang Q, Zhang W, *et al*. G-protein-coupled estrogen receptor activation upregulates interleukin-1 receptor antagonist in the hippocampus after global cerebral ischemia: implications for neuronal self-defense. *J Neuroinflamm*. 2020;17(1):45.

doi: 10.1186/s12974-020-1715-x

57. Pan MX, Tang JC, Liu R, Feng YG, Wan Q. Effects of estrogen receptor GPR30 agonist G1 on neuronal apoptosis and microglia polarization in traumatic brain injury rats. *Chin J Traumatol*. 2018;21(4):224-228.

doi: 10.1016/j.cjtee.2018.04.003

58. Zhang Z, Qin P, Deng Y, *et al*. The novel estrogenic receptor GPR30 alleviates ischemic injury by inhibiting TLR4-mediated microglial inflammation. *J Neuroinflamm*. 2018;15(1):206.

doi: 10.1186/s12974-018-1246-x

59. Peng J, Yu Z, Xiao R, Hu X, Xia Y. Exosomal ZEB1 derived from neural stem cells reduces inflammation injury in OGD/R-treated microglia via the GPR30-TLR4-NF-κB axis. *Neurochem Res*. 2023;48(6):1811-1821.

doi: 10.1007/s11064-023-03866-3

60. Wang XS, Yue J, Hu LN, *et al*. Activation of G protein-coupled receptor 30 protects neurons by regulating autophagy in astrocytes. *Glia*. 2020;68(1):27-43.

doi: 10.1002/glia.23697

61. Yuan LJ, Zhang M, Chen S, Chen WF. Anti-inflammatory effect of IGF-1 is mediated by IGF-1R cross talk with GPER in MPTP/MPP+-induced astrocyte activation. *Mol Cell Endocrinol*. 2021;519:l111053.

doi: 10.1016/j.mce.2020.111053

62. Yin J, Zhang B, Yu Z, *et al*. Ameliorative effect of dietary tryptophan on neurodegeneration and inflammation in d-galactose-induced aging mice with the potential mechanism relying on AMPK/SIRT1/PGC-1α pathway and gut microbiota. *J Agric Food Chem*. 2021;69(16):4732-4744.

doi: 10.1021/acs.jafc.1c00706

63. Yin J, Zhang Y, Liu X, *et al*. Gut microbiota-derived indole derivatives alleviate neurodegeneration in aging through activating GPR30/AMPK/SIRT1 pathway. *Mol Nutr Food Res*. 2023;67(9):e2200739.

doi: 10.1002/mnfr.202200739

64. Peng J, Zuo Y, Huang L, *et al*. Activation of GPR30 with G1 attenuates neuronal apoptosis via src/EGFR/stat3 signaling pathway after subarachnoid hemorrhage in male rats. *Exp Neurol*. 2019;320:113008.

doi: 10.1016/j.expneurol.2019.113008

65. Shen F, Wang J, Gao F, Wang J, Zhu G. Ginsenoside Rg1 prevents cognitive impairment and hippocampal neuronal apoptosis in experimental vascular dementia

mice by promoting GPR30 expression. *Neural Plast*. 2021;2021:2412220.

doi: 10.1155/2021/2412220

66. Kim J, Szinte JS, Boulware MI, Frick KM. 17β-estradiol and agonism of G-protein-coupled estrogen receptor enhance hippocampal memory via different cell-signaling mechanisms. *J Neurosci*. 2016;36(11):3309-3321.

doi: 10.1523/JNEUROSCI.0257-15.2016

67. Kim J, Schalk JC, Koss WA, *et al*. Dorsal hippocampal actin polymerization is necessary for activation of G-protein-coupled estrogen receptor (GPER) to increase CA1 dendritic spine density and enhance memory consolidation. *J Neurosci*. 2019;39(48):9598-9610.

doi: 10.1523/JNEUROSCI.2687-18.2019

68. Wang L, Liu J, Xu J, Zhang W, Wang R. Coupling of GPR30 mediated neurogenesis and protection with astroglial Aromatase-STAT3 signaling in rat hippocampus after global cerebral ischemia. *Mol Cell Endocrinol*. 2021;535:111394.

doi: 10.1016/j.mce.2021.111394

69. Wang X, Jiang Y, Feng B, *et al*. PJA1 mediates the effects of astrocytic GPR30 on learning and memory in female mice. *J Clin Invest*. 2023;133(18):e165812.

doi: 10.1172/JCI165812

70. Machado GDB, Schnitzler AL, Fleischer AW, Beamish SB, Frick KM. G protein-coupled estrogen receptor (GPER) in the dorsal hippocampus regulates memory consolidation in gonadectomized male mice, likely via different signaling mechanisms than in female mice. *Horm Behav*. 2024;161:105516.

doi: 10.1016/j.yhbeh.2024.105516

71. Rishabh, Rohilla M, Bansal S, *et al*. Estrogen signalling and Alzheimer's disease: Decoding molecular mechanisms for therapeutic breakthrough. Eur J Neurosci. 2024;60(1):3466-3490.

doi: 10.1111/ejn.16360

72. Luo W, Yan Y, Cao Y, Zhang Y, Zhang Z. The effects of GPER on age-associated memory impairment induced by decreased estrogen levels. *Front Mol Biosci*. 2023;10:1097018.

doi: 10.3389/fmolb.2023.1097018

73. Kurt AH, Yuksel KZ, Uremis N, *et al*. Protective effects of G protein-coupled estrogen receptor 1 (GPER1) on β-amyloid-induced neurotoxicity: Implications for Alzheimer's disease. *Neurochem J*. 2019;13(1):99-104.

doi: 10.1134/S1819712419010148

74. Deng LJ, Cheng C, Wu J, Wang CH, Zhou HB, Huang J. Oxabicycloheptene sulfonate protects against β-amyloid-induced toxicity by activation of PI3K/Akt and ERK signaling pathways via GPER1 in C6 cells. *Neurochem Res*. 2017;42(8):2246-2256.

doi: 10.1007/s11064-017-2237-5

75. Kubota T, Matsumoto H, Kirino Y. Ameliorative effect of membrane-associated estrogen receptor G protein coupled receptor 30 activation on object recognition memory in mouse models of Alzheimer's disease. *J Pharmacol Sci*. 2016;131(3):219-222.

doi: 10.1016/j.jphs.2016.06.005

76. Meng L, Gui S, Ouyang Z, *et al*. Low-dose bisphenols exposure sex-specifically induces neurodevelopmental toxicity in juvenile rats and the antagonism of EGCG. *J Hazard Mater*. 2023;459:132074.

doi: 10.1016/j.jhazmat.2023.132074

77. Meng L, Liu J, Wang C, *et al*. Sex-specific oxidative damage effects induced by BPA and its analogs on primary hippocampal neurons attenuated by EGCG. *Chemosphere*. 2021;264(Pt 1):128450.

doi: 10.1016/j.chemosphere.2020.128450

78. Kiyama R. Estrogenic biological activity and underlying molecular mechanisms of green tea constituents. *Trends Food Sci Technol*. 2020;95:247-260.

doi: 10.1016/j.tifs.2019.11.014

79. Wang L, Huang C, Li L, Pang Q, Wang C, Fan R. *In vitro* and *in silico* assessment of GPER-dependent neurocytotoxicity of emerging bisphenols. *Sci Total Environ*. 2023;862:160762.

doi: 10.1016/j.scitotenv.2022.160762

80. Moreno-Ulloa A, Mendez-Luna D, Beltran-Partida E, *et al*. The effects of (-)-epicatechin on endothelial cells involve the G protein-coupled estrogen receptor (GPER). *Pharmacol Res*. 2015;100:309-320.

doi: 10.1016/j.phrs.2015.08.014

81. Jia M, Ning F, Wen J, *et al*. Secoisolariciresinol diglucoside attenuates neuroinflammation and cognitive impairment in female Alzheimer's disease mice via modulating gut microbiota metabolism and GPER/CREB/BDNF pathway. *J Neuroinflamm*. 2024;21(1):201.

doi: 10.1186/s12974-024-03195-4

82. Du ZR, Feng XQ, Li N, *et al*. G protein-coupled estrogen receptor is involved in the anti-inflammatory effects of genistein in microglia. *Phytomedicine*. 2018;43:11-20.

doi: 10.1016/j.phymed.2018.03.039

83. Ariyani W, Miyazaki W, Koibuchi N. A novel mechanism of S-equol action in neurons and astrocytes: The possible involvement of GPR30/GPER1. *Int J Mol Sci*. 2019;20(20):5178.

doi: 10.3390/ijms20205178

84. Ariyani W, Amano I, Koibuchi N. Isoflavones mediate dendritogenesis mainly through estrogen receptor α. *Int J Mol Sci*. 2023;24(10):9011.

doi: 10.3390/ijms24109011

85. Moriyama M, Hashimoto A, Satoh H, *et al*. S-equol, a major isoflavone from soybean, inhibits nitric oxide production in lipopolysaccharide-stimulated rat astrocytes partially via the GPR30-mediated pathway. *Int J Inflamm*. 2018;2018:8496973.

doi: 10.1155/2018/8496973

86. Kajta M, Rzemieniec J, Litwa E, *et al*. The key involvement of estrogen receptor β and G-protein-coupled receptor 30 in the neuroprotective action of daidzein. *Neuroscience*. 2013;238:345-360.

doi: 10.1016/j.neuroscience.2013.02.005

87. Valera E, Masliah E. Therapeutic approaches in Parkinson's disease and related disorders. *J Neurochem*. 2016;139 Suppl 1(Suppl 1):346-352.

doi: 10.1111/jnc.13529

88. Morris HR, Spillantini MG, Sue CM, Williams-Gray CH. The pathogenesis of Parkinson's disease. *Lancet*. 2024;403(10423):293-304.

doi: 10.1016/S0140-6736(23)01478-2

89. Cherian A, Divya KP, Vijayaraghavan A. Parkinson's disease - genetic cause. *Curr Opin Neurol*. 2023;36(4):292-301.

doi: 10.1097/WCO.0000000000001167

90. Russillo MC, Andreozzi V, Erro R, *et al*. Sex differences in Parkinson's disease: From bench to bedside. *Brain Sci*. 2022;12(7):917.

doi: 10.3390/brainsci12070917

91. Patel R, Kompoliti K. Sex and gender differences in Parkinson's disease. *Neurol Clin*. 2023;41(2):371-379.

doi: 10.1016/j.ncl.2022.12.001

92. Petrovska S, Dejanova B, Jurisic V. Estrogens: Mechanisms of neuroprotective effects. *J Physiol Biochem*. 2012;68(3):455-460.

doi: 10.1007/s13105-012-0159-x

93. Simpkins JW, Singh M, Brock C, Etgen AM. Neuroprotection and estrogen receptors. *Neuroendocrinology*. 2021;96(2):119-130.

doi: 10.1159/000338409

94. Cersosimo MG, Benarroch EE. Estrogen actions in the nervous system: Complexity and clinical implications. *Neurology*. 2015;85(3):263-273.

doi: 10.1212/WNL.0000000000001776

95. Reekes TH, Higginson CI, Ledbetter CR, Sathivadivel N, Zweig RM, Disbrow EA. Sex specific cognitive differences in Parkinson disease. *NPJ Parkinsons Dis*. 2020;6:7.

doi: 10.1038/s41531-020-0109-1

96. Guan J, Yang B, Fan Y, Zhang J. GPER Agonist G1 attenuates neuroinflammation and dopaminergic neurodegeneration in Parkinson disease. *Neuroimmunomodulation*. 2017;24(1):60-66.

doi: 10.1159/000478908

97. Bourque M, Morissette M, Côté M, Soulet D, Di Paolo T. Implication of GPER1 in neuroprotection in a mouse model of Parkinson's disease. *Neurobiol Aging*. 2013;34(3):887-901.

doi: 10.1016/j.neurobiolaging.2012.05.022

98. Côté M, Bourque M, Poirier AA, *et al*. GPER1-mediated immunomodulation and neuroprotection in the myenteric plexus of a mouse model of Parkinson's disease. *Neurobiol Dis*. 2015;82:99-113.

doi: 10.1016/j.nbd.2015.05.017

99. Mendes-Oliveira J, Lopes Campos F, Videira RA, Baltazar G. GPER activation is effective in protecting against inflammation-induced nigral dopaminergic loss and motor function impairment. *Brain Behav Immun*. 2017;64:296-307.

doi: 10.1016/j.bbi.2017.04.016

100. Poirier AA, Côté M, Bourque M, *et al*. Differential contribution of estrogen receptors to the intestinal therapeutic effects of 17β-estradiol in a murine model of Parkinson's disease. *Brain Res Bull*. 2022;187:85-97.

doi: 10.1016/j.brainresbull.2022.06.019

101. Sergi CM. Epigallocatechin gallate for Parkinson's disease. *Clin Exp Pharmacol Physiol*. 2022;49(10):1029-1041.

doi: 10.1111/1440-1681.13691

102. Du ZR, Gu Y, Xie XM, *et al*. GPER and IGF-1R mediate the anti-inflammatory effect of genistein against lipopolysaccharide (LPS)-induced nigrostriatal injury in rats. *J Steroid Biochem Mol Biol*. 2021;214:105989.

doi: 10.1016/j.jsbmb.2021.105989

103. Chavda VP, Chaudhari AZ, Balar PC, Gholap A, Vora LK. Phytoestrogens: Chemistry, potential health benefits, and their medicinal importance. *Phytother Res*. 2024;38(6):3060-3079.

doi: 10.1002/ptr.8196

104. Calatayud E, Marcén-Román Y, Rodríguez-Roca B, Salavera C, Gasch-Gallen A, Gómez-Soria I. Sex differences on anxiety and depression in older adults and their relationship with cognitive impairment. *Semergen*. 2023;49(4):101923.

doi: 10.1016/j.semerg.2023.101923

105. Di Benedetto MG, Landi P, Mencacci C, Cattaneo A. Depression in women: Potential biological and sociocultural factors driving the sex effect. *Neuropsychobiology*. 2024;83(1):2-16.

doi: 10.1159/000531588

106. Bromet E, Andrade LH, Hwang I, *et al*. Cross-national epidemiology of DSM-IV major depressive episode. *BMC Med*. 2011;9:90.

doi: 10.1186/1741-7015-9-90

107. Silveira PP, Pokhvisneva I, Howard DM, Meaney MJ.

A sex-specific genome-wide association study of depression phenotypes in UK Biobank. *Mol Psychiatry*. 2023;28(6):2469-2479.

doi: 10.1038/s41380-023-01960-0

108. Herreen D, Rice S, Zajac I. Brief assessment of male depression in clinical care: Validation of the male depression risk scale short form in a cross-sectional study of Australian men. *BMJ Open*. 2022;12(3):e053650.

doi: 10.1136/bmjopen-2021-053650

109. Findikli E, Kurutas EB, Camkurt MA, *et al*. Increased serum G protein-coupled estrogen receptor 1 levels and its diagnostic value in drug naïve patients with major depressive disorder. *Clin Psychopharmacol Neurosci*. 2017;15(4):337-342.

doi: 10.9758/cpn.2017.15.4.337

110. Wang R, Kogler L, Derntl B. Sex differences in cortisol levels in depression: A systematic review and meta-analysis. *Front Neuroendocrinol*. 2024;72:101118.

doi: 10.1016/j.yfrne.2023.101118

111. Furlong MA, Barr DB, Wolff MS, Engel SM. Prenatal exposure to pyrethroid pesticides and childhood behavior and executive functioning. *Neurotoxicology*. 2017;62:231-238.

doi: 10.1016/j.neuro.2017.08.005

112. Viel JF, Rouget F, Warembourg C, *et al*. Behavioural disorders in 6-year-old children and pyrethroid insecticide exposure: The PELAGIE mother-child cohort. *Occup Environ Med*. 2017;74(4):275-281.

doi: 10.1136/oemed-2016-104035

113. Kajta M, Wnuk A, Rzemieniec J, *et al*. Depressive-like effect of prenatal exposure to DDT involves global DNA hypomethylation and impairment of GPER1/ESR1 protein levels but not ESR2 and AHR/ARNT signaling. *J Steroid Biochem Mol Biol*. 2017;171:94-109.

doi: 10.1016/j.jsbmb.2017.03.001

114. Kang WC, Lee YS, Park K, *et al*. Paeonol alleviates postmenopause-induced neuropsychiatric symptoms through the modulation of GPR30 in ovariectomized mice. *J Ethnopharmacol*. 2024;327:118063.

doi: 10.1016/j.jep.2024.118063

115. Tongta S, Daendee S, Kalandakanond-Thongsong S. Effects of estrogen receptor β or G protein-coupled receptor 30 activation on anxiety-like behaviors in relation to GABAergic transmission in stress-ovariectomized rats. *Neurosci Lett*. 2022;789:136885.

doi: 10.1016/j.neulet.2022.136885

116. Kastenberger I, Lutsch C, Schwarzer C. Activation of the G-protein-coupled receptor GPR30 induces anxiogenic effects in mice, similar to oestradiol. *Psychopharmacology*. 2012;221(3):527-535.

doi: 10.1007/s00213-011-2599-3

117. Sun Q, Li G, Zhao F, *et al*. Role of estrogen in treatment of female depression. *Aging (Albany NY)*. 2024;16(3):3021-3042.

doi: 10.18632/aging.205507

118. Lei B, Xu L, Zhang X, Peng W, Tang Q, Feng C. The proliferation effects of fluoxetine and amitriptyline on human breast cancer cells and the underlying molecular mechanisms. *Environ Toxicol Pharmacol*. 2021;83:103586.

doi: 10.1016/j.etap.2021.103586

119. Lupu D, Sjödin MOD, Varshney M, Lindberg J, Loghin F, Rüegg J. Fluoxetine modulates sex steroid levels *in vitro*. *Clujul Med*. 2017;90(4):420-424.

doi: 10.15386/cjmed-868

120. Lupu D, Varshney MK, Mucs D, *et al*. Fluoxetine affects differentiation of midbrain dopaminergic neurons *in vitro*. *Mol Pharmacol*. 2018;94(4):1220-1231.

doi: 10.1124/mol.118.112342

121. Koitmäe A, Karsten Y, Li X, Morellini F, Rune GM, Bender RA. GPER1 deficiency causes sex-specific dysregulation of hippocampal plasticity and cognitive function. *J Endocrinol*. 2023;258:3.

doi: 10.1530/JOE-22-0204

122. Ekinci GN, Sanlier N. The relationship between nutrition and depression in the life process: A mini-review. *Exp Gerontol*. 2023;172:112072.

doi: 10.1016/j.exger.2022.112072

123. Ortega MA, Fraile-Martínez Ó, García-Montero C, *et al*. Nutrition, epigenetics, and major depressive disorder: Understanding the connection. *Front Nutr*. 2022;9:867150.

doi: 10.3389/fnut.2022.867150

124. Gamage E, Orr R, Travica N, *et al*. Polyphenols as novel interventions for depression: Exploring the efficacy, mechanisms of action, and implications for future research. *Neurosci Biobehav Rev*. 2023;151:105225.

doi: 10.1016/j.neubiorev.2023.105225

125. Rondanelli M, Opizzi A, Solerte SB, Trotti R, Klersy C, Cazzola R. Administration of a dietary supplement (N-oleyl-phosphatidylethanolamine and epigallocatechin-3-gallate formula) enhances compliance with diet in healthy overweight subjects: A randomized controlled trial. *Br J Nutr*. 2009;101(3):457-464.

doi: 10.1017/S0007114508024008

126. Borgwardt S, Hammann F, Scheffler K, Kreuter M, Drewe J, Beglinger C. Neural effects of green tea extract on dorsolateral prefrontal cortex. *Eur J Clin Nutr*. 2012;66(11):1187-1192.

doi: 10.1038/ejcn.2012.105

127. Li G, Yang J, Wang X, Zhou C, Zheng X, Lin W. Effects of EGCG on depression-related behavior and serotonin

concentration in a rat model of chronic unpredictable mild stress. *Food Funct*. 2020;11(10):8780-8787.

doi: 10.1039/d0fo00524j

128. Macêdo DS, Sanders LLO, das Candeias R, *et al*. G protein-coupled estrogen receptor 1 (GPER) as a novel target for schizophrenia drug treatment. *Schizophrenia Bull Open*. 2020;1(1):sgaa062.

doi: 10.1093/schizbullopen/sgaa062

129. Salehi MA, Zafari R, Mohammadi S, *et al*. Brain-based sex differences in schizophrenia: A systematic review of fMRI studies. *Hum Brain Mapp*. 2024;45(5):e26664.

doi: 10.1002/hbm.26664

130. Hursitoglu O, Orhan F, Kurutas E, Doganer A, Durmuş H, Bozkus O. Evaluation serum levels of G protein-coupled estrogen receptor and its diagnostic value in patients with schizophrenia. *Psychiatry Clin Psychopharmacol*. 2020;30(2):115.

doi: 10.5455/PCP.20200521025152

131. Monte AS, da Silva FER, Lima CNC, *et al*. Sex influences in the preventive effects of N-acetylcysteine in a two-hit animal model of schizophrenia. *J Psychopharmacol*. 2020;34(1):125-136.

doi: 10.1177/0269881119875979

132. da Silva FER, Cordeiro RC, de Carvalho Lima CN, *et al*. Sex and the estrous-cycle phase influence the expression of G protein-coupled estrogen receptor 1 (GPER) in schizophrenia: Translational evidence for a new target. *Mol Neurobiol*. 2023;60(7):3650-3663.

doi: 10.1007/s12035-023-03295-x

133. He J, Wei Y, Li J, *et al*. Sex differences in the association of treatment-resistant schizophrenia and serum interleukin-6 levels. *BMC Psychiatry*. 2023;23(1):470.

doi: 10.1186/s12888-023-04952-0

134. Zhang C, Liu Q, Yu CY, *et al*. G protein-coupled estrogen receptor 1 knockout deteriorates MK-801-induced learning and memory impairment in mice. *Front Behav Neurosci*. 2020;14:157.

doi: 10.3389/fnbeh.2020.00157

135. Matrisciano F. Functional nutrition as integrated intervention for in- and outpatient with schizophrenia. *Curr Neuropharmacol*. 2023;21(12):2409-2423.

doi: 10.2174/1570159X21666230322160259

136. Munawar N, Ahsan K, Ahmad A. Natural molecules in the treatment of schizophrenia. In: Oliveira MR, editor. *Natural Molecules in Neuroprotection and Neurotoxicity*. Netherlands: Elsevier; 2024. p. 259-280.

doi: 10.1016/B978-0-443-23763-8.00011-7

137. Loftis JM, Wilhelm CJ, Huckans M. Effect of epigallocatechin gallate supplementation in schizophrenia and bipolar disorder: An 8-week, randomized, double-blind, placebo-controlled study. *Ther Adv Psychopharmacol*. 2013;3(1):21-27.

doi: 10.1177/2045125312464103

138. Kalpana S. Genestein, a phytoestrogens for the treatment of schizophrenia. *Int J Sci Eng Res*. 2013;4:296-321.

139. Reddy DS, Thompson W, Calderara G. Molecular mechanisms of sex differences in epilepsy and seizure susceptibility in chemical, genetic and acquired epileptogenesis. *Neurosci Lett*. 2021;750:135753.

doi: 10.1016/j.neulet.2021.135753

140. Christensen J, Kjeldsen MJ, Andersen H, Friis ML, Sidenius P. Gender differences in epilepsy. *Epilepsia*. 2005;46(6):956-960.

doi: 10.1111/j.1528-1167.2005.51204.x

141. Betjemann JP, Lowenstein DH. Status epilepticus in adults. *Lancet Neurol*. 2015;14(6):615-624.

doi: 10.1016/S1474-4422(15)00042-3

142. Reddy DS. Brain structural and neuroendocrine basis of sex differences in epilepsy. *Handb Clin Neurol*. 2020;175:223-233.

doi: 10.1016/B978-0-444-64123-6.00016-3

143. Bäckström T. Epileptic seizures in women related to plasma estrogen and progesterone during the menstrual cycle. *Acta Neurol Scand*. 1976;54(4):321-347.

doi: 10.1111/j.1600-0404.1976.tb04363.x

144. Logothetis J, Harner R, Morrell F, Torres F. The role of estrogens in catamenial exacerbation of epilepsy. *Neurology*. 1959;9(5):352-360.

doi: 10.1212/wnl.9.5.352

145. Mastrangelo M, Torres B, de Vita G, *et al*. Neurodevelopmental impairment as the main phenotypic hallmark associated with the translocation t(7;10)(7p22.3;q26.11). *J Pediatr Genet*. 2022;11(1):68-73.

doi: 10.1055/s-0040-1715479

146. Zuo D, Wang F, Rong W, *et al*. The novel estrogen receptor GPER1 decreases epilepsy severity and susceptivity in the hippocampus after status epilepticus. *Neurosci Lett*. 2020;728:134978.

doi: 10.1016/j.neulet.2020.134978

147. Zhang X, Yang Y, Guo L, *et al*. GPER1 modulates synaptic plasticity during the development of temporal lobe epilepsy in rats. *Neurochem Res*. 2021;46(8):2019-2032.

doi: 10.1007/s11064-021-03336-8

148. Kurt AH, Bosnak M, Inan SY, Celik A, Uremis MM. Epileptogenic effects of G protein-coupled estrogen receptor 1 in the rat pentylenetetrazole kindling model of epilepsy. *Pharmacol Rep*. 2016;68(1):66-70.

doi: 10.1016/j.pharep.2015.07.001

149. Wang Z, Huang K, Yang X, *et al*. Downregulated GPR30 expression in the epileptogenic foci of female patients with focal cortical dysplasia type IIb and tuberous sclerosis complex is correlated with 18 F-FDG PET-CT values. *Brain Pathol*. 2021;31(2):346-364.

doi: 10.1111/bpa.12925

150. Ghosh S, Sinha JK, Ghosh S, Sharma H, Bhaskar R, Narayanan KB. A Comprehensive review of emerging trends and innovative therapies in epilepsy management. *Brain Sci*. 2023;13(9):1305.

doi: 10.3390/brainsci13091305

151. Dyńka D, Kowalcze K, Paziewska A. The role of ketogenic diet in the treatment of neurological diseases. Nutrients. 2022;14(23):5003.

doi: 10.3390/nu14235003

152. Arzimanoglou A, Brandl U, Cross JH, *et al*. Epilepsy and cannabidiol: A guide to treatment. *Epileptic Disord*. 2020;22(1):1-14.

doi: 10.1684/epd.2020.1141

153. Wells J, Swaminathan A, Paseka J, Hanson C. Efficacy and safety of a ketogenic diet in children and adolescents with refractory epilepsy-a review. *Nutrients*. 2020;12(6):1809.

doi: 10.3390/nu12061809

154. Wang S, Zhang Z, Wang J, *et al*. Neuronal GPER participates in genistein-mediated neuroprotection in ischemic stroke by inhibiting NLRP3 inflammasome activation in ovariectomized female mice. *Mol Neurobiol*. 2022;59(8):5024-5040.

doi: 10.1007/s12035-022-02894-4

155. Werling DM, Geschwind DH. Sex differences in autism spectrum disorders. *Curr Opin Neurol*. 2013;26(2):146-153.

doi: 10.1097/WCO.0b013e32835ee548

156. Altun H, Kurutaş EB, Şahin N, Sınır H, Fındıklı E. Decreased levels of G protein-coupled estrogen receptor in children with autism spectrum disorders. *Psychiatry Res*. 2017;257:67-71.

doi: 10.1016/j.psychres.2017.06.008

157. Hameed RA, Ahmed EK, Mahmoud AA, Atef AA. G protein-coupled estrogen receptor (GPER) selective agonist G1 attenuates the neurobehavioral, molecular and biochemical alterations induced in a valproic acid rat model of autism. *Life Sci*. 2023;328:121860.

doi: 10.1016/j.lfs.2023.121860

158. Jia Q, Li H, Wang M, *et al*. Transcript levels of 4 genes in umbilical cord blood are predictive of later autism development: A longitudinal follow-up study. J Psychiatry Neurosci. 2023;48(5):E334-E344.

doi: 10.1503/jpn.230046

159. Nøvik TS, Hervas A, Ralston SJ, Dalsgaard S, Rodrigues Pereira R, Lorenzo MJ. Influence of gender on attention-deficit/hyperactivity disorder in Europe--ADORE. *Eur Child Adolesc Psychiatry*. 2006;15 Suppl 1:I15-I24.

doi: 10.1007/s00787-006-1003-z

160. Sahin N, Altun H, Kurutaş EB, Fındıklı E. Evaluation of estrogen and G protein-coupled estrogen receptor 1 (GPER) levels in drug-naïve patients with attention deficit hyperactivity disorder (ADHD). *Bosnian J Basic Med Sci*. 2018;18(2):126-131.

doi: 10.17305/bjbms.2018.2942

161. Xiao G, Zhou X, Huang J, *et al*. Association of GPER gene polymorphism with social function of children with attention deficit hyperactivity disorder. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2018;35(4):587-590.

doi: 10.3760/cma.j.issn.1003-9406.2018.04.028

162. Berkley KJ. Vive la difference. *Trends Neurosci*. 1992;15(9):331-332.

doi: 10.1016/0166-2236(92)90048-d

163. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL. Sex, gender, and pain: A review of recent clinical and experimental findings. *J Pain*. 2009;10(5):447-485.

doi: 10.1016/j.jpain.2008.12.001

164. Pieretti S, Di Giannuario A, Di Giovannandrea R, *et al*. Gender differences in pain and its relief. *Ann Ist Super Sanita*. 2016;52(2):184-189.

doi: 10.4415/ANN_16_02_09

165. Gupta S, McCarson KE, Welch KMA, Berman NEJ. Mechanisms of pain modulation by sex hormones in migraine. *Headache*. 2011;51(6):905-922.

doi: 10.1111/j.1526-4610.2011.01908.x

166. Artero-Morales M, González-Rodríguez S, Ferrer-Montiel A. TRP channels as potential targets for sex-related differences in migraine pain. *Front Mol Biosci*. 2018;5:73.

doi: 10.3389/fmolb.2018.00073

167. Sacco S, Ricci S, Degan D, Carolei A. Migraine in women: The role of hormones and their impact on vascular diseases. *J Headache Pain*. 2012;13(3):177-189.

doi: 10.1007/s10194-012-0424-y

168. Vetvik KG, MacGregor EA. Sex differences in the epidemiology, clinical features, and pathophysiology of migraine. *Lancet Neurol.* 2017;16(1):76-87.

doi: 10.1016/S1474-4422(16)30293-9

169. Jiang M, Sun Y, Lei Y, *et al*. GPR30 receptor promotes preoperative anxiety-induced postoperative hyperalgesia by up-regulating GABAA-α4β1δ subunits in periaqueductal gray in female rats. *BMC Anesthesiol*. 2020;20(1):93.

doi: 10.1186/s12871-020-01017-7

170. Xu JJ, Gao P, Wu Y, *et al*. G protein-coupled estrogen receptor in the rostral ventromedial medulla contributes to the chronification of postoperative pain. *CNS Neurosci Ther*. 2021;27(11):1313-1326.

doi: 10.1111/cns.13704

171. Zheng W, Huang X, Wang J, *et al*. The chronification mechanism of orofacial inflammatory pain: Facilitation by GPER1 and microglia in the rostral ventral medulla. *Front Mol Neurosci*. 2022;15:1078309.

doi: 10.3389/fnmol.2022.1078309

172. Xu Z, Xie W, Feng Y, *et al*. Positive interaction between GPER and β-alanine in the dorsal root ganglion uncovers potential mechanisms: mediating continuous neuronal sensitization and neuroinflammation responses in neuropathic pain. *J Neuroinflamm*. 2022;19(1):164.

doi: 10.1186/s12974-022-02524-9

173. Wang J, Wang S, Fang Z, *et al*. Estrogen receptor GPR30 in the anterior cingulate cortex mediates exacerbated neuropathic pain in ovariectomized mice. *Brain Res*. 2024;1829:148798.

doi: 10.1016/j.brainres.2024.148798

174. Xu E, Xia X, Jiang C, *et al*. GPER1 silencing suppresses the proliferation, migration, and invasion of gastric cancer cells by inhibiting PI3K/AKT-mediated EMT. *Front Cell Dev Biol*. 2020;8:591239.

doi: 10.3389/fcell.2020.591239

175. Araldi D, Ferrari LF, Green P, Levine JD. Marked sexual dimorphism in 5-HT1 receptors mediating pronociceptive effects of sumatriptan. *Neuroscience*. 2017;344:394-405.

doi: 10.1016/j.neuroscience.2016.12.031

176. Jiang M, Liu Y, Wu H, Ma Z, Gu X. High estrogen level modifies postoperative hyperalgesia via GPR30 and MMP-9 in dorsal root ganglia neurons. *Neurochem Res*. 2020;45(7):1661-1673.

doi: 10.1007/s11064-020-03032-z

177. Felix FB, Vago JP, Fernandes DO, *et al*. Biochanin a regulates key steps of inflammation resolution in a model of antigen-induced arthritis via GPR30/PKA-dependent mechanism. *Front Pharmacol*. 2021;12:662308.

doi: 10.3389/fphar.2021.662308

178. Liu H, Duan SR. Prostaglandin E2-mediated upregulation of neuroexcitation and persistent tetrodotoxin-resistant Na(+) currents in Ah-type trigeminal ganglion neurons isolated from adult female rats. *Neuroscience*. 2016;320:194-204.

doi: 10.1016/j.neuroscience.2016.02.008

179. Liu S, Tian Z, Guo Y, Zhang N, Feng B, Zhao M. Activation of GPR30 attenuates chronic pain-related anxiety in ovariectomized mice. *Psychoneuroendocrinology*. 2015; 53:94-107.

doi: 10.1016/j.psyneuen.2014.12.021

180. Liverman CS, Brown JW, Sandhir R, McCarson KE, Berman NEJ. Role of the oestrogen receptors GPR30 and ERalpha in peripheral sensitization: Relevance to trigeminal pain disorders in women. *Cephalalgia*. 2009;29(7):729-741.

doi: 10.1111/j.1468-2982.2008.01789.x

181. Vermeer LMM, Gregory E, Winter MK, McCarson KE, Berman NEJ. Exposure to bisphenol A exacerbates migraine-like behaviors in a multibehavior model of rat migraine. *Toxicol Sci*. 2014;137(2):416-427.

doi: 10.1093/toxsci/kft245

182. Seehusen F, Baumgärtner W. Axonal pathology and loss precede demyelination and accompany chronic lesions in a spontaneously occurring animal model of multiple sclerosis. *Brain Pathol*. 2010;20(3):551-559.

doi: 10.1111/j.1750-3639.2009.00332.x

183. Kingwell E, Marriott JJ, Jetté N, *et al*. Incidence and prevalence of multiple sclerosis in Europe: a systematic review. *BMC Neurol*. 2013;13:128.

doi: 10.1186/1471-2377-13-128

184. Confavreux C, Hutchinson M, Hours MM, Cortinovis-Tourniaire P, Moreau T. Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *N Engl J Med*. 1998;339(5):285-291.

doi: 10.1056/NEJM199807303390501

185. Blasko E, Haskell CA, Leung S, *et al*. Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *J Neuroimmunol*. 2009;214(1-2):67-77.

doi: 10.1016/j.jneuroim.2009.06.023

186. Bodhankar S, Offner H. GPR30 forms an integral part of e2-protective pathway in experimental autoimmune encephalomyelitis. *Immunol Endocr Metab Agents Med Chem*. 2011;11(4):262-274.

doi: 10.2174/1871522211108040262

187. Hirahara Y, Matsuda KI, Yamada H, *et al*. G protein-coupled receptor 30 contributes to improved remyelination after cuprizone-induced demyelination. *Glia*. 2013;61(3):420-431.

doi: 10.1002/glia.22445

188. Wang C, Dehghani B, Li Y, *et al*. Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol*. 2009;182(5):3294-3303.

doi: 10.4049/jimmunol.0803205

189. Yates MA, Li Y, Chlebeck PJ, Offner H. GPR30, but not estrogen receptor-alpha, is crucial in the treatment of experimental autoimmune encephalomyelitis by oral ethinyl estradiol. *BMC Immunol*. 2010;11:20.

doi: 10.1186/1471-2172-11-20

190. Subramanian S, Miller LM, Grafe MR Vandenbark AA, Offner H. Contribution of GPR30 for 1,25 dihydroxyvitamin D_3 protection in EAE. *Metab Brain Dis*. 2012;27(1):29-35.

doi: 10.1007/s11011-011-9266-6

191. Huang C, Yuan P, Wu J, Huang J. Estrogen regulates excitatory amino acid carrier 1 (EAAC1) expression through sphingosine kinase 1 (SphK1) transacting FGFR-mediated ERK signaling in rat C6 astroglial cells. *Neuroscience*. 2016;319:9-22.

doi: 10.1016/j.neuroscience.2016.01.027

192. Kramer H, Han C, Post W, *et al*. Racial/ethnic differences in hypertension and hypertension treatment and control in the multi-ethnic study of atherosclerosis (MESA). *Am J Hypert*. 2004;17(10):963-970.

doi: 10.1016/j.amjhyper.2004.06.001

193. Ma Y, Hua R, Yang Z, Zhong B, Yan L, Xie W. Different hypertension thresholds and cognitive decline: A pooled analysis of three ageing cohorts. BMC medicine. 2021;19(1):287.

doi: 10.1186/s12916-021-02165-4

194. Burt VL, Whelton P, Roccella EJ, *et al*. Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988-1991. *Hypertension*. 1995;25(3):305-313.

doi: 10.1161/01.hyp.25.3.305

195. Rossi GP, Caroccia B, Seccia TM. Role of estrogen receptors in modulating aldosterone biosynthesis and blood pressure. *Steroids*. 2019;152:108486.

doi: 10.1016/j.steroids.2019.108486

196. Fortepiani LA, Zhang H, Racusen L, Roberts LJ, Reckelhoff JF. Characterization of an animal model of postmenopausal hypertension in spontaneously hypertensive rats. *Hypertension*. 2003;41(3 Pt 2):640-645.

doi: 10.1161/01.HYP.0000046924.94886.EF

197. Yanes LL, Reckelhoff JF. Postmenopausal hypertension. *Am J Hypertens*. 2011;24(7):740-749.

doi: 10.1038/ajh.2011.71

198. Benjamin EJ, Muntner P, Alonso A, *et al*. Heart disease and stroke statistics-2019 update: A report from the American heart association. *Circulation*. 2019;139(10):e56-e528.

doi: 10.1161/CIR.0000000000000659

199. Liu S, Ding T, Liu H, Jian L. GPER was associated with hypertension in post-menopausal women. *Open Med* (*Wars*). 2018;13:338-343.

doi: 10.1515/med-2018-0051

200. Alencar AKN, Montes GC, Costa DG, *et al*. Cardioprotection induced by activation of GPER in ovariectomized rats with pulmonary hypertension. *J Gerontol A Biol Sci Med Sci*. 2018;73(9):1158-1166.

doi: 10.1093/gerona/gly068

201. Ghaffari S, Naderi Nabi F, Sugiyama MG, Lee WL. Estrogen inhibits LDL (low-density lipoprotein) transcytosis by human coronary artery endothelial cells via GPER (G-protein-coupled estrogen receptor) and SR-BI (scavenger receptor class B type 1). *Arterioscler Thromb Vasc Biol*. 2018;38(10):2283-2294.

doi: 10.1161/ATVBAHA.118.310792

202. Li F, Yu X, Szynkarski CK, *et al*. Activation of GPER induces differentiation and inhibition of coronary artery smooth muscle cell proliferation. *PLoS One*. 2013;8(6):e64771.

doi: 10.1371/journal.pone.0064771

203. Salehiyeh S, Alborzi N, Azizian H, Esmailidehaj M, Hafizi Barjin Z, Safari F. Sex-related differences in hypertrophy response and cardiac expression of G protein-coupled estrogen receptor in rats with pressure overload. *Gene*. 2024;928:148769.

doi: 10.1016/j.gene.2024.148769

204. da Silva JS, Sun X, Ahmad S, *et al*. G-protein-coupled estrogen receptor agonist G1 improves diastolic function and attenuates cardiac renin-angiotensin system activation in estrogen-deficient hypertensive rats. *J Cardiovasc Pharmacol*. 2019;74(5):443-452.

doi: 10.1097/FJC.0000000000000721

205. Wang H, Zhao Z, Lin M, Groban L. Activation of GPR30 inhibits cardiac fibroblast proliferation. *Mol Cell Biochem*. 2015;405(1-2):135-148.

doi: 10.1007/s11010-015-2405-3

206. Zhao Z, Wang H, Lin M, Groban L. GPR30 decreases cardiac chymase/angiotensin II by inhibiting local mast cell number. *Biochem Biophys Res Commun*. 2015;459(1):131-136.

doi: 10.1016/j.bbrc.2015.02.082

207. Lafferty AR, Torpy DJ, Stowasser M, *et al*. A novel genetic locus for low renin hypertension: Familial hyperaldosteronism type II maps to chromosome 7 (7p22). *J Med Genet*. 2000;37(11):831-835.

doi: 10.1136/jmg.37.11.831

208. Tropea T, de Francesco EM, Rigiracciolo D, *et al*. Pregnancy augments G protein estrogen receptor (GPER) induced vasodilation in rat uterine arteries via the nitric oxide - cGMP signaling pathway. *PLoS One*. 2015;10(11):e0141997.

doi: 10.1371/journal.pone.0141997

209. Meyer MR, Field AS, Kanagy NL, Barton M, Prossnitz ER. GPER regulates endothelin-dependent vascular tone and intracellular calcium. *Life Sci*. 2012;91(13-14):623-627.

doi: 10.1016/j.lfs.2012.01.007

210. Yu X, Zhang Q, Zhao Y, *et al*. Activation of G protein-

coupled estrogen receptor 1 induces coronary artery relaxation via Epac/Rap1-mediated inhibition of RhoA/Rho kinase pathway in parallel with PKA. *PLoS One*. 2017;12(3):e0173085.

doi: 10.1371/journal.pone.0173085

211. Yu X, Stallone JN, Heaps CL, Han G. The activation of G protein-coupled estrogen receptor induces relaxation via cAMP as well as potentiates contraction via EGFR transactivation in porcine coronary arteries. *PLoS One*. 2018;13(1):e0191418.

doi: 10.1371/journal.pone.0191418

212. Bologa CG, Revankar CM, Young SM, *et al*. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol*. 2006;2(4):207-212.

doi: 10.1038/nchembio775

213. Arefin S, Simoncini T, Wieland R, *et al*. Vasodilatory effects of the selective GPER agonist G-1 is maximal in arteries of postmenopausal women. Maturitas. 2014;78(2):123-130.

doi: 10.1016/j.maturitas.2014.04.002

214. Feldman RD, Gros R, Ding Q, *et al*. A common hypofunctional genetic variant of GPER is associated with increased blood pressure in women. *Br J Clin Pharmacol*. 2014;78(6):1441-1452.

doi: 10.1111/bcp.12471

215. Fredette NC, Malik E, Mukhtar ML, Prossnitz ER, Terada N. A hypertension patient-derived iPSC model demonstrates a role for G protein-coupled estrogen receptor in hypertension risk and development. *Am J Physiol Cell Physiol*. 2020;319(5):C825-C838.

doi: 10.1152/ajpcell.00350.2019

216. Fredette NC, Meyer MR, Prossnitz ER. Role of GPER in estrogen-dependent nitric oxide formation and vasodilation. *J Steroid Biochem Mol Biol*. 2018;176:65-72.

doi: 10.1016/j.jsbmb.2017.05.006

217. Meyer MR, Rosemann T, Barton M, Prossnitz ER. GPER mediates functional endothelial aging in renal arteries. *Pharmacology*. 2017;100(3-4):188-193.

doi: 10.1159/000478732

218. Davis GK, Newsome AD, Cole AB, Ojeda NB, Alexander BT. Chronic estrogen supplementation prevents the increase in blood pressure in female intrauterine growth-restricted offspring at 12 months of age. *Hypertension*. 2019;73(5):1128-1136.

doi: 10.1161/HYPERTENSIONAHA.118.12379

219. Magness RR, Parker CR, Rosenfeld CR. Systemic and uterine responses to chronic infusion of estradiol-17 beta. *Am J Physiol*. 1993;265(5 Pt 1):E690-E698.

doi: 10.1152/ajpendo.1993.265.5.E690

220. Đogo A, Dožić B, Vujović S, Srebro D, Dožić I. Effects of continuous-combined oral drospirenone- estradiol on blood pressure, body weight & lipid profile in early menopausal women. *Indian J Med Res*. 2021;154(6):857-865.

doi: 10.4103/ijmr.IJMR_478_20

221. Delgado NTB, Rouver WN, Freitas-Lima LC, *et al*. Sex differences in the vasodilation mediated by G protein-coupled estrogen receptor (GPER) in hypertensive rats. *Front Physiol*. 2021;12:659291.

doi: 10.3389/fphys.2021.659291

222. Lam CSP, Arnott C, Beale AL, *et al*. Sex differences in heart failure. *Eur Heart J*. 2019;40(47):3859-3868c.

doi: 10.1093/eurheartj/ehz835

223. Iorga A, Li J, Sharma S, *et al*. Rescue of pressure overload-induced heart failure by estrogen therapy. *J Am Heart Assoc*. 2016;5:1.

doi: 10.1161/JAHA.115.002482

224. Recchia AG, de Francesco EM, Vivacqua A, *et al*. The G protein-coupled receptor 30 is up-regulated by hypoxia-inducible factor-1alpha (HIF-1alpha) in breast cancer cells and cardiomyocytes. *J Biol Chem*. 2011;286(12):10773-10782.

doi: 10.1074/jbc.M110.172247

225. Bae S, Lim YH, Lee YA, Shin CH, Oh SY, Hong YC. Maternal urinary bisphenol a concentration during midterm pregnancy and children's blood pressure at age 4. *Hypertension*. 2017;69(2):367-374.

doi: 10.1161/HYPERTENSIONAHA.116.08281

226. Lombó M, González-Rojo S, Fernández-Díez C, Herráez MP. Cardiogenesis impairment promoted by bisphenol A exposure is successfully counteracted by epigallocatechin gallate. *Environ Pollut*. 2019;246:1008-1019.

doi: 10.1016/j.envpol.2019.01.004

227. Morselli E, Santos RS, Criollo A, Nelson MD, Palmer BF, Clegg DJ. The effects of oestrogens and their receptors on cardiometabolic health. *Nat Rev Endocrinol*. 2017;13(6):352-364.

doi: 10.1038/nrendo.2017.12

228. Feng Z, Wang C, Yue J, Meng Q, Wu J, Sun H. Kaempferol-induced GPER upregulation attenuates atherosclerosis via the PI3K/AKT/Nrf2 pathway. *Pharm Biol*. 2021;59(1):1106-1116.

doi: 10.1080/13880209.2021.1961823

229. Zhao W, Shan X, Li X, *et al*. Icariin inhibits hypertrophy by regulation of GPER1 and CaMKII/HDAC4/MEF2C signaling crosstalk in ovariectomized mice. *Chem Biol Interact*. 2023;384:110728.

doi: 10.1016/j.cbi.2023.110728

230. Aubin MC, Lajoie C, Clément R, Gosselin H, Calderone A, Perrault LP. Female rats fed a high-fat diet were associated with vascular dysfunction and cardiac fibrosis in the absence of overt obesity and hyperlipidemia: Therapeutic potential

of resveratrol. *J Pharmacol Exp Ther*. 2008;325(3):961-968.

doi: 10.1124/jpet.107.135061

231. Santoro F, Mango F, Mallardi A, *et al*. Arrhythmic risk stratification among patients with hypertrophic cardiomyopathy. *J Clin Med*. 2023;12(10):3397.

doi: 10.3390/jcm12103397

232. Fang Z, Raza U, Song J, *et al.* Systemic aging fuels heart failure: Molecular mechanisms and therapeutic avenues. *ESC Heart Fail*. 2024.

doi: 10.1002/ehf2.14947

233. Kang S, Liu Y, Sun D, *et al*. Chronic activation of the G protein-coupled receptor 30 with agonist G-1 attenuates heart failure. *PLoS One.* 2012;7(10):e48185.

doi: 10.1371/journal.pone.0048185

234. Shao Z, Shen Q, Kong M, Ni H, Hou X. Involvement of GPR30 in protection effect of Dexmedetomidine against myocardial ischemia/reperfusion injury in rat via AKT pathway. *Acta Biochim Pol*. 2021;68(1):119-126.

doi: 10.18388/abp.2020_5473

235. Pei H, Wang W, Zhao DI, Su H, Su G, Zhao Z. G Protein-coupled estrogen receptor 1 inhibits angiotensin II-induced cardiomyocyte hypertrophy via the regulation of PI3K-Akt-mTOR signalling and autophagy. *Int J Biol Sci*. 2019;15(1):81-92.

doi: 10.7150/ijbs.28304

236. Patten RD, Pourati I, Aronovitz MJ, *et al*. 17beta-estradiol reduces cardiomyocyte apoptosis *in vivo* and *in vitro* via activation of phospho-inositide-3 kinase/Akt signaling. *Circ Res*. 2004;95(7):692-699.

doi: 10.1161/01.RES.0000144126.57786.89

237. Lenhart PM, Broselid S, Barrick CJ, Leeb-Lundberg LMF, Caron KM. G-protein-coupled receptor 30 interacts with receptor activity-modifying protein 3 and confers sex-dependent cardioprotection. *J Mol Endocrinol*. 2013;51(1):191-202.

doi: 10.1530/JME-13-0021

238. Watanabe H, Takahashi E, Kobayashi M, *et al*. The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor. *J Mol Endocrinol*. 2006;36(1):81-89.

doi: 10.1677/jme.1.01825

239. Barrick CJ, Lenhart PM, Dackor RT, Nagle E, Caron KM. Loss of receptor activity-modifying protein 3 exacerbates cardiac hypertrophy and transition to heart failure in a sex-dependent manner. *J Mol Cell Cardiol*. 2012;52(1):165-174.

doi: 10.1016/j.yjmcc.2011.10.021

240. De Francesco EM, Angelone T, Pasqua T, Pupo M, Cerra MC, Maggiolini M. GPER mediates cardiotropic effects in spontaneously hypertensive rat hearts. *PLoS One*. 2013;8(8):e69322.

doi: 10.1371/journal.pone.0069322

241. Kabir ME, Singh H, Lu R, Olde B, Leeb-Lundberg LMF, Bopassa JC. G Protein-coupled estrogen receptor 1 mediates acute estrogen-induced cardioprotection via MEK/ERK/GSK-3β pathway after ischemia/reperfusion. *PLoS One*. 2015;10(9):e0135988.

doi: 10.1371/journal.pone.0135988

242. Roubille F, Combes S, Leal-Sanchez J, *et al*. Myocardial expression of a dominant-negative form of Daxx decreases infarct size and attenuates apoptosis in an in vivo mouse model of ischemia/reperfusion injury. *Circulation*. 2007;116(23):2709-2717.

doi: 10.1161/CIRCULATIONAHA.107.694844

243. Li WL, Xiang W, Ping Y. Activation of novel estrogen receptor GPER results in inhibition of cardiocyte apoptosis and cardioprotection. *Mol Med Rep.* 2015;12(2):2425-2430.

doi: 10.3892/mmr.2015.3674

244. Algoet M, Janssens S, Himmelreich U, *et al*. Myocardial ischemia-reperfusion injury and the influence of inflammation. *Trends Cardiovasc Med*. 2023;33(6):357-366.

doi: 10.1016/j.tcm.2022.02.005

245. Wang X, Lu L, Tan Y, *et al*. GPR 30 reduces myocardial infarct area and fibrosis in female ovariectomized mice by activating the PI3K/AKT pathway. *Life Sci*. 2019;226:22-32.

doi: 10.1016/j.lfs.2019.03.049

246. Krishnan SM, Ling YH, Huuskes BM, *et al*. Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage, and dysfunction in salt-sensitive hypertension. *Cardiovasc Res*. 2019;115(4):776-787.

doi: 10.1093/cvr/cvy252

247. van Hout GPJ, Bosch L, Ellenbroek GHJM, *et al*. The selective NLRP3-inflammasome inhibitor MCC950 reduces infarct size and preserves cardiac function in a pig model of myocardial infarction. *Eur Heart J*. 2017;38(11):828-836.

doi: 10.1093/eurheartj/ehw247

248. Carvalho FS, Burgeiro A, Garcia R, Moreno AJ, Carvalho RA, Oliveira PJ. Doxorubicin-induced cardiotoxicity: From bioenergetic failure and cell death to cardiomyopathy. *Med Res Rev*. 2014;34(1):106-135.

doi: 10.1002/med.21280

249. De Francesco EM, Rocca C, Scavello F, *et al*. Protective role of GPER agonist G-1 on cardiotoxicity induced by doxorubicin. *J Cell Physiol*. 2017;232(7):1640-1649.

doi: 10.1002/jcp.25585

250. Wang H, Sun X, Lin MS, Ferrario CM, van Remmen H, Groban L. G protein-coupled estrogen receptor (GPER)

deficiency induces cardiac remodeling through oxidative stress. *Transl Res*. 2018;199:39-51.

doi: 10.1016/j.trsl.2018.04.005

251. Kander MC, Cui Y, Liu Z. Gender difference in oxidative stress: A new look at the mechanisms for cardiovascular diseases. *J Cell Mol Med*. 2017;21(5):1024-1032.

doi: 10.1111/jcmm.13038

252. Ide T, Tsutsui H, Ohashi N, *et al*. Greater oxidative stress in healthy young men compared with premenopausal women. *Arterioscler Thromb Vasc Biol*. 2002;22(3):438-442.

doi: 10.1161/hq0302.104515

253. Bopassa JC, Eghbali M, Toro L, Stefani E. A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2010;298(1):H16-H23.

doi: 10.1152/ajpheart.00588.2009

254. Adu-Amankwaah J, Adekunle AO, Tang Z, *et al*. Estradiol contributes to sex differences in resilience to sepsis-induced metabolic dysregulation and dysfunction in the heart via GPER-1-mediated PPARδ/NLRP3 signaling. *Metabolism*. 2024;156:155934.

doi: 10.1016/j.metabol.2024.155934

255. Sharma M, Bhatt LK. Emerging therapeutic targets for heart failure. *Curr Cardiol Rep*. 2022;24(11):1737-1754.

doi: 10.1007/s11886-022-01789-z

256. Linz D, Gawalko M, Betz K, *et al*. Atrial fibrillation: Epidemiology, screening and digital health. *Lancet Reg Health Europe*. 2024;37:100786.

doi: 10.1016/j.lanepe.2023.100786

257. Gopinathannair R, Chen LY, Chung MK, *et al*. Managing atrial fibrillation in patients with heart failure and reduced ejection fraction: A scientific statement from the American Heart Association. *Circ Arrhythm Electrophysiol*. 2021;14(6):HAE0000000000000078.

doi: 10.1161/HAE.0000000000000078

258. Soliman EZ, Safford MM, Muntner P, *et al*. Atrial fibrillation and the risk of myocardial infarction. *JAMA Intern Med*. 2014;174(1):107-114.

doi: 10.1001/jamainternmed.2013.11912

259. Chao TF, Huang YC, Liu CJ, *et al*. Acute myocardial infarction in patients with atrial fibrillation with a CHA2DS2-VASc score of 0 or 1: A nationwide cohort study. *Heart Rhythm*. 2014;11(11):1941-1947.

doi: 10.1016/j.hrthm.2014.08.003

260. Pierdomenico SD, Coccina F. Atrial cardiopathy and hypertension: A deadly association. *Am J Hypertens*. 2023;36(1):14-16.

doi: 10.1093/ajh/hpac095

261. Bagchi RA, Weeks KL. Histone deacetylases in cardiovascular and metabolic diseases. *J Mol Cell Cardiol*. 2019;130:151-159.

doi: 10.1016/j.yjmcc.2019.04.003

262. Sawa Y, Matsushita N, Sato S, *et al*. Chronic HDAC6 activation induces atrial fibrillation through atrial electrical and structural remodeling in transgenic mice. *Int Heart J*. 2021;62(3):616-626.

doi: 10.1536/ihj.20-703

263. Sugishita K, Uchida M, Ikeda M, *et al*. Gender difference in subjective symptoms related to paroxysmal atrial fibrillation is also detected in postmenopausal women. *Int Heart J*. 2005;46(4):669-678.

doi: 10.1536/ihj.46.669

264. Liu D, Zhan Y, Ono K, *et al*. Pharmacological activation of estrogenic receptor G protein-coupled receptor 30 attenuates angiotensin II-induced atrial fibrosis in ovariectomized mice by modulating TGF-β1/smad pathway. *Mol Biol Rep*. 2022;49(7):6341-6355.

doi: 10.1007/s11033-022-07444-8

265. Baik SH, Baye F, McDonald CJ. Use of menopausal hormone therapy beyond age 65 years and its effects on women's health outcomes by types, routes, and doses. *Menopause*. 2024;31(5):363-371.

doi: 10.1097/GME.0000000000002335

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Single-nucleotide polymorphism rs670 in the promoter region of the apolipoprotein A-I gene

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Abstract

Apolipoprotein A-I (Apo A-I) plays a central role in the function of high-density lipoprotein (HDL). The rs670 single-nucleotide polymorphism (SNP) was identified as a cytosine-to-thymine (C>T) transition in a cytosine–phosphate–guanine site within the promoter region of *APOA1*. Given its location, studies have hypothesized that this polymorphism may influence *APOA1* expression, potentially impacting lipid and carbohydrate metabolism. The aim of this integrative review was to summarize all observational and experimental studies on the rs670 SNP available in the Scopus, ScienceDirect, dbSNP, and LitVar2 databases up to July 2024. In total, 162 articles were identified, of which 28 met the inclusion criteria. Most studies originated from Asia and were published between 2009 and 2023. Of the selected studies, 14 (50%) examined the association of the rs670 SNP with metabolic disorders. Among these, eight (28.57%) reported an association between the polymorphic allele and alterations in lipid profiles, proinflammatory markers, and insulin resistance. The remaining studies explored associations between the polymorphic allele and ischemic events, pharmacological therapies, diet, neurodegenerative diseases, pancreatitis, and breast cancer. The rs670 SNP occurs in more than 20% of the global population. Its position within the promoter region enables it to potentially regulate the *APO* cluster on chromosome 11. This review underscores the need for further investigation into the impact of this polymorphism on gene expression and its role in the pathogenesis of metabolic syndrome, cardiovascular disease, and cancer.

Keywords: Apolipoprotein A-I; High-density lipoprotein; Lipid metabolism; Single-nucleotide polymorphism

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1. Introduction

The proper functioning of lipid metabolism involves several key components, such as lipoproteins, apolipoproteins (Apo), enzymes, and tissue receptors. Apo play crucial roles in the orientation and stabilization of lipoproteins, serving as ligands for receptors and acting as enzyme activators or inhibitors. *Apo A-I*, predominantly synthesized in

the liver and small intestine, is present in very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). It is the primary protein component of HDL, acting as a ligand for scavenger receptor B Type 1 (SR-B1) in hepatocytes and macrophages and as a cofactor for lecithin cholesterol acyltransferase.[1] Genetic alterations in *APOA1* are associated with lipid metabolism disorders, particularly hypoalphalipoproteinemia, hypertriglyceridemia, and familial systemic amyloidosis.[2]

APOA1 spans 2,200 bases and is located on chromosome 11q23.3. Its transcription yields four exons that are processed during splicing to code for pre-pro-Apo A-I, a protein with 267 amino acid residues. Within the cell, 18 NH_2-terminal residues are cleaved to form Pro-Apo A-I (249 residues), which is then secreted into the plasma. Subsequently, six additional NH_2-terminal residues are removed to produce mature Apo A-I, comprising 243 amino acid residues.[3] *APOA1* is part of the *APO* cluster on chromosome 11, which encodes Apo A-I, Apo C-III, Apo A-IV, and Apo A-V. This cluster underscores the relevance of genetic alterations in influencing lipid metabolism.[4]

In 1984, a cytosine–phosphate–guanine (CpG) site in the *APOA1* promoter region was identified as a "hot spot" for polymorphisms using the MspI restriction enzyme.[5] Due to the high methylation rate of CpG sites in promoter regions, a cytosine (C) to thymine (T) transition, likely caused by the spontaneous deamination of 5-methylcytosine, resulted in the SNP rs670 (C>T) at position 75 upstream of the gene start region.[5] Over time, the exact position of this polymorphism has been described using various denominations. Initially reported as a guanine (G) to adenine (A) substitution in the DNA template strand at positions −78 (−78G > A), −76 (−76G > A), and −75 (−75G > A),[6-8] it was later defined as a −75G > A substitution on the template strand and rs670 (C>T) on the coding strand.[9]

Structural and expression changes in Apo A-I caused by genetic polymorphisms have been linked to lipid metabolism disorders.[2] The rs670 SNP was initially associated with altered Apo A-I expression and HDL cholesterol (HDL-c) levels. Over time, its associations with various metabolic disorders, cardiovascular diseases, neurodegenerative diseases, and cancers have been studied.[6,7,10-13] Given the importance of understanding how SNPs interact with environmental factors and their potential impact on population health, this review aimed to compile and describe key findings related to the rs670 SNP, with special emphasis on its potential role in regulating gene expression within the *APO* cluster on chromosome 11.

2. Methods

This integrative review comprehensively analyzed studies on the rs670 polymorphism of *APOA1* available up to July 2024. The databases used were as follows: database of single-nucleotide polymorphisms (dbSNP; www.ncbi.nlm.nih.gov/snp/), LitVar2 (www.ncbi.nlm.nih.gov/research/litvar2/),[14] Scopus (www.scopus.com/), and ScienceDirect (www.sciencedirect.com/). Searches in the Scopus and ScienceDirect databases were performed using the following search string: ["rs670" OR "−75GA" AND "APOA-I" OR "APOA1"]. The dbSNP and LitVar2 databases, which index PubMed articles, provided a curated list of articles related to the rs670 polymorphism.

The inclusion criteria applied to the searches were as follows: (1) full-text articles to allow for critical analysis and (2) articles including either minor allele frequency (MAF) or a contingency table with genotype distributions. Review articles, meta-analyses, genome-wide association studies (GWASs), and duplicate articles were excluded. Eligible studies were selected regardless of their publication language or year.

3. Results

Of the 162 articles retrieved during the searches, 21 were not available in full text, 36 were review articles and/or meta-analyses, 12 were GWAS, and 24 did not report MAF or genotype distributions. After applying the inclusion criteria, 69 articles remained, and following the removal of duplicates, 28 articles were selected for data collection. The extracted data included study type, year of publication, research location, population sample size, allelic and genotypic frequencies, and conclusions regarding the rs670 polymorphism. A flowchart illustrating the selection process is provided in Figure 1, whereas the key findings are summarized in Table 1.

The 28 articles selected for data collection were published between 2009 and 2023, and they comprised 14 (50.0%) case–control studies, 10 (35.72%) cross-sectional studies, 2 (7.14%) cohort studies, and 2 (7.14%) clinical trials. Geographically, the studies were distributed across Asia (15 studies; 53.57%), America (8 studies; 28.57%), Europe (4 studies; 14.29%), and Africa (1 study; 3.57%), as illustrated in Figure 2.

Regarding the clinical associations of the rs670 polymorphism, 14 studies (50.0%) focused on lipid metabolism disorders. Six studies found no association between the polymorphism and metabolic syndromes, including obesity, dyslipidemia, hyperglycemia, and hypertension. However, eight studies reported significant associations with metabolic syndromes (odds ratio

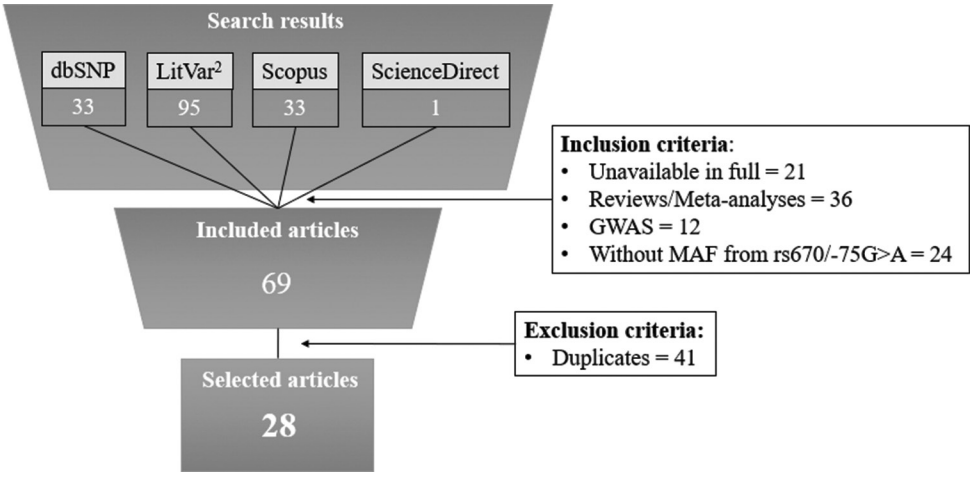

Figure 1. Flowchart of article searches and selection
Abbreviations: dbSNP: Database of single-nucleotide polymorphisms; GWAS: Genome-wide association study; MAF: Minor allele frequency.

Figure 2. Geographical distribution of articles. Adapted from Wikimedia Commons (https://commons.wikimedia.org/).

[OR] = 1.31; $P < 0.001$) and lipid parameters such as total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), VLDL-c, HDL-c (OR = 1.34; $P = 0.026$), triglycerides (TG), high-sensitivity C-reactive protein (hsCRP), insulin, and homeostasis model assessment-estimated insulin resistance (HOMA-IR). In addition, three studies (10.71%) found an association between the polymorphic allele and an increased risk of ischemic events, such as stroke (OR = 2.28; $P < 0.001$) and acute myocardial infarction (relative risk [RR] = 2.40; $P = 0.034$; OR = 1.77; $P = 0.042$).

Two studies (7.14%) explored the pharmacogenetic aspects of the rs670 polymorphism. They found that the homozygous polymorphic genotype was associated with improved responses to statins but not to fenofibrate. Three studies (10.71%) assessed gene-diet interactions. In diets high in saturated fat, the polymorphic allele was associated with elevated TC levels. Conversely, in low-calorie diets rich in polyunsaturated fats, the allele correlated with increased HDL-c levels and reduced LDL-c, insulin, and HOMA-IR levels.

The remaining studies investigated the association of the rs670 polymorphism with other conditions. No significant associations were found with neurological diseases, such as schizophrenia and Alzheimer's, pancreatitis, or benign breast cancer. However, in malignant breast cancer, the homozygous polymorphic genotype was linked to higher risks of recurrence (RR = 3.12; $P = 0.012$) and mortality (RR = 4.36; $P = 0.006$).

Table 1. Description of the selected articles in chronological order

Authors	Study type	Year	Country	*n*	MAF	Relative risk	Odds ratio	Conclusions
Liu *et al.*[50]	Cross-sectional	2009	USA	861	16%	-	-	No association was found between the polymorphism and changes in lipid metabolism in response to fenofibrate treatment
Block *et al.*[13]	Cohort	2009	USA	129	21%	2.40 (1.07 – 5.40); $P = 0.034$	-	The polymorphic allele was associated with a higher risk of cardiovascular events in black post-myocardial infarction patients
Yang *et al.*[11]	Case–control	2010	South Korea	185	24%	-	1.32 (0.87 – 1.99); $P = 0.191$	The polymorphic allele was not associated with the risk of schizophrenia
Smach *et al.*[12]	Case–control	2011	Tunisia	173	22.5%	-	1.31 (0.82 – 2.06); $P = 0.258$	The polymorphic allele was not associated with the risk of Alzheimer
Mattei *et al.*[51]	Cross-sectional	2011	USA	821	15%	-	-	The polymorphic allele in individuals who consumed<31% fat was associated with greater abdominal circumference
Hsu *et al.*[52]	Case–control	2013	China	262	27%	-	0.94 (0.61 – 1.44); $P = 0.774$	The polymorphic allele was not associated with the risk of obesity
Al-Bustan *et al.*[53]	Cross-sectional	2013	Kuwait	549	19%	-	-	The polymorphic allele was associated with high total cholesterol and LDL-c levels
Hsu *et al.*[48]	Cohort	2013	China	223	35%	3.12 (1.29 – 7.56); $P = 0.012$ and 4.36 (1.52 – 12.47); $P = 0.006$	-	The polymorphic homozygote genotype was associated with a greater risk of recurrence and mortality in patients with breast cancer
Rudkowska *et al.*[54]	Cross-sectional	2013	Canada	553	17%	-	-	The polymorphic allele associated with the amount of saturated fat predicted high total cholesterol levels
Song *et al.*[55]	Cross-sectional	2014	China	180	27%	-	-	The polymorphic allele was associated with a higher level of systolic blood pressure and glycemia
Hosseini-Esfahani *et al.*[56]	Case–control	2015	Iran	414	29%	-	1.11 (0.85 – 1.46); $P = 0.443$	The polymorphic allele was not associated with the risk of metabolic syndromes
Włodarczyk *et al.*[57]	Cross-sectional	2016	Poland	621	22%	-	-	The polymorphic allele was not associated with lipid metabolism parameters
Wu *et al.*[10]	Case–control	2016	China	3,340	26%	-	1.31 (1.14 – 1.50); $P < 0.001$	The polymorphic allele was associated with a higher risk of metabolic syndromes
Feng *et al.*[58]	Case–control	2016	China	340	14%	-	1.17 (0.84 – 1.64); $P = 0.341$	The polymorphic allele was not associated with the risk of dyslipidemia

(Cont'd...)

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

Authors	Study type	Year	Country	*n*	MAF	Relative risk	Odds ratio	Conclusions
Vorobyeva *et al.*[59]	Cross-sectional	2017	Russia	58	47%	-	-	The polymorphic homozygote genotype was associated with the best response to statins
Wang *et al.*[60]	Case–control	2017	China	424	17%	-	1.34 (1.03 – 1.74); $P = 0.026$	The polymorphic allele was associated with a higher risk of low HDL-c
Wang *et al.*[61]	Case–control	2017	China	812	28%	-	2.28 (1.52 – 3.44); $P < 0.001$	The polymorphic homozygote genotype was associated with a higher risk of ischemic stroke
Hosseini-Esfahani *et al.*[62]	Case–control	2017	Iran	414	29%	-	1.11 (0.85 – 1.46); $P = 0.443$	The polymorphic allele was not associated with the risk of metabolic syndromes
Kovtun and Ustyuzhanina[63]	Case–control	2018	Russia	69	24%	-	2.14 (0.95 – 4.79); $P = 0.066$	The polymorphic allele was not associated with the risk of obesity and hypertension
Luis *et al.*[64]	Cross-sectional	2018	Spain	63	15%	-	-	The polymorphic allele was associated with higher levels of HDL-c and lower levels of insulin and HOMA-IR
Wang *et al.*[65]	Case–control	2018	China	1,433	16%	-	1.34 (1.06 – 1.69); $P = 0.014$	The polymorphic allele was associated with a higher risk of dyslipidemia
Luis *et al.*[47]	Clinical trial	2019	Spain	360	17%	-	-	The polymorphic allele ligated with the low-calorie diet with high levels of polyunsaturated fatty acids was associated with higher levels of HDL-c and lower levels of LDL-c, insulin, and HOMA-IR
Ściskalska and Milnerowicz[66]	Case–control	2022	Poland	44	37%	-	1.54 (0.68 – 3.49); $P = 0.304$	The polymorphic allele was not associated with the risk of pancreatitis
Supajaree *et al.*[67]	Cross-sectional	2022	Thailand	355	28%	-	-	The polymorphic allele was associated with higher levels of HDL-c and lower levels of hsCRP
Domínguez-Díaz *et al.*[68]	Case–control	2022	Mexico	19	31%	-	2.03 (0.69 – 5.92); $P = 0.196$	The polymorphic allele was not associated with the risk of benign breast disease
Casillas *et al.*[69]	Case–control	2022	Mexico	130	31%	-	1.77 (1.02 – 3.08); $P = 0.042$	The polymorphic allele was associated with a higher risk of myocardial infarction
Pérez-Beltrán *et al.*[70]	Clinical trial	2023	Mexico	101	27%	-	-	The polymorphic allele predicted lower levels of triglycerides and VLDL-c
Krishnamurthy *et al.*[71]	Cross-sectional	2023	USA	184	19%	-	-	The polymorphic allele was associated with reduced myeloperoxidase activity and NT-pro-BNP levels

Abbreviations: HDL-c: High-density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; hsCRP: High-sensitivity C-reactive protein; LDL-c: Low-density lipoprotein cholesterol; MAF: Minor allele frequency; NT-pro-BNP: N-terminal fragment brain natriuretic peptides; USA: United States of America; VLDL-c: Very low-density lipoprotein cholesterol; *P*-value refers to a relative risk test or odds ratio for the presence of the polymorphic allele. Statistical significance $P < 0.05$.

The frequency of the minor allele varied across continents. In Asia, the frequency ranged from 14% to 47% with an average of 26%. In America, it ranged from 15% to 31% with an average of 22.125%, while in Europe, it ranged from 15% to 37% with an average of 22.75%. In Africa, only one study reported a frequency of 22.5%. Although minor allele frequencies are influenced by sample size, it is evident that the rs670 SNP is prevalent in the global population, with an average frequency exceeding 20%.

4. Discussion

HDL is involved in reverse cholesterol transport and plays a critical role in removing excess cholesterol from peripheral tissues, redirecting it to the liver for excretion or recycling. The antiatherogenic role of HDL is achieved by refluxing cholesterol from macrophages through SR-B1 receptors in the subendothelial layer, preventing their differentiation into foam cells initiating atherosclerotic plaque formation.[15] In addition, HDL exhibits antioxidant and anti-inflammatory activities mediated by paraoxonase-1 and Apo A-I. These mechanisms enable HDL to reduce cholesterol oxidation and regulate macrophage activity, thereby mitigating oxidative stress and vascular inflammation.[16]

Apo A-I and its mimetic peptides exert anti-inflammatory and antiatherogenic effects by lowering adhesion molecule expression, suppressing macrophage proptosis, and inhibiting monocyte chemotaxis.[17,18] The mechanisms of Apo A-I include cholesterol efflux through SR-B1 receptors and ATP-binding cassette subfamily A member 1 (ABCA1), which inhibit macrophage activation and reduce proinflammatory cytokine expression through Janus kinase 2/signal transducer and activator of transcription 3 signaling.[19,20] Furthermore, Apo A-I disrupts membrane lipid rafts expressing Toll-like 4 receptors, thereby decreasing the activation of the phosphatidyl inositol 3-kinase/PI3K-activated serine-threonine kinase (PI3K/Akt) signaling pathway in macrophages.[17,21]

Structural changes in Apo A-I are notably linked to a loss of anti-inflammatory activity, ranging from altered affinities for phospholipid structures to conformational changes induced by the non-enzymatic glycation of lysine residues.[22,23] Increased modified Apo A-I levels promote cardiovascular events, dyslipidemia, and diabetes.[17] *In vivo* studies show that Apo A-I and its mimetic peptides influence glycemic control by enhancing insulin secretion and sensitivity, facilitating insulin-independent glucose uptake, and suppressing gluconeogenesis.[24] Its mechanisms appear reliant on SR-B1 and ABCA1 receptors to activate the PI3K/Akt and AMPK (AMP protein kinase) pathways in skeletal muscle cells, leading to greater GLUT4 transporter translocation.[25,26]

SR-B1 receptors also contribute to HDL's antiatherogenic effects through Apo A-I as binding to these receptors results in cholesterol efflux from macrophages and reduction of LDL transcytosis by endothelial cells – key processes in atherosclerosis pathophysiology.[27] However, SR-B1 receptor overexpression in malignant cells correlates with increased tumor size and reduced survival. *In vitro* and *in vivo* studies suggest that the levels of HDL and its cancer cell uptake through SR-B1 receptors are associated with tumor progression.[28,29] This uptake is hypothesized to serve as an escape mechanism from oxidative stress-mediated immune responses, as it increases antioxidant and anti-inflammatory agents in the tumor microenvironment. Based on this mechanism, alternative antitumor therapies involve HDL-functionalized nanoparticles delivering small interfering RNA targeting SR-B1 receptors.[30]

Environmental factors, such as diet and drugs, influence Apo A-I synthesis and secretion primarily by modulating peroxisome proliferator-activated receptor alpha (PPARα). PPARα activates the transcription of genes involved in lipid and carbohydrate metabolism, including *APOA1*.[31] PPARα agonists have emerged as a therapeutic option for managing metabolic disorders such as dyslipidemia and diabetes, improving reverse cholesterol transport, reducing TG, and exerting antidiabetic effects.[32,33] *In vivo* studies suggest that PPARα activation enhances mitochondrial activity and provides protection against diabetic keratopathy and non-alcoholic fatty liver disease (NAFLD).[34,35]

Apo A-I, through hepatic PPARα activation, is protective against high-fat diet-induced NAFLD.[36] Dietary factors also regulate nuclear signaling via PPARα for Apo A-I synthesis. For instance, a low-choline diet is linked to methylation of CpG sites in the promoter region of *PPARA*, reducing *PPARA* and *APOA1* expression in pregnant mice.[37] Other dietary components, such as ellagic acid, n-3 polyunsaturated fatty acids, short-chain fatty acids, and tryptophan, activate PPARα and increase hepatic Apo A-I levels.[38-41] Gene-diet interactions have identified two SNPs in the 3' untranslated region of *PPARA* (rs6008259 G > A and rs3892755 C > T) that interact with n-3 and n-6 long-chain fatty acid-rich diets, reducing TC and LDL-c levels.[42]

Over 150 genetic variations have been identified in *APOA1*.[43] The first reported variation, Apo A-I Milano, involves an Arg173Cys substitution (rs28931573 G>A) that changes the codon CGC to UGC.[44,45] Another SNP, rs670, located in the promoter region of *APOA1*, alters a CpG site and may affect gene expression rates, thereby influencing Apo A-I synthesis.[5] The rs670 polymorphism is associated with increased Apo A-I and HDL-c levels, potentially producing HDL particles with higher protein content and enhanced reverse cholesterol transport, antioxidant, and

anti-inflammatory activities, as well as improved glycemic control.[6]

Beyond the promoter region of *APOA1*, rs670 SNP impacts the intronic region of the long non-coding RNA antisense to *APOA1* (APOA1-AS). APOA1-AS negatively regulates *APOA1* expression in tissues like the testicle, ovary, cervix, and thyroid. *In vitro* studies have shown that APOA1-AS modulates histone methylation patterns, regulating active and inactive states of gene expression. This action not only represses *APOA1* but also neighboring *APOC3* and *APOA4*. Silencing APOA1-AS with small antisense oligonucleotides increased *APOA1* expression, confirming its regulatory role in the *APO* cluster on chromosome 11.[46]

Research into the effect of the rs670 SNP on the *APO* cluster offers insights into personalized medicine, including cardiovascular risk variations, insulin resistance, and dietary interventions for lipid metabolism. It may also inform the prognosis of cancers overexpressing SR-B1 receptors.[47,48] However, existing studies are limited by sample size and ethnic diversity, hindering the generalization of findings. Thus, further research must account for factors like ethnicity, age, sex, nutrition, and lifestyle to understand the role of the rs670 SNP in metabolic syndrome development.[49]

5. Conclusion

The rs670 SNP is present in a significant proportion of the global population and has been studied extensively, particularly in Asian countries. Positioned in the promoter region of *APOA1*, this polymorphism has the potential to alter gene expression, affecting lipid and carbohydrate metabolism, oxidative stress, immune response, and tumor development. This review underscores the need to investigate the actual impact of this polymorphism on gene expression and its relationship with metabolic syndrome, cardiovascular disease, and cancer as well as examines environmental factors that may influence epigenetic profiles.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

This integrative review was carried out to comprehensively analyze the available studies on the rs670 polymorphism of the *APOA1* gene up to July 2024. The databases used were dbSNP–database of single-nucleotide polymorphisms (www.ncbi.nlm.nih.gov/snp/), LitVar2 (www.ncbi.nlm.nih.gov/research/litvar2/),[14] Scopus (www.scopus.com/), and ScienceDirect (www.sciencedirect.com/). The searches were carried out in the Scopus and ScienceDirect databases using the following search string ["rs670" OR "−75GA" AND "APOA-I" OR "APOA1"], while the dbSNP and LitVar2 databases, which share PubMed articles, provide a list of articles deposited on the rs670 polymorphism.

The inclusion criteria applied to the searches were as follows: (1) the article was available in full to enable a critical analysis; (2) it was not a review article and/or meta-analysis to avoid duplicate articles; (3) it was not a GWAS, as it does not provide allele and genotype frequencies; and (4) it had the MAF or a contingency table with the genotype distributions. Duplicate articles were then excluded. The articles selected could be in any language and year of publication.

References

1. Feingold KR. *Introduction to Lipids and Lipoproteins*. *Endotext*. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305896 [Last accessed on 2024 Jul 08].
2. Zanoni P, Von Eckardstein A. Inborn errors of apolipoprotein A-I metabolism: Implications for disease, research and development. *Curr Opin Lipidol*. 2020;31(2):62-70.

   doi: 10.1097/MOL.0000000000000667
3. Zannis VI, Karathanasis SK, Keutmann HT, Goldberger G,

Breslow JL. Intracellular and extracellular processing of human apolipoprotein A-I: Secreted apolipoprotein A-I isoprotein 2 is a propeptide. *Proc Natl Acad Sci U S A*. 1983;80(9):2574-2578.

doi: 10.1073/PNAS.80.9.2574

4. Baldanzi G, Park B, Lee YJ, *et al*. APOA1/C3/A4/A5 gene cluster at 11q23.3 and lipid metabolism disorders: From epigenetic mechanisms to clinical practices. *Biomedicines*. 2024;12(6):1224.

doi: 10.3390/BIOMEDICINES12061224

5. Seilhamer JJ, Protter AA, Frossard P, Levy-Wilson B. Isolation and DNA sequence of full-length cDNA and of the entire gene for human apolipoprotein AI-discovery of a new genetic polymorphism in the apo AI Gene. *DNA*. 1984;3(4):309-317.

doi: 10.1089/DNA.1.1984.3.309

6. Pagani F, Sidoli A, Giudici GA, Barenghi L, Vergani C, Baralle FE. Human apolipoprotein A-I gene promoter polymorphism: Association with hyperalphalipoproteinemia. *J Lipid Res*. 1990;31(8):1371-1377.

doi: 10.1016/s0022-2275(20)42608-2

7. Smith JD, Brinton EA, Breslow JL. Polymorphism in the human apolipoprotein A-I gene promoter region. Association of the minor allele with decreased production rate *in vivo* and promoter activity *in vitro*. *J Clin Invest*. 1992;89(6):1796-1800.

doi: 10.1172/JCI115783

8. Jeenah M, Kessling A, Miller N, Humphries S. G to A substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI and high density lipoprotein cholesterol concentrations. *Mol Biol Med*. 1990;7(3):233-241.

9. *RefSNP Report - dbSNP - NCBI - rs670*. Available from: https://www.ncbi.nlm.nih.gov/snp/rs670 [Last accessed on 2024 Jul 02].

10. Wu Y, Yu Y, Zhao T, *et al*. Interactions of environmental factors and APOA1-APOC3-APOA4-APOA5 gene cluster gene polymorphisms with metabolic syndrome. *PLoS One*. 2016;11(1):e0147946.

doi: 10.1371/JOURNAL.PONE.0147946

11. Yang JM, Park JK, Kim SS, Chun W, Lee HJ. Association between apolipoprotein A1 (APOA1) single-nucleotide polymorphisms and schizophrenia in a Korean population. *Clin Psychopharmacol Neurosci*. 2010;8(1):45-48.

12. Smach MA, Edziri H, Charfeddine B, *et al*. Polymorphism in apoA1 influences high-density lipoprotein cholesterol levels but is not a major risk factor of Alzheimer's disease. *Dement Geriatr Cogn Dis Extra*. 2011;1(1):249-257.

doi: 10.1159/000329910

13. Block R, Corsetti J, Goldenberg I, *et al*. The common apolipoprotein A-1 polymorphism −75A>G is associated with ethnic differences in recurrent coronary events after recovery from an acute myocardial infarction. *Heart Int*. 2009;4(1):hi.2009.e8.

doi: 10.4081/HI.2009.E8

14. Allot A, Wei CH, Phan L, *et al*. Tracking genetic variants in the biomedical literature using LitVar 2.0. *Nat Genet*. 2023;55(6):901-903.

doi: 10.1038/s41588-023-01414-x

15. Ouimet M, Barrett TJ, Fisher EA. HDL and reverse cholesterol transport: Basic mechanisms and their roles in vascular health and disease. *Circ Res*. 2019;124(10):1505.

doi: 10.1161/CIRCRESAHA.119.312617

16. Ruiz-Ramie JJ, Barber JL, Sarzynski MA. Effects of exercise on HDL functionality. *Curr Opin Lipidol*. 2019;30(1):16.

doi: 10.1097/MOL.0000000000000568

17. Bhale AS, Venkataraman K. Leveraging knowledge of HDLs major protein ApoA1: Structure, function, mutations, and potential therapeutics. *Biomed Pharmacother*. 2022;154:113634.

doi: 10.1016/J.BIOPHA.2022.113634

18. Kluck GEG, Qian AS, Sakarya EH, Quach H, Deng YD, Trigatti BL. Apolipoprotein A1 protects against necrotic core development in atherosclerotic plaques: PDZK1-dependent high-density lipoprotein suppression of necroptosis in macrophages. *Arterioscler Thromb Vasc Biol*. 2023;43(1):45-63.

doi: 10.1161/ATVBAHA.122.318062

19. Tang C, Houston BA, Storey C, Leboeuf RC. Both STAT3 activation and cholesterol efflux contribute to the anti-inflammatory effect of apoA-I/ABCA1 interaction in macrophages. *J Lipid Res*. 2016;57(5):848-857.

doi: 10.1194/JLR.M065797

20. Wang JL, Gong D, Hu XY, *et al*. ApoA-1 mimetic peptide ELK-2A2K2E decreases inflammatory factor levels through the ABCA1-JAK2-STAT3-TTP axis in THP-1-derived macrophages. *J Cardiovasc Pharmacol*. 2018;72(1):60-67.

doi: 10.1097/FJC.0000000000000594

21. Iqbal AJ, Barrett TJ, Taylor L, *et al*. Acute exposure to apolipoprotein a1 inhibits macrophage chemotaxis *in vitro* and monocyte recruitment *in vivo*. *Elife*. 2016;5:e15190.

doi: 10.7554/ELIFE.15190

22. Liu D, Ji L, Zhao M, *et al*. Lysine glycation of apolipoprotein A-I impairs its anti-inflammatory function in type 2 diabetes mellitus. *J Mol Cell Cardiol*. 2018;122:47-57.

doi: 10.1016/J.YJMCC.2018.08.001

23. Anantharamaiah GM, Mishra VK, Garber DW, *et al*. Structural requirements for antioxidative and anti-

inflammatory properties of apolipoprotein A-I mimetic peptides. *J Lipid Res*. 2007;48(9):1915-1923.

doi: 10.1194/JLR.R700010-JLR200

24. King TW, Cochran BJ, Rye KA. ApoA-I and diabetes. *Arterioscler Thromb Vasc Biol*. 2023;43(8):1362-1368.

doi: 10.1161/ATVBAHA.123.318267

25. Tang S, Tabet F, Cochran BJ, *et al.* Apolipoprotein A-I enhances insulin-dependent and insulin-independent glucose uptake by skeletal muscle. *Sci Rep.* 2019;9(1):1-9.

doi: 10.1038/s41598-018-38014-3

26. Cochran BJ, Bisoendial RJ, Hou L, *et al*. Apolipoprotein A-I increases insulin secretion and production from pancreatic β-cells via a G-protein-cAMPPKA-FoxO1-dependent mechanism. *Arterioscler Thromb Vasc Biol*. 2014;34(10):2261-2267.

doi: 10.1161/ATVBAHA.114.304131/-/DC1

27. Fung KYY, Ho TWW, Xu Z, *et al*. Apolipoprotein A1 and high-density lipoprotein limit low-density lipoprotein transcytosis by binding SR-B1. *J Lipid Res*. 2024;65(4):100530.

doi: 10.1016/J.JLR.2024.100530

28. Alicia Traughber C, Opoku E, Brubaker G, *et al*. Uptake of high-density lipoprotein by scavenger receptor class B type 1 is associated with prostate cancer proliferation and tumor progression in mice. *J Biol Chem*. 2020;295(24):8252-8261.

doi: 10.1074/JBC.RA120.013694

29. Oberle R, Kührer K, Österreicher T, *et al.* The HDL particle composition determines its antitumor activity in pancreatic cancer. *Life Sci Alliance*. 2022;5(9):e202101317.

doi: 10.26508/LSA.202101317

30. Masimov R, Büyükköroğlu G. HDL-chitosan nanoparticles for siRNA delivery as an SR-B1 receptor targeted system. *Comb Chem High Throughput Screen*. 2023;26(14):2541-2553.

doi: 10.2174/1386207326666230406124524

31. Shah A, Rader DJ, Millar JS. The effect of PPAR-α agonism on apolipoprotein metabolism in humans. *Atherosclerosis*. 2010;210(1):35-40.

doi: 10.1016/j.atherosclerosis.2009.11.010

32. Khera AV, Millar JS, Ruotolo G, Wang MD, Rader DJ. Potent peroxisome proliferator-activated receptor-α agonist treatment increases cholesterol efflux capacity in humans with the metabolic syndrome. *Eur Heart J*. 2015;36(43):3020-3022.

doi: 10.1093/EURHEARTJ/EHV291

33. Kim MK, Chae YN, Son MH, *et al.* PAR-5359, a well-balanced PPARα/γ dual agonist, exhibits equivalent antidiabetic and hypolipidemic activities *in vitro* and *in vivo*. *Eur J Pharmacol*. 2008;595(1-3):119-125.

doi: 10.1016/J.EJPHAR.2008.07.066

34. Liang W, Huang L, Whelchel A, *et al*. Peroxisome proliferator-activated receptor-α (PPARα) regulates wound healing and mitochondrial metabolism in the cornea. *Proc Natl Acad Sci U S A*. 2023;120(13):e2217576120.

doi: 10.1073/PNAS.2217576120

35. Wang J, Zhang Y, Zhuo Q, *et al*. TET1 promotes fatty acid oxidation and inhibits NAFLD progression by hydroxymethylation of PPARα promoter. *Nutr Metab (Lond)*. 2020;17(1):1-11.

doi: 10.1186/S12986-020-00466-8

36. Chen C, Li H, Song J, *et al*. Role of apolipoprotein A1 in PPAR signaling pathway for nonalcoholic fatty liver disease. *PPAR Res*. 2022;2022(1):4709300.

doi: 10.1155/2022/4709300

37. Mikael LG, Pancer J, Wu Q, Rozen R. Disturbed one-carbon metabolism causing adverse reproductive outcomes in mice is associated with altered expression of apolipoprotein AI and inflammatory mediators PPARα, interferon-γ, and interleukin-10., *J Nutr*. 2012;142(3):411-418.

doi: 10.3945/JN.111.151753

38. Yoshimura Y, Nishii S, Zaima N, Moriyama T, Kawamura Y. Ellagic acid improves hepatic steatosis and serum lipid composition through reduction of serum resistin levels and transcriptional activation of hepatic ppara in obese, diabetic KK-Ay mice. *Biochem Biophys Res Commun*. 2013;434(3):486-491.

doi: 10.1016/J.BBRC.2013.03.100

39. Xie X, Zhang T, Zhao S, *et al.* Effects of n-3 polyunsaturated fatty acids high fat diet intervention on the synthesis of hepatic high-density lipoprotein cholesterol in obesity-insulin resistance rats. *Lipids Health Dis*. 2016;15(1):1-7.

doi: 10.1186/S12944-016-0250-3/TABLES/3

40. Tayyeb JZ, Popeijus HE, Mensink RP, Konings MCJM, Mokhtar FBA, Plat J. Short-chain fatty acids (except hexanoic acid) lower NF-kB transactivation, which rescues inflammation-induced decreased apolipoprotein A-I transcription in HepG2 cells. *Int J Mol Sci*. 2020;21(14):5088.

doi: 10.3390/IJMS21145088

41. Tayyeb JZ, Popeijus HE, van de Sanden J, Zwaan W, Mensink RP, Plat J. Effects of individual amino acids on PPARα transactivation, mTORC1 activation, ApoA-I transcription and pro-ApoA-I secretion. *Int J Mol Sci*. 2022;23(11):6071.

doi: 10.3390/IJMS23116071/S1

42. Volcik KA, Nettleton JA, Ballantyne CM, Boerwinkle E. Peroxisome proliferator-activated receptor α genetic variation interacts with n-6 and long-chain n-3 fatty acid intake to affect total cholesterol and LDL-cholesterol concentrations in the Atherosclerosis Risk in Communities Study. *Am J Clin Nutr*. 2008;87(6):1926-1931.

doi: 10.1093/AJCN/87.6.1926

43. *All Variants in the APOA1 Gene - Global Variome Shared LOVD*. Available from: https://databases.lovd.nl/shared/variants/APOA1 [Last accessed on 2024 Jun 26].

44. *RefSNP Report - dbSNP - NCBI - rs28931573*. Available from: https://www.ncbi.nlm.nih.gov/snp/rs28931573#clinical_significance [Last accessed on 2024 Jun 26].

45. Weisgraber KH, Rall SC, Bersot TP. Apolipoprotein A-IMilano. Detection of normal A-I in affected subjects and evidence for a cysteine for arginine substitution in the variant A-I. *J Biol Chem*. 1983;258(4):2508-2513.

doi: 10.1016/S0021-9258(18)32955-7

46. Halley P, Kadakkuzha BM, Faghihi MA, *et al.* Regulation of the apolipoprotein gene cluster by a long noncoding RNA. *Cell Rep*. 2014;6(1):222-230.

doi: 10.1016/J.CELREP.2013.12.015

47. Luis DA de, Izaola O, Primo D, *et al.* Influencia de la variante rs670 del gen APOA1 en la respuesta HDL sérica a una dieta hipocalórica enriquecida con grasas poliinsaturadas frente a una enriquecida con grasas monoinsaturadas. *Nutr Hosp*. 2019;36(6):1288-1295.

doi: 10.20960/NH.02390

48. Hsu MC, Lee KT, Hsiao WC, *et al*. The dyslipidemia-associated SNP on the APOA1/C3/A5 gene cluster predicts post-surgery poor outcome in Taiwanese breast cancer patients: A 10-year follow-up study. *BMC Cancer*. 2013;13(1):1-11.

doi: 10.1186/1471-2407-13-330/TABLES/7

49. Kolovou GD, Anagnostopoulou KK, Salpea KD, Mikhailidis DP. The prevalence of metabolic syndrome in various populations. *Am J Med Sci*. 2007;333(6):362-371.

doi: 10.1097/MAJ.0B013E318065C3A1

50. Liu Y, Ordovas JM, Gao G, *et al.* Pharmacogenetic association of the APOA1/C3/A4/A5 gene cluster and lipid responses to fenofibrate: The genetics of lipid-lowering drugs and diet network study. *Pharmacogenet Genomics*. 2009;19(2):161-169.

doi: 10.1097/FPC.0B013E32831E030E

51. Mattei J, Demissie S, Tucker KL, Ordovas JM. The APOA1/C3/A4/A5 cluster and markers of allostatic load in the Boston Puerto Rican Health Study. *Nutr Metab Cardiovasc Dis*. 2011;21(11):862-870.

doi: 10.1016/J.NUMECD.2010.02.024

52. Hsu MC, Chang CS, Lee KT, *et al*. Central obesity in males affected by a dyslipidemia-associated genetic polymorphism on APOA1/C3/A4/A5 gene cluster. *Nutr Diabetes*. 2013;3(3):e61.

doi: 10.1038/nutd.2013.2

53. Al-Bustan SA, Al-Serri AE, Annice BG, Alnaqeeb MA, Ebrahim GA. Re-sequencing of the APOAI promoter region and the genetic association of the -75G > A polymorphism with increased cholesterol and low density lipoprotein levels among a sample of the Kuwaiti population. *BMC Med Genet*. 2013;14(1):1-11.

doi: 10.1186/1471-2350-14-90/TABLES/5

54. Rudkowska I, Dewailly E, Hegele RA, *et al.* Gene-diet interactions on plasma lipid levels in the Inuit population. *Br J Nutr*. 2013;109(5):953-961.

doi: 10.1017/S0007114512002231

55. Song YY, Gong RR, Zhang Z, *et al.* Effects of apolipoprotein A1 Gene rs670 and rs5069 polymorphisms on the plasma lipid profiles in healthy adolescents with different body mass index. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*. 2014;36(4):369-376.

doi: 10.3881/J.ISSN.1000-503X.2014.04.005

56. Hosseini-Esfahani F, Mirmiran P, Daneshpour MS, *et al*. Dietary patterns interact with APOA1/APOC3 polymorphisms to alter the risk of the metabolic syndrome: The Tehran Lipid and Glucose Study. *Br J Nutr*. 2015;113(4):644-653.

doi: 10.1017/S0007114514003687

57. Włodarczyk M, Wrzosek M, Nowicka G, Jabłonowska-Lietz B. Impact of variants in CETP and apo AI genes on serum HDL cholesterol levels in men and women from the Polish population. *Arch Med Sci*. 2016;12(6):1188-1198.

doi: 10.5114/AOMS.2016.60870

58. Feng DW, Ma RL, Guo H, *et al.* Association of APOA1 gene polymorphisms (rs670, rs5069, and rs2070665) with dyslipidemia in the Kazakhs of Xinjiang. *Genet Mol Res*. 2016;15(2):???.

doi: 10.4238/GMR.15028094

59. Vorobyeva MA, Azova MM, Gigani OO, Garmash IV, Villevalde SV, Kobalava ZD. Genetic predictors of intensive lipid-lowering therapy efficacy and its anti-inflammatory effects in very high cardiovascular risk patients. *Rational Pharmacother Cardiol*. 2017;13(4):525-531.

doi: 10.20996/1819-6446-2017-13-4-525-531

60. Wang X, He J, Guo H, *et al*. Interactions of six SNPs in APOA1 gene and types of obesity on low HDL-C disease in Xinjiang pastoral area of China. *Lipids Health Dis*. 2017;16(1):1-7.

doi: 10.1186/S12944-017-0581-8/TABLES/4

61. Wang Y, Liu F, Li L, Deng S, He Z. The association between apolipoprotein A1-C3-A5 gene cluster promoter polymorphisms and risk of ischemic stroke in the northern Chinese Han population. *J Int Med Res*. 2017;45(6):2042-2052.

doi: 10.1177/0300060517713517

62. Hosseini-Esfahani F, Mirmiran P, Daneshpour MS,

Mottaghi A, Azizi F. The effect of interactions of single nucleotide polymorphisms of APOA1/APOC3 with food group intakes on the risk of metabolic syndrome. *Avicenna J Med Biotechnol*. 2017;9(2):94.

63. Kovtun OP, Ustyuzhanina MA. Polymorphism of PPARG (P12A), APOA1 (G75A), and APOE (C112A and A158C) genes in children with obesity and arterial hypertension: A case-control study. *Curr Pediatr*. 2018;17(4):307-315.

doi: 10.15690/VSP.V17I4.1924

64. Luis DA, Pacheco D, Izaola O, Primo D, Aller R. The association of the rs670 variant of APOA1 gene with insulin resistance and lipid profile in morbid obese patients after a biliopancreatic diversion surgery. *Eur Rev Med Pharmacol Sci*. 2018;22(23):8472-8479.

doi: 10.26355/EURREV_201812_16547

65. Wang X, Guo H, Li Y, *et al.* Interactions among genes involved in reverse cholesterol transport and in the response to environmental factors in dyslipidemia in subjects from the Xinjiang rural area. *PLoS One*. 2018;13(5):e0196042.

doi: 10.1371/JOURNAL.PONE.0196042

66. Ściskalska M, Milnerowicz H. Importance of polymorphisms in the gene of paraoxonase-1 (SNP rs662) and apolipoprotein A-I (SNP rs670 and rs5069) in non-smoking and smoking healthy subjects and patients with acute pancreatitis. *Genes (Basel)*. 2022;13(11):1968.

doi: 10.3390/GENES13111968/S1

67. Supajaree P, Chanprasertyothin S, Shantavasinkul PC, Sritara P, Sirivarasai J. Association between ApoA1 Gene, plasma lipid profile, hsCRP level, and risk of arterial stiffness in thai elderly. *Adv Prev Med*. 2022;2022(1):4930033.

doi: 10.1155/2022/4930033

68. Domínguez-Díaz C, Morán-Moguel MC, Navarro-Hernandez RE, Romo-Vázquez R, Mendizabal-Ruiz AP. Association of SNP rs5069 in APOA1 with benign breast diseases in a Mexican population. *Genes (Basel)*. 2022;13(5):738.

doi: 10.3390/GENES13050738/S1

69. Casillas FA, Fernández DEM, Valle Y, *et al*. APOA1 (-75 G>A and 83 C>T) and APOB (2488 C>T) polymorphisms and their association with myocardial infarction, lipids and apolipoproteins in patients with type 2 diabetes mellitus. *Arch Med Sci*. 2022;18(6):1438-1445.

doi: 10.5114/AOMS/108674

70. Pérez-Beltrán YE, González-Becerra K, Rivera-Iñiguez I, *et al*. A nutrigenetic strategy for reducing blood lipids and low-grade inflammation in adults with obesity and overweight. *Nutrients*. 2023;15(20):4324.

doi: 10.3390/NU15204324/S1

71. Krishnamurthy HK, Balaguru UM, Pereira M, *et al*. Influence of genetic polymorphisms on serum biomarkers of cardiac health. *Medicine (Baltimore)*. 2023;102(23):E33953.

doi: 10.1097/MD.0000000000033953

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Gene & Protein in Disease

REVIEW ARTICLE

Sickle cell disease: A 75-year journey

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Abstract

Sickle cell disease (SCD) is a disorder characterized by the polymerization of hemoglobin chains in the deoxy-form, sickling of red blood cells, and hence vaso-occlusive crisis, multiple organ damage, and increased mortality due to an inherited defect in hemoglobin structure. SCD can also lead to a host of complications, which include acute chest syndrome, avascular necrosis, stroke, pulmonary hypertension, splenic sequestration, gallstones, deep vein thrombosis, pregnancy complications, and end-organ damage. Complications are of varying complexities and can be as grave as life-threatening. According to a report in 2005, the median life expectancy for male and female patients with SCD in the United States (US) was around 42 and 38 years, respectively. However, the survival rate of SCD patients in high-income countries has steadily improved. Treatment options that were mainly for symptomatic relief and led to better well-being of the patient, containment of complication recurrence, and decrease in mortality rates have evolved into curative treatment options such as stem cell transplantations and gene therapy. The present paper is a review of the disease, its complications and implications on the community, and a historical tracking of the evolution of treatment options up to modern-day gene therapy.

Keywords: Sickle cell disease; Treatments; Myeloablative transplantation; Gene therapy; Casgevy; Lentiviral

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1. Introduction

Sickle cell disease (SCD) is a disorder characterized by the polymerization of hemoglobin chains in the deoxy-form, sickling of red blood cells (RBCs), and hence vaso-occlusive crisis, multiple organ damage, and increased mortality due to an inherited (autosomal recessive) defect in hemoglobin structure. Normal adult hemoglobin (HbA) in erythrocytes and reticulocytes is a tetrameric protein composed of four globin subunits (two α-globin and two β-globin). In SCD, the hemoglobin (HbS) is defective with an

E6V resulting from a missense mutation on the beta chain gene on both chromosomes, resulting in sickle-shaped erythrocytes.[1-3] SCD can also lead to a host of complications, including acute chest syndrome (ACS), avascular necrosis, stroke, pulmonary hypertension, splenic sequestration, gallstones, deep vein thrombosis (DVT), pregnancy complications, and end-organ damage.[4,5] Complications are of varying complexities and can be as grave as life-threatening. Treatment options available were mainly used for symptomatic relief, better well-being of the patient, containment of complication recurrence as well as decrease in mortality rates.[4-6] These have evolved into curative treatment options such as stem cell transplantations and gene therapy. The present review provides an update about the disease, its complications, and implications on the community, as well as a historical tracking of the evolution of treatment options up to modern-day gene therapy.

2. Classification

The predominant type of SCD is the homozygous HbSS also called sickle cell anemia. The next most common type of SCD is HbSC, which is a coinheritance of HbS and HbC. Sickle beta thalassemia (HbS/βo or HbS/β+) results from coinheritance with beta-thalassemia and, depending on the genetic defect on the beta thalassemia component, is mild or as severe as HbSS.[7]

3. Epidemiology

Globally, millions of individuals are affected by SCD, and it is common among those whose ancestry is from the malaria-affected regions of the world, namely, Sub-Saharan Africa, South America, the Caribbean, Central America, Saudi Arabia, India, and Mediterranean countries such as Turkey, Greece, and Italy. In the US, where around 100,000 individuals are affected by SCD, 90% of the affected individuals are from the non-Hispanic or African American population (one in 365 births), and 3 – 9% are Hispanic (one in 16,300 births) or Latinos.[8]

The gene frequency is highest in Western Africa where the carrier frequency for HbS is 25 – 30% and where the prevalence of HbSC is also maximum.[7] Around one in 13 Black or African American births is a carrier.[8]

4. Pathophysiology

SCD is the most common monogenic disorder caused by point mutation worldwide,[9,10] with significant variation in disease manifestation due to several influencing factors, and the coinheritance of which can alter the severity of symptoms.[9-16] Although polymerization is identified as the root cause of the pathogenesis in SCD, this alone cannot entirely explain the pleiotropy of SCD, namely, how often a patient experiences vaso-occlusive crisis, severity of complications, rate of hemolysis, age of onset, to name a few. This is because of the variable expressivity of SCD even among genotypically similar patients and even among homozygous individuals, which has been attributed to modifier gene effect, social factors, and environmental factors. For instance, increasing levels of fetal hemoglobin, coinheritance with thalassemia, and certain other genetic factors are known to potentially alleviate complications, while on the other hand, inheritance of thrombophilic genetic factors may accentuate them because of the enhanced hypercoagulable state.[9-17] Figure 1 summarizes the sequence of events that highlight the pathophysiology of SCD. While polymerization results in the sickling of RBCs, the pathophysiology of complications also involves vascular endothelial dysfunction, resulting in a pro-inflammatory milieu and an inflammatory response. The inflammatory response activates signaling pathways in RBCs through activation of adhesion receptors. Hypoxia and stress are also known to modulate the RBC signaling pathways by increasing the formation of deoxy-Hb S.[13,18-23]

An intrinsic mechanism heightening the complications in SCD involves the scavenging of nitric oxide by the free hemoglobin released during hemolysis.[24,25] Sickle RBCs have lower activity of arginase-1, the enzyme necessary for nitric oxide synthesis, blocking *de novo* nitric oxide synthesis. Further, the cell-free plasma hemoglobin limits nitric oxide bioavailability[26,27] and also leads to the generation of reactive oxygen species (ROS).[28]

The role of polymerization-induced ion channel activation has been implicated in a vicious cycle of polymerization–ion channel activation–polymerization, eventually leading to hemolysis. Loss of potassium ions from the RBCs and cellular dehydration follow the activation of the K^+-Cl^- cotransport system and the Gardos channel (Ca^{2+}-dependent K^+ channel).[28] This is ensued by an increase in the concentration of erythrocytic hemoglobin that triggers more deoxygenated-Hb polymer formation. The resulting changes in sequence – denaturation of hemoglobin, accumulation of hemichromes on the inside of the cell membrane next to cytoskeletal proteins (*e.g.*, Band 3), loss of heme, and release of Fe^{3+} – create an oxidizing micromilieu. Subsequently, disruption of the membrane phospholipid asymmetry and clustering of anti-band 3 IgGs on cytoskeletal Band 3 protein due to negative charges on anionic phosphatidylserine exposed to the cell surface take place. These changes lead to erythrophagocytosis by macrophages and the production of microparticles.[29,30] The role of other factors, namely, coexisting hypercoagulability or concomitant inheritance of thrombophilic genetic variants, intervening environmental factors, infections,

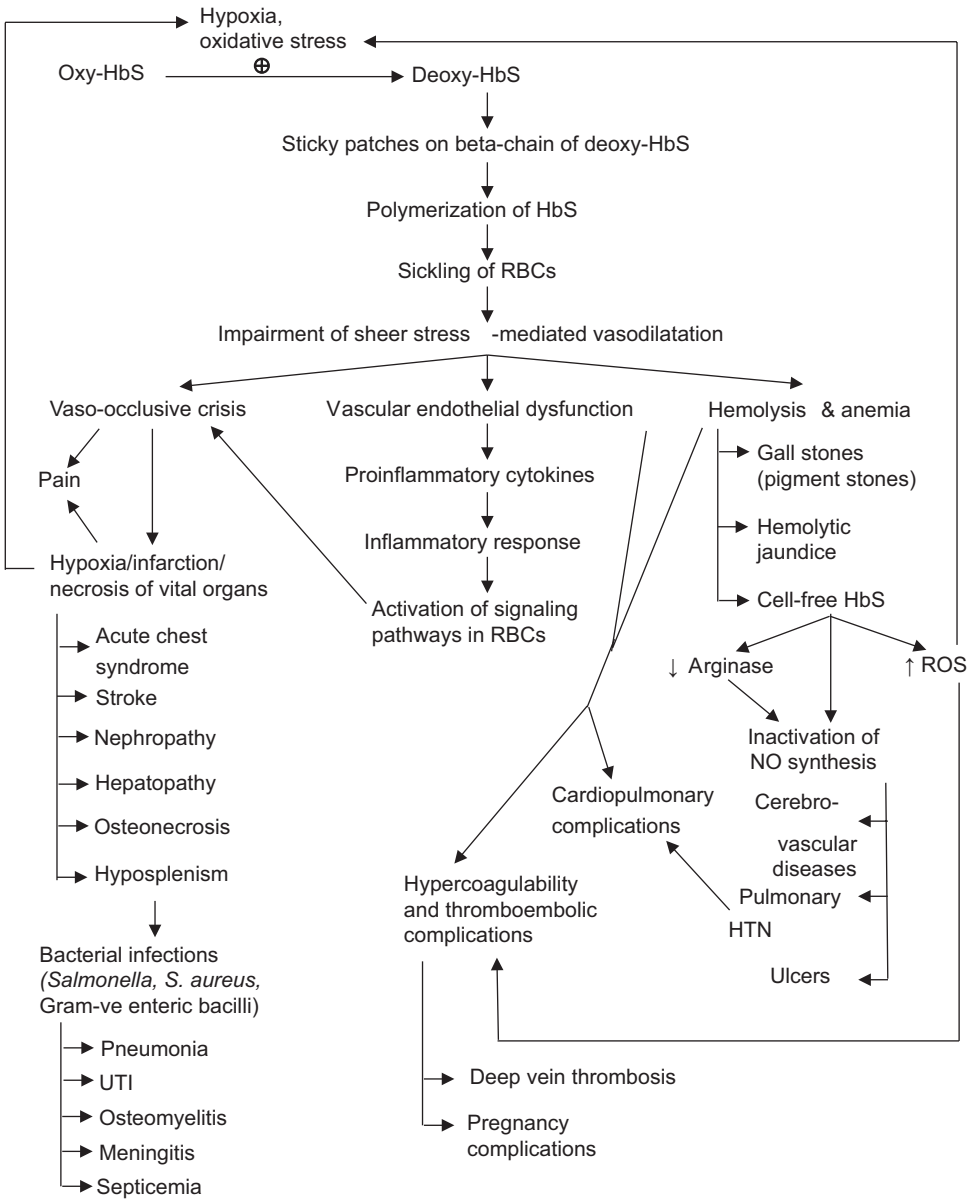

Figure 1. Pathophysiology in sickle cell disease
Abbreviations: Gram -ve: Gram negative; HTN: Hypertension; RBCs: Red blood cells; ROS: Reactive oxygen species; UTI: Urinary tract infection.

and behavioral and psychosocial factors, are implicated in the disease manifestation and complication.[31]

SCD is marked by increased oxidative stress. This is resulted primarily from the reduced bioavailability of nitric oxide, as already explained above. Second, the antioxidant defense mechanism consisting of both enzymatic and non-enzymatic antioxidants is defective in SCD. Third, the presence of cell-free HbS and heme due to hemolysis is known to increase ROS.[25,32] The resulting oxidative stress adds to the proinflammatory state and vasculopathy events. The role of the mitochondrial antioxidant enzyme superoxide dismutase (SOD) and its isoform (SOD2), as well as the variant of the isoform ($SOD2^{V16A}$), is implicated in mitochondrial function inhibition and vascular dysfunction in SCD.[33] The presence of the SOD isoform variant mentioned above is attributed to reduced pulmonary and cardiovascular functions, increased anemia and hemolysis, and decreased ability toward physical activities and exercise. Cardiopulmonary complications may be attributed to the fact that chronic anemia decreases the oxygen-carrying capacity of blood, causing a compensatory increase in stroke volume and hence cardiac output. This along

with endothelial activation and vaso-occlusion poses a strain on the cardiovascular system. In addition, the high cardiac output-induced increase in pulmonary pressure, the sickling-induced hypoxemia, and the chronic volume overload intensify left ventricular failure as well as pulmonary venous hypertension, resulting in pulmonary hypertrophy. The role of free heme released due to hemolysis has been implicated in pulmonary arterial vasculopathy and the role of chronic stress (due to anemia, endothelial dysfunction, and chronic inflammation) has been implicated in pathologic remodeling of the heart in SCD patients, characterized by chamber structure changes, diffuse fibrosis, and diastolic dysfunction. All of the aforementioned pathophysiological changes contribute to the onset of cardiopulmonary dysfunction and poor outcomes in SCD patients.[21,23,27,30,33]

Hypercoagulability is one of the cardinal features of SCD. This is attributed to multiple factors, with HbS being the major one. The HbS induces hemolysis and the prevalent oxidative stress activates hemostasis, coagulation cascade, and fibrinolysis and depletes coagulation inhibitors. A chronic activation of coagulation takes place in patients with SCD as against normal individuals with HbA, due to increased production and hence increased plasma levels of prothrombin fragment 1.2, fibrinopeptide A, thrombin-antithrombin complexes, D-dimers and plasmin-antiplasmin complexes.[34-41] Among these, it is the D-dimer that is reported to show a significant increase during pain crises as compared to the time without acute events in the previous year.[42]

5. Clinical features

The most common manifestations of SCD (homozygous) as anemia, vaso-occlusion, and hemolysis, which can be exacerbated by oxidative stress, hypercoagulable state, inflammatory response, and defective arginine metabolism. Infants with homozygous mutation are asymptomatic in the first few months of life because of the effect of HbF but once the disease is expressed, they will be fraught with the above-mentioned complications for life and worsening with age. In addition to the vaso-occlusive crisis, hemolysis, and anemia, SCD includes a host of complications such as ACS, avascular necrosis, splenic sequestration, stroke, pulmonary hypertension, gallstones, thromboembolic complications, and end-organ damage, which involve virtually every organ and organ system.[4,5,7,17,43-50]

5.1. Vaso-occlusive pain crisis

The most common complication of SCD is the unpredictable, episodic, recurring, excruciating pain of bone and joints caused by vaso-occlusion. Sickle RBCs occlude microvasculature, and the micro-occlusion stimulates nociceptive fibers resulting in pain. Pain begins from early infancy and continues in childhood as well as adulthood and is the major cause of hospitalizations as well as a negative impact on the quality of life of SCD patients. Some patients present with as high as 6 (or more) episodes in a year, whereas others may suffer the complication less frequently or not at all. In addition to pain, micro-occlusion also results in ischemia, edema, necrosis, and organ damage. The edema and pain of extremities result in one of the cardinal presentations in infancy, the "hand-foot syndrome." While infants express the pain through irritability and what appear to be features of regression (*e.g.*, no weight bearing, no walking or crawling), vaso-occlusive pain in older children and adults could affect any part of the body. The pain is unpredictable in terms of both onset and resolution, but some known triggers are fever, dehydration, infections, acidosis, abrupt weather changes or pollutants, and any factor stabilizing deoxyhemoglobin. Acute pain progresses into chronic pain.[1,3,7,9,13,17]

5.2. Hemolysis and anemia

Symptomatic anemia is the most common symptom in SCD and more so in HbSS patients. Hemoglobin levels for asymptomatic patients vary according to phenotype with steady-state levels ranging from 60 – 80 g/L in HbSS and $HbS\beta_0$ to 100 – 110 g/L in HbSC and $HbS\beta_+$. It is the fall from the steady state levels that triggers hypoxic symptoms such as aplastic crisis or shock-like states like splenic sequestration crisis.[7]

The most common cause of an aplastic crisis in SCD children is a parvovirus B19 infection. HbAA children with parvovirus 19 infection might experience a slight drop in hematocrit, but HbSS children might face a significant drop in hematocrit due to the decreased RBC life span of 10 – 20 days, and the viral infection takes 4 – 7 days to resolve, necessitating a transfusion.[7,31]

Acute splenic sequestration crisis in SCD follows from a cycle of hypoxia, RBC polymerization, and reduced blood flow in the narrow capillaries of the splenic bed, resulting in splenomegaly, sudden pooling of blood in the capillary bed and hence shock and circulatory failure. This emergency condition of splenic sequestration needs immediate attention, as it could cause death within 1 – 2 h due to circulatory failure.[7,11,17,19,20,49]

5.3. ACS

ACS is a dangerous complication of SCD seen as new radiodensity/densities on chest imaging along with respiratory symptoms and fever. Around 50% of SCD patients experience more than one episode of ACS, and

the peak incidence occurs in the second to fourth year of life. ACS in affected adults is most often (around 78% of affected cases) secondary to vaso-occlusive pain episodes. ACS is the most common cause of death in SCD patients (nearly 25% of all deaths in SCD), with a mortality rate of 4.3% in adults and 1.1% in children.[51]

ACS results from vaso-occlusion within the pulmonary vasculature in SCD patients, followed by deoxygenation of hemoglobin, sickling of RBCs, further vaso-occlusion (by fat and bone marrow released into venous circulation due to vaso-occlusion and traveling to the lungs), ischemia and endothelial injury. Pulmonary infarction and fat embolism are large contributors to ACS. Infection, asthma, factors causing hypoxemia, and post-operative complications are other known causes that incite ACS in SCD patients. Patients hospitalized for vaso-occlusive pain in the spine, ribs, and abdomen are at a greater risk of developing ACS because the pain in these regions can lead to hypoventilation and hence alveolar hypoxia when opioids are used.[49,51] Children affected by ACS present with wheezing, coughing, shortness of breath, and fever, whereas adults affected by the same condition present with chest pain, pain in extremities, dyspnea, or vaso-occlusive signs in other parts of the body.[49,51,52-65]

5.4. Infections

SCD patients are highly likely to contract bacterial infections such as pneumonia, urinary tract infections, osteomyelitis, meningitis, and septicemia, probably due to hyposplenism, defective opsonization, decreased immune response and complement pathway, defective leukocyte function, and cellular immunity. *S. aureus*, *Salmonella,* and Gram-negative enteric bacteria are reported to be the most common causative organisms. Children under the age of five are at increased risk of life-threatening pneumococcal infection due to hyposplenism.[48,66,67]

5.5. Neurological complications

Neurological complications in SCD are not uncommon. There have been reports that silent cerebral infarcts were seen in 39% of patients by 18 years of age, acute and chronic headaches in 36% of children with SCD, ischemic strokes in 9 – 11% of children with sickle cell anemia without screening, hemorrhagic stroke affected 3% of children and 10% of adults with SCD. Intracranial hemorrhage and aneurysms are also seen in SCD. Asymptomatic silent cerebral infarcts in early life can progress to neurocognitive impairments and reduced academic performance.[68-72]

5.6. Cardiopulmonary complications

Studies have indicated that 60% of SCD patients exhibited some cardiac abnormality, 40% succumbed prematurely to SCD, and cardiopulmonary complications were cited as the most common cause of death in SCD adults. ACS has been already discussed in a separate subsection above. In addition, eccentric hypertrophy, diastolic dysfunction, left ventricular dysfunction, and right ventricular dysfunction due to pulmonary hypertension are commonly reported cardiac complications of SCD.[73-80] Compared to age- and race-matched children, SCD children have lower lung capacity and expiratory flows in general. Compromised pulmonary function, asthma, wheezing, and sleep-disordered breathing are associated with increased episodes of vaso-occlusive crisis and ACS. Sleep-disordered breathing along with chronic pulmonary disease and hypoxemia is also associated with the development of pulmonary hypertension and ventricular dysfunction. Most treatments in SCD patients are administered per guidelines for the general population and need to be monitored in a multidisciplinary setting by specialists.[76]

5.7. Genitourinary complications

The kidney is one of the most commonly affected organs in SCD. Ischemia-reperfusion injury to the kidney due to vaso-occlusion leads to hematuria, proteinuria, tubular disturbances, and complications, namely, sickle cell nephropathy, painful priapism, papillary necrosis, and renal medullary carcinoma.[81-89] Microalbuminuria is the earliest sign of sickle cell nephropathy, and its prevalence increases with the age of the SCD patients. Hyperphosphatemia, hyperuricemia, and increased GFR are seen in SCD patients. Acute kidney injury due to the resulting volume depletion, rhabdomyolysis, renal vein thrombosis, papillary necrosis, and clot obstructions of the urinary tract are reported in 10% of SCD patients. Chronic kidney injury is reported in 30% of SCD patients, whereas 4 – 12% of the patients develop end-stage renal disease, whose mortality rate is thrice as high as in patients without SCD.[82-86]

Ischemic priapism is not common in sickle cell trait but has a prevalence rate of as high as 42% in SCD males. It is characterized by a rigid, painful erection lasting more than 4 h, unrelated to orgasm and not involving the glans penis, is often seen in SCD children and adults, and is a medical emergency that needs to be treated even before treating the disease itself.[83]

5.8. Hepatobiliary complications

Liver dysfunction is estimated to be prevalent in about 10% of SCD adults, and the prevalence is expected to increase with age. Acute manifestations of hepatic dysfunction in SCD include hepatic crisis, intrahepatic cholestasis, and hepatic sequestration. Cholelithiasis, cholangiopathy, auto-immune hepatitis, viral hepatitis, and iron overload

are among the chronic manifestations. Owing to possible confusion between hemolysis and hepatic markers and the resulting misinterpretation of results, severe hepatic diseases, although rare in SCD patients, could be underdiagnosed in SCD children.[90,91] In a retrospective study on 616 patients in a university hospital, as high as 40% had a history of liver or biliary manifestations. Gallstones were found in 42% of them, although almost half of them were incidental discoveries. Iron overload was seen in 3% of the children. Severe hepatic crisis was also noted among some, although the incidence rate was less than the general reported rate of 10%.[90]

5.9. Thromboembolic complications

Venous thromboembolism (VTE) is one of the common complications in SCD. The prevalence of VTE in SCD patients (25%) matches that with families affected by strong thrombophilic defects. Incidence of VTE is cumulative, increases with age and the mean age ranges from 24 to 37 years.[34-42] Prevalence data point toward a higher prevalence in females, although some studies have found that sex is not a risk factor for VTE. Prevalence of DVT in a retrospective case–control study was seen in more than 50% of the cases as compared with isolated pulmonary embolisms.[36] The high prevalence of VTE in SCD patients has been attributed to a hypercoagulable state, increased hospitalizations, asplenia, central venous catheterization, prevalence of ACS, and infection.

The rate of VTE has been reported to be 1.5 – 2.5 times greater in pregnant than in non-pregnant SCD females. Hence, prophylactic anticoagulation is strongly recommended in SCD females who conceive.[36]

5.10. Psychosocial impact

SCD patients generally have a low quality of life due to the significant psychosocial impact of the disease on them. While medical complications and frequent hospitalization negatively impact their life, these patients also face several psychosocial challenges.[7,92]

The cardinal feature in SCD, namely pain due to vaso-occlusive crises, is challenging to manage for several reasons and increases psychosocial distress in these patients. First, opioids are the primary choice of treatment for chronic pain in SCD, but they have their own adverse effects, namely, constipation, addiction (leading to substance abuse), mast cell activation, and respiratory depression. Second, SCD patients experience different levels of neuropathic pain, including hyperalgesia and allodynia. Thirdly, pain affects behavior, mood, and emotions and even induces catastrophizing in pediatric as well as adult SCD patients. Understanding the complex interplay between pain, mental health outcomes, and the disease itself is very important to ensure the well-being of SCD patients. To minimize substance abuse, exploring non-opioid options for pain management is very crucial in these patients.[92]

Depression and anxiety tremendously affect SCD patients and, consequently, increase pain and opioid use, decrease compliance with treatment, and disrupt the dynamics of personal life. Nearly 20 – 50% of SCD patients experience depression, and this has been attributed to the chronic nature of the disease, psychosocial stressors, or the severity of the disease symptoms. Addressing mental disorders in SCD patients is imperative for holistic management and improving their quality of life for a better outcome.[92]

Research has shown the increased prevalence of snoring, sleep-disordered breathing, and obstructive sleep apnea in SCD patients. These can cause cognitive and behavioral problems and hence affect physical health. Sleep assessments and interventions are hence important to be incorporated into comprehensive health-care testing for SCD patients.[92]

Stigmatization, influenced by opioid use for pain relief, being black, having delayed growth or puberty due to the disease or its management, a socioeconomic status further worsened by the cost of lifelong treatment and severity of the disease, is another challenge faced by SCD patients. It can come from unexpected quarters, namely health-care professionals, family, and friends, further adding to the emotional and mood disturbances in SCD. Compromised interpersonal relationships with family members and peers, functional impairment and neurocognitive deficits, and workplace discrimination are among other psychosocial challenges faced by SCD patients and their caretakers and need to be considered while planning comprehensive care for such patients.[92]

6. Treatment and management

SCD treatment and management requires a multidisciplinary approach and comprehensive care by an interprofessional team of health-care experts such as hemoglobinopathy teams to not only educate patients and caregivers about SCD but also guide them on treatment options such as standard treatment, curative treatment, psychological support, and social support. They also coordinate pediatric screening through transcranial Doppler ultrasound, detections of iron overload or allo-antibodies during transfusion programs, and referral to specialists for complications such as organ dysfunction. The following subsections discuss the different standard treatments and curative treatments in SCD. Gene therapy in SCD is discussed in a separate section.

6.1. Standard treatments

In most cases, the mainstay of treatment is to prevent or control symptoms and complications, particularly pain, hence early intervention to that effect is imperative. While over-the-counter pain medications such as acetaminophen or ibuprofen help treat mild or moderate pain, prescription medications are necessary for severe pain. General care, hydroxyurea, and transfusion have been the standard disease-modifying treatments for years now. In children with SCD, penicillin prophylaxis has been recommended by the American Academy of Pediatricians to prevent pneumococcal infections. In addition, in the past seven years, three new disease-modifying treatments were approved, namely, L-glutamine in 2017 and crizanlizumab and voxelotor in 2019.[93,94]

6.1.1. General care

Self-care at home by SCD patients and caretakers is imperative in pain management and prevention of pain crises, particularly in young adults because pain episodes are most reported from ages 19 – 39. Dehydration is most often the triggering factor for painful crises, alongside exposure to cold, infection, or high altitude. Hence, preventing pain episodes, maintaining good hydration, and preventing infections are the mainstay of self-care. Self-care strategies include regular check-ups, staying hydrated, eating healthily, getting adequate rest, avoiding extremes of temperatures, avoiding high-altitude activities (*e.g.*, trekking and mountain climbing), and frequent hand-washing throughout the day.[7,11] Penicillin prophylaxis and pneumococcal polysaccharide vaccine to prevent infection are discussed more at the end of this section.

6.1.2. Hydroxyurea

Hydroxyurea, a myelosuppressive agent, is the only effective proven drug for decreasing the frequency of episodes of painful crises and one of the major current therapies available for SCD. Hydroxyurea works by inducing the formation of fetal hemoglobin, improving hydration in RBCs, causing neutropenia, and decreasing leukocyte adhesion and pro-inflammatory markers. It induces vasodilation by serving as a donor of nitric oxide.[95-99]

The standard therapy is a single undivided dose daily of 10 – 15 mg/kg body weight/day. Dividing the dose is likely to decrease compliance. The dose may be escalated by 5 mg/kg every 4 – 6 weeks until a maximum of 35 mg/kg body weight/day is reached, or the maximum tolerated dose is evident, or an absolute neutrophil count of 3000 – 4000 is achieved. The above-mentioned standard dose of hydroxyurea is the gold standard of treatment over the past 37 years. It is known to decrease the frequency of painful episodes in adults and children. It preserves spleen function in infants and is beneficial in children with organ damage due to sickling. It also decreases the frequency of ACS episodes and transfusions by 50% in adults.

However, it also causes short-term adverse effects in patients, with the most common among them being myelosuppression, neutropenia, reticulocytopenia, decreased platelet count, and gastrointestinal suppression. Hence, it is very important for physicians to know that hydroxyurea is contraindicated in pregnancy.[99]

A platelet count of <80000, hemoglobin <6 g/dL, and neutrophil count of <2500 can be dose-limiting on hydroxyurea.[99] Agrawal *et al.*[99] reviewed that despite evidence of the efficacy of hydroxyurea in treating SCD, the drug has not yet translated into effective therapy for the disease because of a lack of knowledge and unrealistic apprehensions. The authors explain that although cytopenia is a commonly reported adverse effect, severe cytopenia is rare even at high doses in toxicity studies, and that although discussions are ongoing about the benefits versus risks, the risks of treatment are more acceptable than the risks of not treating the disease.

6.1.3. Transfusion

In addition to general patient care, blood transfusion remains an essential supportive and preventive measure for SCD and strokes.[52-65,81-87] Blood transfusion improves the oxygen-carrying capacity of hemoglobin and hence microvascular perfusion because it decreases the percentage of HbS. However, the availability of fully compatible blood is a limiting factor as is the risk of iron overload, alloimmunization of RBCs, or hemolytic transfusion reaction, more so with repeated transfusions.

The goal of transfusion is to reduce the patient's HbS by 30% and maintain the hematocrit (Hct) at <30%. Transfusion can be a simple, manual exchange or automated exchange. The choice depends on whether the indication is acute or chronic. Indications and contraindications of transfusion are discussed at the end of this section.[99]

Simple transfusion is the transfusion of allogeneic-packed red cells without autologous red cell removal. It is the preferred method of transfusion, especially in acute clinical scenarios because of the ease of using peripheral venous access and standard nursing. In adults, for a desired increase in hemoglobin of every gram per deciliter, a simple transfusion of one red cell unit is the recommended dose. In children, dosing is recommended by weight and or a desired increase in Hb of 2 – 3 g/dL; a simple transfusion of 10 – 15 mL/kg is the recommended dose. In a chronic simple transfusion, three units of packed cells are the

maximum recommended dose per transfusion episode. In a chronic simple transfusion, ideally, the hematocrit (Hct) desired to be achieved is used as a reference to calculate the volume of red cells needed, as per the formula: Volume of red cells for transfusion = ([Desired Hct - Starting Hct] × Total volume of patient blood)/Hct of red cell unit.[100]

Manual red cell exchange (RCE) is the autologous removal of whole blood alternating with transfusion of allogeneic packed red cells and fluid infusion. Automated RCE involves the use of an apheresis machine for autologous RBC removal and concurrent replacement with allogeneic-packed red cells. Exchange transfusion, whether manual or automated, requires expertise and supplies and the use of a central venous line. Manual RCE takes more time due to non-concurrent replacement and runs a higher risk of causing adverse events in users due to fluid infusion-induced volume shifts. Automated RCE, on the other hand, has the advantage that it enables the programming of targeted parameters, namely, %HbS required hematocrit, and fluid homeostasis. A pre- and post-CBS, as well as hemoglobin fractionation, is recommended to achieve accurate programming.[100]

Indications for transfusion in SCD patients are either acute or chronic. Acute stroke, severe ACS or rapid progressive ACS, acute multisystem organ failure, intrahepatic cholestasis, hepatic/splenic sequestration, and priapism are examples of acute indications. Chronic indications include stroke prophylaxis, silent infarcts, recurrent ACS, recurrent painful episodes, and complicated pregnancy.[100]

Transfusion is contraindicated in SCD patients with acute vaso-occlusive pain episodes and asymptomatic anemia. The former is managed with support, hydration, opioids, and psychological, social, or behavioral interventions. Asymptomatic anemia is managed by treating the underlying cause.[100]

Possible complications of transfusion include alloimmunization, infections, and iron overload. It is important to note that SCD patients are assessed with special blood-matching tests to decrease the possibility of alloimmunization.[101]

6.1.4. L-Glutamine

L-Glutamine as an oral powder formulation is currently a U.S. Food and Drug Administration (FDA)-approved treatment since 2017 for acute complications in both adult and pediatric SCD patients. L-Glutamine is a precursor of nicotinamide adenine dinucleotide (NAD), which counters the oxidant-induced pathophysiology in SCD. Sickle erythrocytes have low levels of NAD, a condition where L-glutamine serves as an effective treatment. Patients on L-glutamine have shown a significant reduction in vaso-occlusive crisis, and hospitalization.[7] L-glutamine treatment has also shown a significant reduction in ACS, improvement in hemoglobin and hematocrit, reduction in a median number of blood transfusions and transfusion-related adverse events or complications, a significant decrease in reticulocyte counts, and a mean reduction in WBC counts in SCD patients.[102] It has also been noted that unlike some newer drugs used in SCD (discussed in separate sub-sections below), L-glutamine showed a good correlation between improvement of painful crisis and improvement of hemolytic parameters. Reported adverse effects are constitutive, and the recommended dose is an oral intake of 10 – 30 g/day (based on body weight) twice daily.[102]

6.1.5. Voxelotor

Inhibitors of deoxyHbS polymerization such as voxelotor stabilize oxyHb and are known to reduce sickling and blood viscosity as well as increase erythrocyte deformability, thereby increasing erythrocyte half-life and decreasing hemolytic anemic episodes. It was FDA-approved in 2019 for the treatment of SCD in adults and pediatric patients. In phase 3 of the HOPE (hemoglobin oxygen affinity modulation to inhibit HbS polymerization) trial, voxelotor treatment significantly increased hemoglobin levels and decreased markers of hemolysis in 51% of the patients under trial. However, there was no significant improvement in vaso-occlusive pain episodes. In the *post hoc* assessment of HOPE findings, the same results were observed, and improvement was also seen clinically in the leg ulcers of SCD patients.[94,103] Voxelotor was copyrighted to Pfizer Inc. under the brand name Oxbryta. However, in its recent September 2024 news release, the FDA has notified the general public and health-care professionals that Pfizer has voluntarily withdrawn Oxbryta from the market because, in the post-marketing clinical trials, Pfizer observed a higher rate of vaso-occlusive crisis and more deaths in Oxbryta-treated group as against placebo. The higher rate of vaso-occlusive crisis in the Oxbryta-treated group has also been reported by Pfizer in two real-world registry studies. Consequently, the FDA has also initiated a safety review of the post-marketing clinical trial data for Oxbryta in addition to real-world registry studies and post-marketing data from the FDA adverse event reporting system.[104]

6.1.6. Crizanlizumab

Crizanlizumab is a humanized monoclonal antibody that inhibits P-selectin that inhibits endothelial adhesion molecules, namely, vascular cell adhesion molecule and intercellular adhesion molecule, and thereby reduces adhesion of vascular cells to the endothelial surface. Thus,

there is an abrogation of hypoxia-induced vaso-occlusion in SCD patients. Crizanlizumab was approved by the FDA in 2019 as a treatment for vaso-occlusive pain in SCD patients aged 16 or above following the multicenter clinical trial SUSTAIN. It has been shown to decrease the frequency of pain crises in SCD, with no significant change in hemolytic parameters. In a systematic review of the SUSTAIN trial, *post hoc* studies, retrospective cohort studies, reviews, and case reports, a significant reduction in the rate of vaso-occlusive crisis, longer time for onset of first and second crises, and a decrease in hospitalization were noted. However, there was no significant change to the patients' requirement for transfusion, opioids, or emergency room visits. Reported side effects are constitutive, and the recommendation is an intravenous dose of 5 mg/kg over a period of 30 min at weeks 0, 2, and every 4 weeks thereafter.[94,105]

6.1.7. Other potential candidates for treatment in SCD

Rivipansel is a pan-selectin inhibitor that was subjected to the initial phase of clinical trials without much promising results. However, the *post hoc* tests revealed that in patients who presented to hospitals within 26 h of onset of a vaso-occlusive episode, rivipansel shortened the length of hospital stay and the duration of intravenous opioid use, as well as significantly decreased the time of readiness-to-discharge; therefore, it has been designated as an orphan drug by FDA.[105,106]

L-Arginine is a substrate for nitric oxide generation. L-Arginine supplements decreased oxidative stress and hemolysis in mice by increasing nitric oxide bioavailability. It also significantly reduced opioid use by >50% in SCD patients enrolled in a phase 2 trial. A Nigerian study involving randomized controlled trials showed that arginine significantly decreased the time to resolution of vaso-occlusive pain, the length of stay in hospital, and the total use of analgesics. It was also shown to improve hemodynamics in pulmonary hypertension in SCD. Thus, arginine could be an inexpensive treatment for pain and pulmonary hypertension in SCD.[105,107]

Phosphodiesterase 9 (PDE9) is an enzyme that degrades cGMP and is found in the neutrophils and RBCs of patients with SCD. IMR-687, a selective PDE9 inhibitor, has been shown to increase levels of cGMP and fetal hemoglobin F *in vitro* and is currently being investigated for the treatment of SCD.[108,109]

6.1.8. Penicillin V potassium prophylaxis in children

Due to hyposplenism, the case fatality rate of pneumococcal infection in pediatric SCD patients <3 years of age is reported to be as high as 24%. As recommended by the American Academy of Pediatricians, all SCD children below 5 years of age are required to get a penicillin prophylaxis against life-threatening pneumococcal infection. Children above 5 years of age who have undergone splenectomy need to continue this prophylaxis. Penicillin prophylaxis is also recommended in adults who have undergone splenectomy as well as in SCD females who have conceived. As a standard practice, pediatric SCD patients aged 0 to 3 years are given 125 mg penicillin V potassium twice daily. From 3 to 5 years of age, the dose is increased to 250 mg orally twice daily. At 2 years or above, the 23-valent *Streptococcus pneumoniae* polysaccharide vaccine (PPV-23) is also recommended.[110,111]

6.2. Curative treatments

Although newborn screening, penicillin prophylaxis, and disease-modifying treatments discussed above have lowered the rates of infant and pediatric mortality, and more than 94% of SCD children survive into adulthood, they are still affected by chronic complications, reduced quality of life as well as increased mortality risk in adulthood. Hence, curing SCD has become the prime goal for hemoglobinopathy teams. The only curative treatment that was available till the FDA approval of gene therapy recently for SCD was hematopoietic stem cell transplantation (HSCT). HSCT could be autologous where a patient's stem cells are genetically modified to correct the point mutation, or can be allogeneic where defective stem cells are replaced with healthy stem cells from the donor. Autologous HSCT was not found therapeutically effective in SCD until the emergence of the recently approved gene therapies for SCD. Allogeneic HSCT can involve myeloablative or non-myeloablative procedures.[112,113] The more recent and promising curative therapies include the FDA-approved gene therapies Casgevy and Lyfgenia, both of which employ autologous transplantation of genetically modified stem cells.[114]

6.2.1. Allogeneic stem cell transplantation

Allogeneic HSCT is the only time-tested curative treatment for SCD till date, but several limitations lie in how SCD patients can be eligible for the treatment.

First, it involves a not-so-easy decision-making and warrants a thorough discussion on benefits versus risks such as graft versus host disease, infections, organ injury, or unexpected effects on preexisting cardiopulmonary or renal diseases.

Second, HLA typing is necessary to identify a matching donor. In fact, in 2014, the National Heart Lung and Blood Institute issued clear guidelines that more research is needed on the selection procedures for patient–donor

matching and determining the optimal regimen before HSCT availability as a curative option could be made public. Usually, a fully matched donor such as a sibling is sought. However, in select cases, alternate donors such as haploidentical and matched unrelated donors can be considered. It has been estimated that in the U.S., there is only a 10 – 15% chance of finding an HLA-identical donor.[112]

Third, concern regarding patient autonomy in the pediatric setting is a limitation of recommending HSCT as a curative option. The socioeconomic barrier to transplantation also needs to be considered during the HSCT option discussion.[112,113]

The myeloablative procedure, a commonly used regimen, combines whole-body irradiation and chemotherapy (busulfan + cyclophosphamide, or busulfan + fludarabine, are most commonly employed). Following transfusion, the patient is assessed for engraftment and donor chimerism (≥20% is considered an assurance for cure). Thereafter, recipients are immunized and initiated on prophylaxis against graft-versus-host disease and infections. The use of granulocyte-colony stimulating factor is a contraindication post-transplantation as it is reported to cause fatal sickle cell crisis.[112] Children younger than 16 years old with sickle cell are recommended curative treatment through myeloablative allo-HSCT.[115,116] There are no reliable indicators that a patient should undergo myeloablative allo-HSCT, and hence it is performed in pediatric patients who are not expected to or did not benefit from hydroxyurea.[116] Myeloablative allo-HSCT presents high risks in adults, such as graft failure and organ toxicity.

Non-myeloablative stem cell–cell transplantation has been developed to provide treatment to adults because of fewer transplant complications while having the same results as myeloablative allo-HSCT, offering an alternative to patients who are not able to find HLA-matched donors. Patients not only receive hydroxyurea, total body irradiation, immunosuppressive therapy, and standard antimicrobial prophylaxis with penicillin V potassium but also platelet transfusions as preparation for non-myeloablative stem cell transplantation.[117]

6.2.2. Gene therapy

As a very recent development in the history of SCD treatments, gene therapy is singled out for discussion in a separate section.

7. Gene therapy in SCD

In a landmark decision on December 8, 2023, the U.S. FDA approved two gene therapies, namely, Casgevy and Lyfgenia, for SCD patients aged 12 years or above. Casgevy was approved by Vertex Pharmaceuticals Inc., whereas Lyfgenia was approved by Bluebird Bio Inc. Both drugs were granted priority review, as well as designations as an orphan drug and fast track and tegenerative medicine advanced therapy.[114] Both drugs are made from autologous hematopoietic stem cells which are modified and transplanted back into SCD patients as a one-time single-dose infusion. Once stem cells are collected from patients for genome-editing, patients undergo myeloablative conditioning to remove the defective stem cells from the bone marrow for replacement with the modified Casgevy or Lyfgenia cells.

7.1. Casgevy

Casgevy is a cell-based therapy and is approved for SCD patients aged 12 years or above and with recurrent vaso-occlusive episodes. It is the first FDA-approved treatment involving the genome editing technology CRISPR/Cas9. With this technology, the patient's hematopoietic stem cells are genome-edited by directing CRISPR/Cas9 to cleave targeted regions of DNA and edit the DNA at the cleaved region. These genome-edited cells are transplanted back into the patient for engrafting within the bone marrow and increasing the production of fetal hemoglobin HbF.[114]

Casgevy was approved after an ongoing single-arm multicenter trial involving 44 adult and adolescent SCD patients. The included subjects had a history of at least two severe vaso-occlusive episodes as defined by the protocols, during each of the two years before the screening. The efficacy outcome was a complete resolution of severe vaso-occlusive episodes for a year during the 2-year follow-up period. Of the 44 patients enrolled in the study, 31 were evaluable with sufficient follow-up time. Of these, 29 patients (93.5%) achieved the efficacy outcome. Successful engraftment was seen in all treated patients and graft failure/rejection was not detected.[114] Nausea and vomiting, headache, musculoskeletal pain, abdominal pain, mouth sores, itching, thrombocytopenia, leukocytopenia, and febrile neutropenia were the most commonly reported side effects.

7.2. Lyfgenia

Lyfgenia, like Casgevy, is a cell-based therapy approved for SCD patients aged 12 years or above and with recurrent vaso-occlusive episodes. This therapy, like Casgevy, genome-modifies a patient's stem cells to produce HbA^{T87Q}, a genetically modified hemoglobin functionally similar to HbA. Erythrocytes with HbA^{T87Q} have a lower risk of sickling, and hence patients treated with Lyfgenia are freed from vaso-occlusive episodes.[114]

Lyfgenia was approved for gene therapy of SCD after a 24-month single-arm multicenter study involving SCD

Table 1. Sickle cell disease: The 75-year journey

Year	Landmark development in SCD	Contributor(s)
1948	Fetal hemoglobin does not sickle	Brooklyn pediatrician Janet Watson
1949	Discovery of the sickle cell defect	Linus Pauling and associates
1956	Genetic change pinpointed in sickle hemoglobin	Vernon Ingram, University of Cambridge, U.K.
1972–1990	Fetal hemoglobin makes sickle cell milder	Different scientists over the years
1980	The development of gene delivery vectors	• David Williams, MD, Boston Children's Hospital • Hospital for Sick Children in Toronto
1994	Fetal hemoglobin boosts life expectancy	Orah Platt, MD, Boston Children's Hospital
1998	FDA approves hydroxyurea as a sickle cell drug	Platt and David Nathan, Boston Children's Hospital
2008	Discovery of fetal hemoglobin silencer: *BCL11A*	• Orkin and Joel Hirschhorn, MD, PhD, Boston Children's Hospital • Orkin and Vijay Sankaran, MD, PhD, at the Dana-Farber/Boston Children's Cancer and Blood Disorders Center
2011	Curing sickle cell in mice by inactivating *BCL11A*	• Orkin *et al.*, Boston Children's Hospital
2013 – 2015	A safer way to enhance fetal hemoglobin (CRISPR editing)	• Orkin and Daniel Bauer, MD, PhD, at Dana-Farber/Boston Children's Hospital
2014	Early trial for the efficacy of glutamine in SCD	• Nihara Y. and Stark C.W., University of South California
2016	First gene therapy vector targeting *BCL11A*	• David Williams, MD, Dana-Farber/ Boston Children's Hospital
2017	First sickle cell gene transfer trial opens	• Williams and Erica Esrick, MD, Boston Children's Hospital
2017	Phase 3 trial for L-glutamine in SCD	• Nihara Y. and Stark C.W., Emmaus Medical
2017	FDA approval of L-glutamine powder for SCD	• Sponsored by Emmaus Medical Inc.
2018	First sickle cell gene editing trial opens	• Sponsored by CRISPR Therapeutics and Vertex Pharmaceuticals • Based on the work by Orkin, Bauer *et al.*
2019	FDA approval of voxelotor for SCD	• Sponsored by Global Blood Therapeutics
2019	FDA approval of crizanlizumab for SCD	• Sponsored by Novartis
January 2021	First clinical trial results	• Orkin and Bauer • Esrick and Williams
July 2022	GRASP trial opens	• Sponsored by Williams
November/December 2023	First sickle cell gene therapy approvals (Casgevy and Lyfgenia)	• Casgevy: Sponsored by Vertex Pharmaceuticals Inc. • Lyfgenia: Sponsored by Bluebird Biotech Inc.

Source: https://answers.childrenshospital.org/sickle-cell-fetal-hemoglobin-timeline/ (main source), and FDA website.
Abbreviations: FDA: Food and drug administration; SCD: Sickle cell disease.

patients aged 12 – 50 years and with a history of vaso-occlusive episodes. A successful efficacy outcome was the complete resolution of vaso-occlusive crisis between 6 and 18 months after Lyfgenia infusion. This outcome was achieved in 28 of 32 patients (88%).[114]

The most commonly reported side effects of Lyfgenia were stomatitis, pancytopenia, and febrile neutropenia. Noteworthy is the fact that hematologic malignancy has occurred in patients treated with Lyfgenia, and hence patients who have received Lyfgenia need to undergo lifelong monitoring. Also, the label for Lyfgenia includes a black-box warning about this risk.[114]

8. SCD – A journey of 75 years

Table 1 sums up multiple discoveries in the area of SCD, which led to the most recent gene therapy (CRISPR gene editing). Table 1 is mainly adopted from information available on the website of Boston Children's hospital, one of the pioneers in the development of Casgevy.[118]

9. Conclusion

SCD has been treated since the 1960s, and since then, the scientific community has made advances that could potentially cure this condition. In a 2005 report, the median life expectancy for women and men with SCD in the United States (US) was approximately 42 and 38 years, respectively.[119] However, the survival rate of SCD patients in high-income countries has steadily improved.[119,120] To deliver new treatments and better means of prevention and strategies for genetic and non-genetic therapies worldwide is the need of the hour to improve quality of life and reduce death in SCD patients in the future. Gene therapy seems to be a promising solution in the curative treatment of SCD.

The FDA-approved gene therapies – Casgevy, Lyfgenia, and the lentiviral gene therapy, as already discussed above – have only demonstrated short-term results, and the FDA on its website has specified that "patients who received Casgevy or Lyfgenia will be followed in a long-term study to evaluate each product's safety and effectiveness."

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References

1. Kato GJ, Piel FB, Reid CD, *et al*. *Sickle Cell Disease. Nature News*; 2018. Available from: https://www.nature.com/articles/nrdp201810 [Last accessed on 2024 Apr 03].
2. Billett HH. *Hemoglobin and Hematocrit. Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd ed; 1990. Available from: https://www.ncbi.nlm.nih.gov/books/NBK259 [Last accessed on 2024 Apr 06].
3. Mayo Foundation for Medical Education and Research. *Sickle Cell Anemia. Mayo Clinic*; (n.d.). Available from: https://www.mayoclinic.org/diseases-conditions/sickle-cell-anemia/symptoms-causes/syc-20355876 [Last accessed on 2024 Jun 29].
4. Bender MA, Carlberg K. Sickle cell disease. *In: Adam MP, Feldman J, Mirzaa GM, Pagon RA, Wallace SE, Amemiya A, editors. GeneReviews®*. Seattle, WA: University of Washington, Seattle; 1993-2025.
5. Serjeant GR. The natural history of sickle cell disease. *Cold Spring Harb Perspect Med*. 2013;3:a011783.

   doi: 10.1101/cshperspect.a011783
6. Grosse SD, Odame I, Atrash HK, Amendah DD, Piel FB, Williams TN. Sickle cell disease in Africa: A neglected cause of early childhood mortality. *Am J Prev Med*. 2011;41(6):S398-S405.

   doi: 10.1016/j.amepre.2011.09.013
7. Inusa BPD, Hsu LL, Kohli N, *et al*. Sickle cell disease-genetics, pathophysiology, clinical presentation and treatment. *Int J Neonatal Screen*. 2019;5(2):20.

   doi: 10.3390/ijns5020020
8. US Centers for Disease Controls and Prevention (CDC). *Sickle Cell Disease*. Available from: https://www.cdc.gov/sickle-cell/data/index.html [Last accessed on 2024 May 15].
9. Ansari J, Moufarrej YE, Pawlinski R, Gavins FN. Sickle cell disease: A malady beyond a hemoglobin defect in cerebrovascular disease. *Expert Rev Hematol*. 2018;11:45-55.

   doi: 10.1080/17474086.2018.1407240
10. Piel FB, Tatem AJ, Huang Z, Gupta S, Williams TN, Weatherall DJ. Global migration and the changing distribution of sickle haemoglobin: A quantitative study of temporal trends between 1960 and 2000. *Lancet Glob Health*. 2014;2:e80-e89.

    doi: 10.1016/S2214-109X(13)70150-5
11. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet*. 2010;376:2018-2031.

    doi: 10.1016/S0140-6736(10)61029-X
12. Steinberg MH, Sebastiani P. Genetic modifiers of sickle cell disease. *Am J Hematol*. 2012;87:795-803.

    doi: 10.1002/ajh.23232
13. Mohandas N, Evans E. Adherence of sickle erythrocytes to vascular endothelial cells: Requirement for both cell membrane changes and plasma factors. *Blood*. 1984;64:282-287.
14. Fawaz NA, Bashawery L, Al-Sheikh I, Qatari A, Al-Othman SS, Almawi WY. Factor V-Leiden, prothrombin G20210A, and MTHFR C677T mutations among patients with sickle cell disease in Eastern Saudi Arabia. *Am J Hematol*. 2004;76:307-309.

    doi: 10.1002/ajh.20087
15. Pandey SK, Meena A, Kishor K, Mishra RM, Pandey S, Saxena R. Prevalence of factor V leiden G1691A, MTHFR

C677T, and prothrombin G20210A among Asian Indian sickle cell patients. *Clin Appl Thromb*. 2011;18:320-323.

doi: 10.1177/1076029611425830

16. Moreira Neto F, Lourenço DM, Noguti MAE, *et al*. The clinical impact of MTHFR polymorphism on the vascular complications of sickle cell disease. *Braz J Med Biol Res*. 2006;39:1291-1295.

doi: 10.1590/s0100-879x2006001000004

17. Horan J, Lerner N. Prediction of adverse outcomes in children with sickle cell disease. *N Engl J Med*. 2000;342:1612-1613.

doi: 10.1056/NEJM200005253422114

18. Kaul D, Hebbel R. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. *J Clin Investig*. 2000;106:411-420.

doi: 10.1172/JCI9225

19. Chiang EY, Frenette PS. Sickle cell vaso-occlusion. *Hematol Oncol Clin N Am*. 2002;9:771-794, 5.

doi: 10.1016/j.hoc.2005.08.002

20. Frenette PS, Atweh GF. Sickle cell disease: Old discoveries, new concepts, and future promise. *J Clin Investig*. 2007;117:850-858.

doi: 10.1172/JCI30920

21. Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: Inflammation and a chronic vasculopathy. *Microcirculation*. 2004;11:129-151.

22. Elion JE, Brun M, Odievre MH, Lapoumeroulie CL, Krishnamoorthy R. Vaso-occlusion in sickle cell anemia: Role of interactions between blood cells and endothelium. *Hematol J*. 2004;5:S195-S198.

doi: 10.1038/sj.thj.6200452

23. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med*. 1997;337:1584-1590.

doi: 10.1056/NEJM199711273372203

24. Mack AK, Kato GJ. Sickle cell disease and nitric oxide: A paradigm shift? *Int J Biochem Cell Biol*. 2006;38:1237-1243.

doi: 10.1016/j.biocel.2006.01.010

25. Kato GJ. Defective nitric oxide metabolism in sickle cell disease. *Pediatr Blood Cancer*. 2014;62:373-374.

doi: 10.1002/pbc.25297

26. Reiter CD, Wang X, Tanus-Santos JE, *et al*. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8:1383-1389.

doi: 10.1038/nm1202-799

27. Morris CR, Kato GJ, Poljakovic M, *et al*. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA*. 2005;294:81-90.

doi: 10.1001/jama.294.1.81

28. Green D. Thrombogenesis in sickle cell disease. *J Lab Clin Med*. 1999;134:329-330.

29. Stuart MJ, Nagel RL. Sickle-cell disease. *Lancet*. 2004;364:1343-1360.

doi: 10.1016/S0140-6736(04)17192-4

30. Odièvre MH, Verger E, Silva-Pinto AC, Elion J. Pathophysiological insights in sickle cell disease. *Indian J Med Res*. 2011;134:532-537.

31. Booth C, Inusa B, Obaro SK. Infection in sickle cell disease: A review. *Int J Infect Dis*. 2010;14:e2-e12.

doi: 10.1016/j.ijid.2009.03.010

32. Silva DGH, Junior EB, De Almeida EA, Bonini-Domingos CR. Oxidative stress in sickle cell disease: An overview of erythrocyte redox metabolism and current antioxidant therapeutic strategies. *Free Radic Biol Med*. 2013;65:1101-1109.

doi: 10.1016/j.freeradbiomed.2013.08.181

33. Messonnier LA. Mitochondrial function in sickle cell disease. *Blood*. 2022;139:1616-1617.

doi: 10.1182/blood.2021015216

34. Kurantsin-Mills J, Ofosu FA, Safa TK, Siegel RS, Lessin LS. Plasma factor VII and thrombin-antithrombin III levels indicate increased tissue factor activity in sickle cell patients. *Br J Haematol*. 1992;81:539-544.

doi: 10.1111/j.1365-2141.1992.tb02989.x

35. Peters M., Plaat BE, Cate HT, Wolters HJ, Weening RS, Brandjes DP. Enhanced thrombin generation in children with sickle cell disease. *Thromb Haemost*. 1994;71:169-172.

36. Alkindi S, Al-Ghadani AR, Al-Zeheimi SR, *et al*. Predicting risk factors for thromboembolic complications in patients with sickle cell anaemia-lessons learned for prophylaxis. *J Int Med Res*. 2021;49:3000605211055385.

37. Nsiri B, Gritli N, Bayoudh F, Messaoud T, Fattoum S, Machghoul S. Abnormalities of coagulation and fibrinolysis in homozygous sickle cell disease. *Hematol Cell Ther*. 1996;38:279-284.

doi: 10.1007/s00282-996-0279-2

38. Key NS, Slungaard A, Dandelet L, *et al*. Whole blood tissue factor procoagulant activity is elevated in patients with sickle cell disease. *Blood*. 1998;91:4216-4223.

39. Westerman MP, Green D, Gilman-Sachs A, *et al*. Antiphospholipid antibodies, proteins C and S, and coagulation changes in sickle cell disease. *J Lab Clin Med*. 1999;134:352-362.

doi: 10.1016/s0022-2143(99)90149-x

40. Setty BNY, Rao AK, Stuart MJ. Thrombophilia in sickle cell

disease: The red cell connection. *Blood*. 2001;98:3228-3233.

doi: 10.1182/blood.v98.12.3228

41. Noubouossie D, Key NS, Ataga KI. Coagulation abnormalities of sickle cell disease: Relationship with clinical outcomes and the effect of disease modifying therapies. *Blood Rev*. 2016;30:245-256.

doi: 10.1016/j.blre.2015.12.003

42. Tomer A, Harker LA, Kasey S, Eckman JR. Thrombogenesis in sickle cell disease. *J Lab Clin Med*. 2001;137:398-407.

doi: 10.1067/mlc.2001.115450

43. Adekile A, Gupta R, Yacoub F, Sinan T, Al-Bloushi M, Haider M. Avascular necrosis of the hip in children with sickle cell disease and high Hb F: Magnetic resonance imaging findings and influence of alpha-thalassemia trait. *Acta Haematol*. 2001;105:27-31.

doi: 10.1159/000046529

44. Marouf R, Gupta R, Haider M, Al-Wazzan H, Adekile A. Avascular necrosis of the femoral head in adult Kuwaiti sickle cell disease patients. *Acta Haematol*. 2003;110:11-15.

doi: 10.1159/000072406

45. Almeida A, Roberts I. Bone involvement in sickle cell disease. *Br J Haematol*. 2005;129:482-490.

doi: 10.1111/j.1365-2141.2005.05476.x

46. Mahadeo KM, Oyeku S, Taragin B, *et al*. Increased prevalence of osteonecrosis of the femoral head in children and adolescents with sickle-cell disease. *Am J Hematol*. 2011;86:806-808.

doi: 10.1002/ajh.22103

47. Tantawy AA, Ibrahim SW, Abdel-Aziz TT, Rabie AN, Makkeyah SM, Ragab IA. Inner ear complications in children and adolescents with sickle cell disease. *Hemoglobin*. 2020;44:411-417.

doi: 10.1080/03630269.2020.1837863

48. Burnett MW, Bass JW, Cook BA. Etiology of osteomyelitis complicating sickle cell disease. *Pediatrics*. 1998;101:296-297.

doi: 10.1542/peds.101.2.296

49. Neonato MG, Guilloud-Bataille M, Beauvais P, *et al*. Acute clinical events in 299 homozygous sickle cell patients living in France. French study group on sickle cell disease. *Eur J Haematol*. 2000;65:155-164.

doi: 10.1034/j.1600-0609.2000.90210.x

50. Kirkham FJ, DeBaun MR. Stroke in children with sickle cell disease. *Curr Treat Options Neurol*. 2004;6:357-375.

doi: 10.1007/s11940-996-0028-4

51. Friend A, Settelmeyer TP, Girzadas D. Acute Chest Syndrome. In: *StatPearls*. Treasure Island, FL: StatPearls Publishing; 2024. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441872 [Last accessed on 2023 Nov 25].

52. Charache S. "Acute chest syndrome" in adults with sickle cell anemia. Microbiology, treatment, and prevention. *Arch Intern Med*. 1979;139:67-69.

doi: 10.1001/archinte.1979.03630380047016

53. Barrett-Connor E. Acute pulmonary disease and sickle cell anemia. *Am Rev Respir Dis*. 1971;104:159-165.

doi: 10.1164/arrd.1971.104.2.159

54. Gray A, Anionwu EN, Davies SC, Brozovic M. Patterns of mortality in sickle cell disease in the United Kingdom. *J Clin Pathol*. 1991;44:459-463.

doi: 10.1136/jcp.44.6.459

55. Babiker MA, Obeid HA, Ashong EF. Acute reversible pulmonary ischemia. A cause of the acute chest syndrome in sickle cell disease. *Clin Pediatr (Phila)*. 1985;24:716-718.

doi: 10.1177/000992288502401209

56. Aldrich TK, Dhuper SK, Patwa NS, *et al*. Pulmonary entrapment of sickle cells: The role of regional alveolar hypoxia. *J Appl Physiol*. 1996;80:531-539.

doi: 10.1152/jappl.1996.80.2.531

57. Chaturvedi S, Ghafuri DL, Glassberg J, *et al*. Rapidly progressive acute chest syndrome in individuals with sickle cell anemia: A distinct acute chest syndrome phenotype. *Am J Hematol*. 2016;91:1185-1190.

doi: 10.1002/ajh.24539

58. Bhalla M, Abboud MR, McLoud TC, *et al*. Acute chest syndrome in sickle cell disease: CT evidence of microvascular occlusion. *Radiology*. 1993;187:45-49.

doi: 10.1148/radiology.187.1.8451435

59. Nur E, Gaartman AE, Van Tuijn CFJ, Tang MW, Biemond BJ. Vaso-occlusive crisis and acute chest syndrome in sickle cell disease due to 2019 novel coronavirus disease (COVID-19). *Am J Hematol*. 2020;95:725-726.

doi: 10.1002/ajh.25821

60. Vichinsky EP, Neumayr LD, Earles AN, *et al*. Causes and outcomes of the acute chest syndrome in sickle cell disease. National acute chest syndrome study group. *N Engl J Med*. 2000;342:1855-1865.

doi: 10.1056/NEJM200006223422502

61. Graham LM Jr. The effect of sickle cell disease on the lung. *Clin Pulm Med*. 2004;11:369.

62. Powars D, Weidman JA, Odom-Maryon T, Niland JC, Johnson C. Sickle cell chronic lung disease: Prior morbidity and the risk of pulmonary failure. *Medicine (Baltimore)*. 1988;67:66-76.

63. Castro O, Brambilla DJ, Thorington B, *et al*. The acute chest syndrome in sickle cell disease: Incidence and risk factors. The cooperative study of sickle cell disease. *Blood*.

1994;84:643-649.

64. Vichinsky EP, Styles LA, Colangelo LH, *et al*. Acute chest syndrome in sickle cell disease: Clinical presentation and course. Cooperative study of sickle cell disease. *Blood*. 1997;89:1787.

65. Howard J, Hart N, Roberts-Harewood M, Cummins M, Awogbade M, Davis B. The BCSH committee guideline on the management of acute chest syndrome in sickle cell disease. *Br J Haematol*. 2015;169:492-505.

66. Obaro SK, Iroh Tam PY. Preventing infections in sickle cell disease: The unfinished business. *Pediat Blood Cancer*. 2016;63:781-785.

doi: 10.1002/pbc.25911

67. De Ceulaer K, Pagliuca A, Forbes M, Maude G, Serjeant B, Serjeant G. Recurrent infections in sickle cell disease: Haematological and immune studies. *Clin Chim Acta*. 1985;148:161-165.

doi: 10.1016/0009-8981(85)90142-1

68. Switzer JA, Hess DC, Nichols FT, Adams RJ. Pathophysiology and treatment of stroke in sickle-cell disease: Present and future. *Lancet Neurol*. 2006;5:501-512.

doi: 10.1016/S1474-4422(06)70469-0

69. Anson JA, Koshy M, Ferguson L, Crowell RM. Subarachnoid hemorrhage in sickle-cell disease. *J Neurosurg*. 1991;75:552-558.

doi: 10.3171/jns.1991.75.4.0552

70. De Baun MR, Kirkham FJ. Central nervous system complications and management in sickle cell disease. *Blood*. 2016;127:829-838.

doi: 10.1182/blood-2015-09-618579

71. De Baun MR, Armstrong FD, McKinstry RC, Ware RE, Vichinsky E, Kirkham F. Silent cerebral infarcts: A review on a prevalent and progressive cause of neurologic injury in sickle cell anemia. *Blood*. 2012;119:4587-4596.

doi: 10.1182/blood-2011-02-272682

72. Ohene-Frempong K, Weiner SJ, Sleeper LA, *et al*. Cerebrovascular accidents in sickle cell disease: Rates and risk factors. *Blood*. 1998;91:288-294.

73. Sachdev V, Rosing DR, Thein SL. Cardiovascular complications of sickle cell disease. *Trends Cardiovasc Med*. 2020;31:187-193.

doi: 10.1016/j.tcm.2020.02.002

74. Palomarez A, Jha M, Medina Romero X, Horton RE. Cardiovascular consequences of sickle cell disease. *Biophys Rev (Melville)*. 2022;3(3):031302.

doi: 10.1063/5.0094650

75. Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Investig*. 1975;56:56-64.

doi: 10.1172/JCI108079

76. Teresa SM, Feld L, Dhar A, *et al*. *Cardiopulmonary Complications of Sickle Cell Disease in Children*. *Current Practices in Sickle Cell Disease*. IntechOpen; 2024. Available from: https://www.intechopen.com/chapters/1182476 [Last accessed on 2024 Aug 11].

doi: 10.5772/intechopen.1005507

77. Balfour IC, Covitz W, Arensman FW, Eubig C, Garrido M, Jones C. Left ventricular filling in sickle cell anemia. *Am J Cardiol*. 1988;61:395-399.

doi: 10.1016/0002-9149(88)90952-6

78. Hankins JS, McCarville MB, Hillenbrand C, *et al*. Ventricular diastolic dysfunction in sickle cell anemia is common but not associated with myocardial iron deposition. *Pediatr Blood Cancer*. 2010;55:495-500.

doi: 10.1002/pbc.22587

79. Johnson MC, Kirkham F, Redline S, *et al*. Left ventricular hypertrophy and diastolic dysfunction in children with sickle cell disease are related to asleep and waking oxygen desaturation. *Blood*. 2010;116:16-21.

doi: 10.1182/blood-2009-06-227447

80. Chinawa JM, Chukwu BF, Chinawa AT, *et al*. Right ventricular function among South East Nigeria children with sickle cell anaemia. *BMC Pediatr*. 2020;20:240.

doi: 10.1186/s12887-020-02143-4

81. Osegbe DN. Haematuria and sickle cell disease. A report of 12 cases and review of the literature. *Trop Geogr Med*. 1990;42:22-27.

82. Scheinman JI. Sickle cell disease and the kidney. *Nat Clin Pract Nephrol*. 2009;5:78-88.

doi: 10.1038/ncpneph1008

83. Gebreselassie S, Simmons MN, Montague DK. Genitourinary manifestations of sickle cell disease. *Cleve Clin J Med*. 2015;82(10):679-683.

doi: 10.3949/ccjm.82a.14029

84. Odita JC, Ugbodaga CI, Okafor LA, Ojogwu LI, Ogisi OA. Urographic changes in homozygous sickle cell disease. *Diagn Imaging*. 1983;52:259-263.

85. Walker TM, Serjeant GR. Increased renal reflectivity in sickle cell disease: Prevalence and characteristics. *Clin Radiol*. 1995;50:56669.

doi: 10.1016/s0009-9260(05)83194-0

86. Baddam S, Aban I, Hilliard L, Howard T, Askenazi D, Lebensburger JD. Acute kidney injury during a pediatric sickle cell vaso-occlusive pain crisis. *Pediatr Nephrol*. 2017;32:1451-1456.

doi: 10.1007/s00467-017-3623-6

87. Anele U, Burnett AL. Erectile dysfunction after sickle cell

disease-associated recurrent ischemic priapism: Profile and risk factors. *J Sex Med*. 2015;12:713-719.

doi: 10.1111/jsm.12816

88. Mantadakis E, Cavender JD, Rogers ZR, Ewalt DH, Buchanan GR. Prevalence of priapism in children and adolescents with sickle cell anemia. *J Pediatr Hematol*. 1999;21:518-522.

89. Kato GJ. Priapism in sickle-cell disease: A hematologist's perspective. *J Sex Med*. 2012;9:70-78.

doi: 10.1111/j.1743-6109.2011.02287.x

90. Allali S, De Montalembert M, Brousse V, *et al*. Hepatobiliary complications in children with sickle cell disease: A retrospective review of medical records from 616 patients. *J Clin Med*. 2019;8(9):1481.

doi: 10.3390/jcm8091481

91. Martins RA, Soares RS, De Vito FB, *et al*. Cholelithiasis and its complications in sickle cell disease in a university hospital. *Rev Bras Hematol Hemoter*. 2017;39:28-31.

doi: 10.1016/j.bjhh.2016.09.009

92. Essien EA, Winter-Eteng BF, Onukogu CU, Nkangha DD, Daniel FM. Psychosocial challenges of persons with sickle cell anemia: A narrative review. *Medicine (Baltimore)*. 2023;102(47):e36147.

doi: 10.1097/MD.0000000000036147

93. Vinjamur DS, Bauer DE, Orkin SH. Recent progress in understanding and manipulating haemoglobin switching for the haemoglobinopathies. *Br J Haematol*. 2018;180:630-643.

doi: 10.1111/bjh.15038

94. Migotsky M, Beestrum M, Badawy SM. Recent Advances in sickle-cell disease therapies: A review of voxelotor, crizanlizumab, and l-glutamine. *Pharmacy (Basel)*. 2022;10(5):123.

doi: 10.3390/pharmacy10050123

95. King SB. N-hydroxyurea and acyl nitroso compounds as nitroxyl (HNO) and nitric oxide (NO) donors. *Curr Top Med Chem*. 2005;5:665-673.

doi: 10.2174/1568026054679362

96. Eaton WA, Bunn HF. Treating sickle cell disease by targeting HbS polymerization. *Blood*. 2017;129:2719-2726.

doi: 10.1182/blood-2017-02-765891

97. Čokić V, Smith RD, Beleslin-Cokic BB, *et al*. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. *J Clin Investig*. 2003;111:231-239.

doi: 10.1172/JCI16672.

98. Nevitt SJ, Jones AP, Howard J. Hydroxyurea (hydroxycarbamide) for sickle cell disease. *Cochrane Database Syst Rev*. 2017;2017:CD002202.

doi: 10.1002/14651858.CD002202.pub2

99. Agrawal RK, Patel RK, Shah V, Nainiwal L, Trivedi B. Hydroxyurea in sickle cell disease: Drug review. *Indian J Hematol Blood Transfus*. 2014;30(2):91-96.

doi: 10.1007/s12288-013-0261-4

100. Tanhehco YC, Shi PA, Schwartz J. Transfusion therapy in sickle cell disease. *Ann Blood*. 2022;7:9.

101. Linder GE, Chou ST. Red cell transfusion and alloimmunization in sickle cell disease. *Haematologica*. 2021;106:1805-1815.

doi: 10.3324/haematol.2020.270546

102. Elenga N, Loko G, Etienne-Julan M, Al-Okka R, Adel AM, Yassin MA. Real-World data on efficacy of L-glutamine in preventing sickle cell disease-related complications in pediatric and adult patients. *Front Med*. 2022;9:931925.

doi: 10.3389/fmed.2022.931925

103. Vichinsky E, Hoppe CC, Ataga KI, *et al*. A phase 3 Randomized trial of voxelotor in sickle cell disease. *N Engl J Med*. 2019;381(6):509-519.

doi: 10.1056/NEJMoa1903212

104. FDA News Release. *FDA is Alerting Patients and Health Care Professionals About the Voluntary Withdrawal of Oxbryta from the Market Due to Safety Concerns*; 2024. Available from: https://www.fda.gov/drugs/drug-safety-and-availability/fda-alerting-patients-and-health-care-professionals-about-voluntary-withdrawal-oxbryta-market-due [Last accessed on 2024 Dec 03].

105. Sagi V, Mittal A, Tran H, Gupta K. Pain in sickle cell disease: Current and potential translational therapies. *Transl Res*. 2021;234:141-158.

doi: 10.1016/j.trsl.2021.03.007

106. Dampier CD, Telen MJ, Wun T, *et al*. A randomized clinical trial of the efficacy and safety of rivipansel for sickle cell vaso-occlusive crisis. *Blood*. 2023;141(2):168-179.

doi: 10.1182/blood.2022015797

107. Rees CA, Brousseau DC, Cohen DM, *et al*. Sickle cell disease treatment with Arginine Therapy (STArT): Study protocol for a phase 3 randomized controlled trial. *Trials*. 2023;24(1):538.

doi: 10.1186/s13063-023-07538-z

108. Goksel E, Ugurel E, Nader E, *et al*. A preliminary study of phosphodiesterases and adenylyl cyclase signaling pathway on red blood cell deformability of sickle cell patients. *Front Physiol*. 2023;14:1215835.

doi: 10.3389/fphys.2023.1215835

109. McArthur JG, Svenstrup N, Chen C, *et al*. A novel, highly potent and selective phosphodiesterase-9 inhibitor for the treatment of sickle cell disease. *Haematologica*. 2020;105:623-631.

doi: 10.3324/haematol.2018.213462

110. Ndefo UA, Maxwell AE, Nguyen H, Chiobi TL. Pharmacological management of sickle cell disease. *P T*. 2008;33(4):238-243.

111. Eastep TG, Kendsersky RM, Zook J, Moore A. Penicillin prophylaxis in patients with sickle cell disease beyond age 5 years. *J Pediatr Pharmacol Ther*. 2023;28(6):519-523.

doi: 10.5863/1551-6776-28.6.519

112. Ashorobi D, Naha K, Bhatt R. *Hematopoietic Stem Cell Transplantation in Sickle Cell Disease*. In: StatPearls; 2023. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538515 [Last accessed on 2024 Nov 07].

113. Leonard A, Tisdale JF. Stem cell transplantation in sickle cell disease: Therapeutic potential and challenges faced. *Expert Rev Hematol*. 2018;11(7):547-565.

doi: 10.1080/17474086.2018.1486703

114. FDA News Release. *FDA Approves First Gene Therapies to Treat Patients with Sickle Cell Disease*; 2023. Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-first-gene-therapies-treat-patients-sickle-cell-disease [Last accessed on 2024 Nov 06].

115. Hsieh MM, Kang EM, Fitzhugh CD, *et al*. Allogeneic hematopoietic stem-cell transplantation for sickle cell disease. *N Engl J Med*. 2009;361(24):2309-2317.

doi: 10.1056/NEJMoa0904971

116. Hsieh MM, Fitzhugh CD, Tisdale JF. Allogeneic hematopoietic stem cell transplantation for sickle cell disease: The time is now. *Blood*. 2011;118(5):1197-1207.

doi: 10.1182/blood-2011-01-332510

117. Saraf SL, Oh AL, Patel PR, *et al*. Nonmyeloablative stem cell transplantation with alemtuzumab/low-dose irradiation to cure and improve the quality of life of adults with sickle cell disease. *Biol Blood Marrow Transplant*. 2016;22(3):441-448.

doi: 10.1016/j.bbmt.2015.08.036

118. Fliesler N. *Sickle cell Gene Therapy and Boosting Fetal Hemoglobin: A 75-Year History*. *Boston Children's Hospital*; 2023. Available from: https://answers.childrenshospital.org/sickle-cell-fetal-hemoglobin-timeline [Last accessed on 2024 Apr 06].

119. Platt OS, Brambilla DJ, Rosse WF, *et al*. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330:1639.

doi: 10.1056/NEJM199406093302303

120. Lanzkron S, Carroll CP, Haywood C Jr. Mortality rates and age at death from sickle cell disease: U.S., 1979-2005. *Public Health Rep*. 2013;128:110-116.

doi: 10.1177/003335491312800206

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Pre-clinical studies for oral enzyme replacement therapy in Pompe disease knockout mice with tobacco seeds expressing human GAA

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Abstract

Genetic deficiency of acid α-glucosidase (GAA) results in Pompe disease (PD) encompassing four clinical subtypes of varying severity. Our objective is to develop an innovative and affordable approach for enzyme replacement therapy (ERT) through oral administration (Oral-ERT) to maintain a sustained therapeutic level of enzyme daily to improve treatment efficacy. Tobacco seeds contain the metabolic machinery compatible with mammalian glycosylation-phosphorylation. We have shown that transgenic tobacco seeds expressing human GAA (tobrhGAA) were enzymatically active and can correct the enzyme deficiency in cultured cells and in *GAA knockout* (*GAAKO*) mice administered IP. We have extended these studies in PD KO mice with ground tobrhGAA seeds. Briefly, in PD knockout mice, Oral-ERT with ground tobrhGAA seeds showed a significant reversal of fore-limb and hind-limb muscle weakness, increased motor coordination/balance/strength/mobility, improved spontaneous learning, increased GAA activity in tissues, reduced glycogen in tissues and negligible serum titers to GAA. Pharmacokinetics showed maximum serum GAA concentration at 8 – 10 h and peak urine excretion at 10 – 12 h post-administration. The tobrhGAA was taken up in PD fibroblast, lymphoid, and myoblast cells. Enzyme kinetics compared favorably to hGAA, plus alglucosidase alfa or other recombinant human GAAs for K_m, V_{max}, pH optima, thermal heat stability, and IC_{50} for inhibitors. The tobrhGAA in seeds was stable for 15 years at room temperature. Thus, Oral-ERT with ground tobrhGAA seeds is an innovative approach that overcomes some of the challenges of alglucosidase alfa-ERT and provides a more effective, safe, and significantly less expensive treatment.

Keywords: Recombinant human acid maltase; Transgenic tobacco plants; Pompe disease; Oral enzyme replacement

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1. Introduction

Genetic deficiency of lysosomal acid maltase or acid α-glucosidase (GAA) results in acid maltase deficiency or Pompe disease (PD) encompassing at least four clinical subtypes of varying severity (infantile, childhood, juvenile, and adult-onset).[1] PD is characterized by the intracellular accumulation of glycogen in multiple tissues, with skeletal muscle being the primary target; moreover, it manifests as myopathy and cardiomyopathy.[2-5] The classic infantile form of PD is characterized by the absence of enzyme activity, muscle weakness, feeding difficulties, and hypertrophic cardiomyopathy, which leads to death in the 1st year.[6-8] The adult-onset form of PD has partial enzyme deficiency that manifests with slow, progressive muscle weakness, which leads to wheelchair/ventilator dependence and premature death from ventilatory insufficiency.[9-11] The incidence of people living with PD is estimated at 1:40,000 (Orpha number: ORPHA365 at www.orpha.net).[12] Park[13] recalculated the carrier frequency (pCF) and predicted genetic prevalence (pGP) using general population databases based on the causative genotype proportions. Total pCF and pGP were 1.3% (1 in 77) and 1:23,232, respectively. Recently, Colburn and Lapidus reviewed the literature for over 11.6 million newborns screened for PD from 29 screening programs across eight countries and four continents. The birth prevalence of PD was 1:18,711, with no evidence of difference across European, Latin American, and Asian populations; however, differences may exist in the PD subtypes.[14]

Enzyme replacement therapy (ERT) with a recombinant human GAA (rhGAA) secreted by Chinese hamster ovary (CHO) cells (alglucosidase alfas; Myozyme from Genzyme; Lumizyme from Sanofi Corp) infused every 2 weeks was the first approved therapy. Although it is efficient in rescuing cardiac abnormalities and extending the life span of infants, the response of skeletal muscle varies (marketed dose of 20 mg/kg/every 2 weeks administered intravenously) through the cation-independent mannose 6-phosphate receptor.[15-18] In infants, muscle pathology and glycogen deposition degree are correlated with symptom severity, and the earlier ERT is introduced, the better the chance of response.[15] In patients with adult-onset PD, mild improvements in motor and respiratory functions have been achieved, which are unsatisfactory for the reversal of skeletal muscle pathology.[15-18] In patients with adult-onset PD, the ability of muscles to metabolize extralysosomal as well as intralysosomal glycogen is impaired. Lysosomal glycogen accumulation results in multiple secondary abnormalities (autophagy, lipofuscin, mitochondria, trafficking, and signaling) that may improve following long-term therapy. ERT is usually initiated when the patients are symptomatic and these secondary problems are already present, which contributes to inefficient delivery and uptake of alglucosidase alfa in the muscles.[19] The outcome of infantile patients is determined by numerous factors, including age, disease severity at ERT commencement, genotype, genotype-dependent cross-reactive immunological material (CRIM) status, and antibody response level. High and sustained antibody titers need to be prevented to achieve a good response to ERT; however, titers vary substantially among patients and do not strictly correlate with the patients' CRIM status. Approximately 40% of infantile patients are CRIM-negative, whereas the remaining 60% are CRIM-positive. The immune response may be minimized by the early commencement of ERT and pretreatment with an immune tolerance induction regimen, such as using a combination of rituximab, methotrexate, bortezomib, and IV immunoglobulins. Up to 20% of adult patients develop high titers when receiving ERT, 40% develop intermediate titer levels, and 40% develop none or low titers.[20-37] In most patients with adult-onset PD, antibody formation does not interfere with rhGAA efficacy.[38-41] Gutschmidt *et al.*[41] found that the clinical outcomes of ERT in patients with late-onset PD, particularly lung function, muscle strength, and walking capability, tend to deteriorate over time, indicating that additional efforts must be made to improve the

effectiveness of ERT and supplement currently available therapies. Korlimarla *et al.*[42] used standardized behavior checklists as a screening tool for the early identification and treatment of behavior, emotional, and social concerns in children with PD. Although alglucosidase alfa is an effective first-line treatment for individuals with PD, subtle aspects must be addressed for successful treatment.[19,41,43-51] ERT has identified previously unrecognized clinical manifestations, including tracheobronchomalacia, vascular aneurysms, and gastrointestinal (GI) discomfort, which impact smooth muscle. Persistent smooth muscle pathology has a substantial impact on quality of life and can lead to life-threatening complications. In addition to airway smooth muscle weakness, vascular deterioration, GI discomfort, and loss of genitourinary control have been observed. Cerebral and aortic aneurysms may cause microhemorrhages that lead to symptoms ranging from headaches and numbness to coma and death.[52-55] Kenney-Jung *et al.*[56] found that the median age of the onset of seizures and encephalopathy was 11.9 years in six patients with infantile-onset PD (IOPD) treated with ERT for 12 – 15 years. All patients were noted to have extensive white matter hyperintensities in the brain MRIs and very high Fazekas scores, which preceded the onset of neurological symptoms. Longitudinal IQ scores from four of these patients suggested developmental plateauing. PD results in the subsequent pathology in smooth muscle cells and may lead to life-threatening complications if not treated properly.[57] In GAA-knockout (GAAKO) mice, there is increased glycogen in the smooth muscle cells of the aorta, trachea, esophagus, stomach, and bladder as well as increased lysosome membrane protein and autophagosome membrane protein (LC3). Importantly, lifelong treatment ($250,000 – 600,000/year for an adult) can be prohibitively expensive, resulting in the reluctance of insurance companies to reimburse costs for adult patients[58]; thus, more affordable and novel delivery strategies are needed for the treatment of PD.

The technological platform involving the accumulation of recombinant proteins in seeds warrants improved availability of the products and allows for the long-term storage of biomass for processing.[59-61] Transgenic plants, seeds, and cultured plant cells are potentially one of the most economical systems for the large-scale production of recombinant enzymes for pharmaceutical use.[61-69] Seeds are particularly attractive because of their high rates of protein synthesis and their ability to remain viable in a mature-dry state as a stable repository.[61,68-79] More than one-third of approved pharmaceutical proteins are glycoproteins,[73-82] and even minor differences in N-glycan structures can change the distribution, activity, or longevity of recombinant proteins compared with their native counterparts, thereby altering their therapeutic efficacy.[60,61,82-85] Protalix Biotherapeutics and Shaaltiel *et al.*[85,86] produced glucocerebrosidase for ERT of Gaucher's disease using a plant cell system and an exploratory, open-label study to evaluate the safety of PRX-112 and the pharmacokinetics (PK) of oral prGCD in patients receiving 250 mL of resuspended carrot cells. Administration of PRX-112 using carrot cells as a carrier vehicle overcomes absorption, degradation, and uptake in the small intestine and can be found in the bloodstream in an active form.[87-89]

Previously, we expressed human *GAA* cDNA in the chloroplasts, callus, and leaves of transgenic tomato and tobacco plants, but this was not enzymatically active. However, *hGAA* was expressed in tobacco seeds expressing human GAA (tobrhGAA) by targeting the endoplasmic reticulum using the signal peptide sequence/promoter of soybean β-conglycinin.[90] The tobrhGAA from transgenic plant seeds was enzymatically active, taken up by PD fibroblasts and white blood cells to reverse the enzyme defect, and bound to the affinity matrix Sephadex G100, which was eluted by maltose. A crude lysate of transgenic seeds administered intraperitoneally to GAA KO mice increased GAA activity by 10% – 20% in the tissues, most notably in the heart, skeletal muscle, and diaphragm.[90-95]

This study is an extension of these pre-clinical studies in PD KO mice with ground tobrhGAA seeds that support the Oral-ERT proof-of-concept for future clinical trials. In brief, Oral-ERT with various preparations of tobrhGAA showed a significant reversal of forelimb and hindlimb muscle weakness; increased motor coordination, balance, strength, and mobility; improved spontaneous learning; increased GAA baseline activity in tissues; reduced glycogen in tissues; and negligible GAA serum titers in GAA KO mice. PK analysis revealed the maximum serum GAA concentration at 8 – 10 h and peak urine excretion at 10 – 12 h. Similar to human placenta GAA and rhGAA, tobrhGAA was taken up in fibroblast and lymphoid cells from infantile, juvenile, and adult-onset patients. Exposure of tobrhGAA to a human PD myoblast cell line increased GAA to 24% – 35% of the normal level. Enzyme kinetics for tobrhGAA *versus* placental hGAA, alglucosidase alfa, or other rhGAAs for K_m, V_{max}, optimal pH, thermal heat stability, and IC_{50} for inhibitors revealed that tobrhGAA was comparable to placental GAA and superior to alglucosidase alfa and other rhGAAs. The tobrhGAA in seeds stored for 15 years at room temperature showed <15% loss in GAA activity, thereby indicating extreme stability. Oral-ERT with tobrhGAA is an innovative approach that overcomes some of the challenges of alglucosidase alfa-ERT and provides an effective, safe, and affordable treatment option. Oral-ERT with tobrhGAA is an innovative approach that overcomes some of the challenges of alglucosidase alfa-ERT and

provides an effective, safe, and affordable treatment option as a plant-made pharmaceutical (PMP).[82,87-89,94]

2. Methods

2.1. Animal approval

A total of 24 wild-type mice and 34 GAA KO mice were used in this experiment. The Institutional Biosafety Committee ratified the project at biosafety level BSL1 containment, and this study was approved by the Institutional Animal Care and Use Committee at Rutgers, The State University of New Jersey (number: PROTO201800168) and at NYU School of Medicine protocol number 131109, New York, NY, USA.

2.2. Hydroponic growth of tobacco plants

Transgenic tobacco plant #3 expressing human GAA in the seeds was grown indoors using a hydroponic system (Active Aqua Grow Flow Kit, Hydrobuilder, Inc., USA) with deionized water, thereby eliminating all water and soil contaminants. Seed pods were harvested and dried in a freeze dryer, followed by separation from the husk by passage through a standard food mesh strainer. To eliminate environmental contamination, seeds were ground for 10 min in a porcelain pestle and mortar until fine powder was obtained, which was then placed in a UV germicidal incubator for 30 min for sterilization.

2.3. Simulated stomach and small intestinal environments

To mimic the stomach and small intestine environment, we exposed 100 mg of whole or milled tobrhGAA seeds to physiologic conditions and times in the stomach with pepsin followed by the conditions and times in the small intestine trypsin/chymotrypsin.[86,95] Samples were added to 300 μg/mL pepsin at pH 4.0 for 60 min, the pH was adjusted to 6.5, and then trypsin at 800 μg/mL and chymotrypsin at 700 μg/mL were added for 60 min at 37°C, followed by a GAA assay.

2.4. Enzyme assay

Ground seeds or tissues were resuspended in 10 mM sodium phosphate (pH 7.5), frozen and thawed 3 times, and centrifuged at 13,000 ×g for 10 min. The supernatants were assayed for GAA for 18 h using the artificial substrate 4-methylumbelliferyl-α-D-glucoside (4-MU-Glyc, 1 mg/mL) at pH 4.0 (0.5 M sodium acetate) and neutral alpha-glucosidase (NAG) at pH 7.5 (0.5 M sodium phosphate) as an internal control. Fluorescence was determined using a fluorometer with an excitation wavelength of −360 nm and an emission wavelength of −460 nm (Sequoia-Turner) as previously described.[96]

2.5. Biochemical/enzyme kinetic analyses

We compared a lysate of tobrhGAA seeds to rhGAA (R&D Systems) and mature placental human GAA for specific activity using 4-MU-Glyc at pH 4.0, maltose and glycogen, optimal pH, inhibitors (acarbose, castanospermine, deoxynojirimycin, miglitol, and voglibose) and heat stability[93,97-107] according to standard enzyme kinetic methods.

2.6. Uptake of tobrhGAA by PD human myoblast, fibroblast, and lymphoid cell lines

Human lymphoid (GM6314, GM13793, and GM14450) or fibroblast (GM4912, GM1935, and GM3329) cell lines from patients with infantile or adult PD were maintained in 15% fetal bovine serum, RPMI 1640, or DMEM supplemented with glutamine, penicillin, and streptomycin at 37°C in a 5% CO_2 environment. Cells were plated at a density of 0.3 – 0.4 × 10^6/well in a 6-well plate in 1.5 mL of media 24 h before the addition of varying amounts of tobrhGAA or other GAA formulations.[91] Cells were harvested after various hours of exposure, washed with PBS, lysed by adding 0.5 mL of 0.01 M sodium phosphate (pH 7.5), frozen and thawed 3 times, and centrifuged at 13,000 rpm for 5 min. The lysate was used for human GAA and NAG assay, as described above. A human PD myoblast cell line (homozygous for IVS1 c.-32 – 13T>G)[108] and normal skeletal muscle cells were plated at a density of 0.3 – 0.4 × 10^6/well in a 6-well plate containing PromoCell skeletal muscle growth media. Cells were exposed to a lysate of tobrhGAA for 48 h and assayed. Mock-treated GAA, normal myoblast cells, and cells treated with equivalent amounts of rhGAA were used as the controls (R&D Systems #8329-GH-025). Simultaneously, we measured cell proliferation using the Cayman MTT cell proliferation assay (#10009365).

2.7. Short-term studies in GAA KO mice

We used PD KO mice with exon 6^{neo} disruption,[109] wild-type BALB/c or 129/J, or mock-treated PD KO mice with PBS. PD KO mice (4 – 6 months old) were orally administered a lysate from 300 mg (approximately 75 μg of tobrhGAA) of transgenic seeds mixed 3:1 with apple juice every other day up to day 7 as a safe, non-invasive oral administration technique,[110] and the grip strength was measured using a grip-strength meter (GSM) (Columbus Inst., OH, USA). On day 7, the mice were sacrificed, and the tissues were assayed for GAA and NAG and compared to the wild-type and mock (PBS)-treated GAA KO mice.

2.8. Long-term treatment by oral gavage in GAA KO mice

We treated two age-matched groups of GAA KO mice (exon 6^{neo}) (n = 3) 3 times a week with either a 1× or 3×

dose (containing either 25 or 75 μg of tobrhGAA per dose) mixed 3:1 with apple juice as a safe, non-invasive oral administration technique[99] and measured the vertical hang-time, as previously described by Raben *et al.*[109]

2.9. Oral-ERT in GAA KO mice

GAA KO mice aged approximately 5 months were fed either 50 mg or 150 mg of tobrhGAA ground seeds daily mixed with peanut butter in Petri dishes (12/12-h light/dark cycle) (B6,129-Gaa^{tm1Rabn}/J mice).[109] Tissues, urine, blood, weight, and serum were collected. Mice were tested every 3 weeks for motor activity using a running wheel (RW-Mini-Mitter Co., Inc., OR, USA); forelimb muscle strength using a GSM (Columbus Inst.); motor coordination and balance using a Rotarod (AJATPA, Expert Industries, India); open-field mobility using a 5-min video to determine distance traveled; and spontaneous alternative learning using a T-maze (Stoelting, IL, USA).

2.10. Determination of antibodies against tobrhGAA using ELISA

In Falcon plates #3912, 1 μg/well of Sephadex G100 purified human placental GAA[111] was added in 100 μL PBS and incubated overnight at 4°C. This was then washed 3 times with PBS and blocked with 200 μL of PBS with 1% bovine serum albumin (BSA) and 0.05% Tween20 for at least 60 min at room temperature. Thereafter, 1 μL of serum in 100 μL of PBS was added and incubated overnight at 4°C. This was washed 3 times with PBS, once with PBS and 0.05% Tween20, and twice with PBS. Then 100 μL of goat anti-mouse IgG-horse radish peroxidase (BioRad #172-1101) was added (diluted 1:500 in PBS/BSA/Tween20) and incubated overnight at 4°C. This was washed 3 times with PBS, once with PBS and 0.05% Tween-20, and twice with PBS. The cells were stained with 100 μL of 3,3',5,5'–tetramethylbenzidine (Sigma #T5525) in 9 mL of 0.05 M phosphate-citrate buffer, pH 5.0 (Sigma #P4922) with 20 μL of 30% H_2O_2 for 30 – 60 min at room temperature. Reactions are stopped with the addition of 50 μL 1 N HCl and read at 450 nm absorption.

2.11. Endotoxin determination

Endotoxin in various amounts of tobrhGAA seed lysate was determined using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (#88282) according to the manufacturer's instructions.[111]

2.12. Bradford protein assay

For the protein assay, 150 μL of reagent (BioRad #500-0006) diluted 1:5 with water in a 96-well microtiter plate. Standards were serial dilutions of BSA from 1,000 to 2 μg/mL and 1 to 25 μL of sample. Samples were read at 595 nm absorbance within 60 min.

2.13. Glycogen content assay

In a 96-well microtiter plate, 25 μL of sample, 25 μL of 0.05 M sodium phosphate pH 6.5, and 0.5 μg of amyloglucosidase (Sigma #A-1602) were added and incubated overnight at 37°C.[112] Glycogen standards (rabbit liver, 2 mg/mL) and D-glucose standards were measured at 400, 200, and 100 μM. Thereafter, 20 μL of Eton Bioscience glucose assay solution (#1200031002) was added and incubated for 30 min at 37°C. The reaction was stopped with the addition of 25 μL of 0.5 M acetic acid and read at 490 nm absorbance.[112]

2.14. Ames mutagenicity test

Escherichia coli (*E. coli*) DH5α and DH5α/pUC19 cells were plated on NZ media with ampicillin and various amounts of tobrhGAA lysate, and the colony-forming units (CFUs) were counted.[113-117]

2.15. Complete blood count differentials

Complete blood count (CBC) differentials were performed on blood smear slides using Giemsa–Wright staining.

2.16. Extraction of DNA and RNA from seeds for NGS and RNA transcriptome profiling

DNA and total RNA were extracted from wild-type (*Nicotiana tobacum L. cv. xanthi*) and tobrhGAA seeds using commercial kits (Thermo Scientific GeneJET Plant RNA Purification Kit-K0801; GeneJET Plant DNA Purification Kit-K0791). The NGS genome sequences and RNA expression profiles were performed at BGI.com, and global proteomic profiling was performed using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) at bioproximity.com.

2.17. Nicotine levels in leaves and seeds

The levels of nicotine in tobrhGAA#3 seeds and leaves were measured by gas chromatography-mass spectroscopy (GC/MS) at Avogado Analytical (LLC, Salem, NH, USA).

2.18. Statistical analysis

The Student's *t*-test (1-tail and 2-equal variance) for probability associated with a population and standard deviation (SD) based upon the population were determined using Microsoft Excel software, and results were considered significant at $P \leq 0.05$.

3. Results

3.1. Nicotine levels in leaves and seeds

The nicotine levels were measured using GC/MS. The tobrhGAA seeds or leaves contained <5 ng of nicotine per

gram in dry conditions, and a GC/MS spectral profile was generated for future comparisons (data not shown).

3.2. Long-term stability

We compared tobrhGAA in seeds stored for 9 and 15 years at room temperature versus freshly harvested seeds. We found there was <15% loss in GAA activity, which indicated extreme stability (old: 0.20 µg tobrhGAA/g seeds *vs*. fresh: 0.25 µg tobrhGAA/g seeds).

3.3. Endotoxin levels

The endotoxin levels were measured in an extract of tobrhGAA#3 seeds using a LAL endotoxin kit and were found to be <0.25 EU/mL endotoxin or ~25 pg/mL. These values are lower than the estimate of 300 mg/kg for oral endotoxin toxicity in mammals and humans.[118-120] Moreover, we found no viable anaerobic or aerobic bacteria (data not shown).

3.4. Effect of the stomach and small intestinal environments

To mimic the stomach and small intestine environments, we exposed tobrhGAA to the physiologic conditions and times of pepsin (stomach) and trypsin/chymotrypsin (small intestine).[86,95] Lysosomal GAA is stable at low pH. None of the enzymes had any effect on tobrhGAA activity, thus demonstrating that the conditions in the digestive tract probably do not affect tobrhGAA (data not shown). We repeated the conditions with 100 mg of whole or milled seeds. Interestingly, more than double the amount of tobrhGAA in the whole and milled seeds (600 µg/g seeds) was lysed, which suggests that the acidic environment of the stomach more thoroughly disrupts and releases tobrhGAA from the whole or milled seed (data not shown).

3.5. Ames mutagenicity test

The Ames mutagenicity test was performed using *E. coli* DH5α and DH5α/pUC19 with various amounts of tobrhGAA seed lysate. We found no additional bacterial or plasmid revertant CFUs generated with the tobrhGAA lysate by exposure to >10^4 bacteria.[113-117]

3.6. *In vitro* studies in PD fibroblast and lymphoid cell lines

We evaluated tobrhGAA uptake in fibroblast and lymphoid cells from infantile, juvenile, and adult-onset patients with PD and compared it to human placenta GAA and rhGAA. Various equivalent concentrations and time points (2, 24, and 48 h) exhibited similar uptake and increases in GAA (mean ± standard error of the mean [SEM]) (Figure 1 and 2). All treatments were significant at $P \leq 0.05$.

3.7. Uptake of tobrhGAA in a human PD myoblast cell line

A human PD myoblast cell line[108] was exposed to equivalent amounts of tobrhGAA seed lysate for 48 h and assayed. Mock-treated GAA and normal skeletal muscle cells as well as cells treated with rhGAA were used as controls. We found that tobrhGAA increased GAA to 24% – 35% of normal (mean ± SD) (Figure 3). All treatments were significant at $P \leq 0.05$. Simultaneously, we measured cell proliferation using the Cayman MTT assay and found that both tobrhGAA doses slightly reduced cell growth by <10% (data not shown).

3.8. Biochemical/enzyme kinetic analyses

We compared the enzyme kinetics for a lysate of tobrhGAA *versus* placental human placental GAA and rhGAA, as well as the limited published data for alglucosidase alfa or other rhGAAs[99-107] for K_m, V_{max}, optimal pH, thermal heat

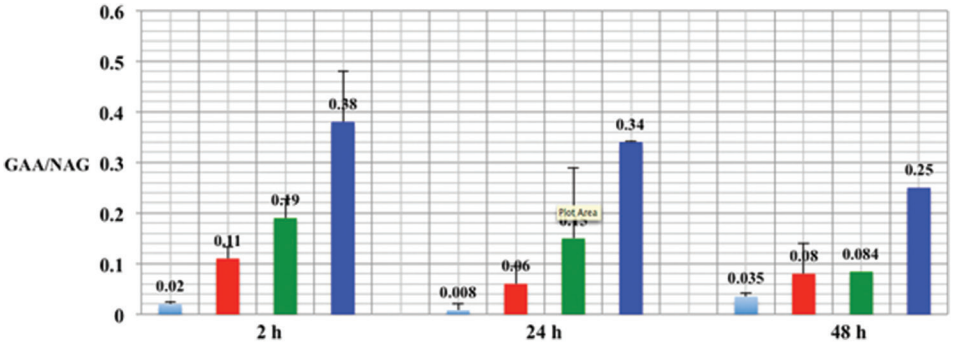

Figure 1. Uptake of tobrhGAA in human lymphoid cell lines. Human lymphoid cell lines from infantile or adult Pompe disease (GM6314, GM13793, and GM14450) were exposed to equivalent amounts of **mock**, **tobrhGAA**, **placental human GAA**, **or a rhGAA**. Cells were harvested after 2, 24, and 48 h and assayed for GAA and NAG (mean ± standard deviation)

Abbreviations: GAA: Acid α-glucosidase; NAG: Neutral α-glucosidase; h: Hours; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; rhGAA: Recombinant human acid α-glucosidase.

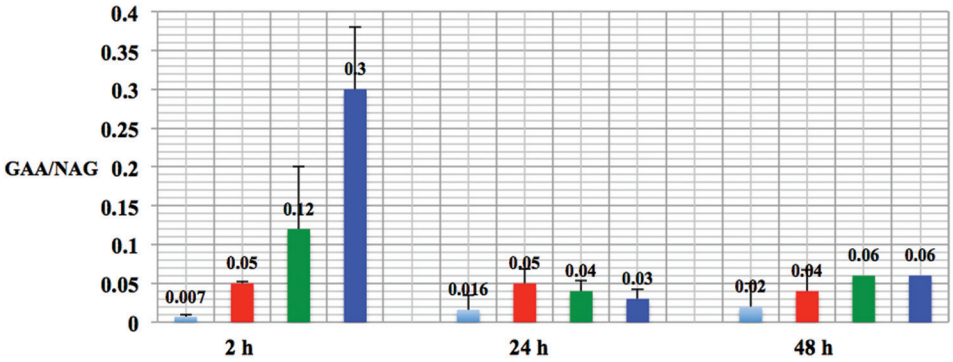

Figure 2. Uptake of tobrhGAA in human fibroblast cell lines. Human fibroblast (GM4912, GM1935, and GM3329) cell lines from infantile or adult Pompe disease were exposed to equivalent amounts **mock**, **tobrhGAA**, **placental human GAA**, **or a rhGAA**. Cells were harvested after 2, 24, and 48 h and assayed for GAA and NAG (mean ± standard deviation)
Abbreviations: GAA: Acid α-glucosidase; NAG: Neutral α-glucosidase; h: Hours; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; rhGAA: Recombinant human acid α-glucosidase.

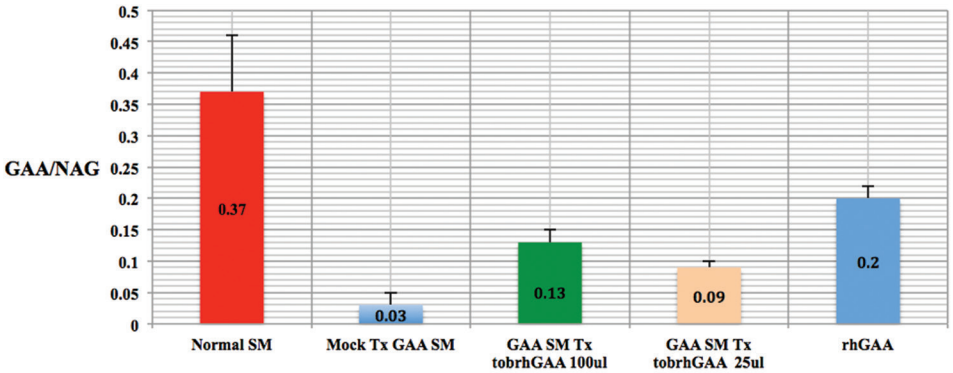

Figure 3. Uptake of tobrhGAA in a human Pompe disease myoblast cell line. A human Pompe disease myoblast cell line was exposed to equivalent amounts of a tobrhGAA seed lysate or rhGAA for 48 h and assayed for GAA and NAG (mean ± standard deviation)
Abbreviations: GAA: Acid α-glucosidase; NAG: Neutral α-glucosidase; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; rhGAA: Recombinant human acid α-glucosidase.

stability, and IC_{50} for inhibitors (acarbose, castanospermine, deoxynojirimycin, miglitol, and voglibose). Data showed that tobrhGAA was comparable to placental GAA and superior to alglucosidase alfa and other rhGAAs (Table 1).[93,97-107,121,122]

3.9. Short-term treatment

Forelimb grip strength was measured using a GMS in GAA KO mice (exon 6^{neo})[109] after three oral administrations with a lysate of tobrhGAA seeds (each dose containing 75 µg of tobrhGAA) every other day up to day 7. Wild-type mice (n = 3) averaged 245 ± 21 lbs (SEM) grip at release. Mock-treated GAA KO mice (n = 3) averaged 92 ± 3 lbs grip at release (3 – 5 attempts/mouse), and tobrhGAA lysate-treated GAA KO mice (n = 3) averaged 105 ± 3 lbs grip at release ($P \leq 0.024$ treated *vs.* mock-treated), which was a 14% improvement in strength. At day 7, mice were sacrificed, and the tissues were assayed for GAA/NAG and compared to the mock-treated GAA KO and wild-type mice. We found an increase in GAA activity in tissues from 8% to 23% of the wild-type mice tissues (heart, skeletal muscle, liver, and diaphragm) *versus* treated GAA KO mice (mean ± SD; with the statistical significance indicated) (Table 2).

3.10. Long-term treatment (203 days)

We treated two groups of GAA KO mice[109] (n = 3) 3 times a week with either a 1× or 3× dose (25 or 75 µg of tobrhGAA, respectively) orally[90] and measured their vertical hang-time over 203 days. Both treatment groups exhibited a steady, significant improvement in vertical hang-time *versus* the mock-treated GAA KO mice. Interestingly, the hang-time of mice in the 1× group gradually increased until it was equal to that of the 3× dose group by day 92, which suggests that the lower dose can be effective for long-term treatment. Both treatment groups showed a 25% – 35% increase *versus* the mock-treated wild-type mice at day 82, but by day 203, both groups were almost equal (89%

Table 1. Biochemical/enzyme kinetic analyses

Enzyme kinetics	From the literature		Human mature placental GAA		tobrhGAA		Myozyme** or other rhGAAs** published data for Myozyme is limited	
Substrate	**Km***	**Vmax***	**Km**	**Vmax**	**Km**	**Vmax**	**Km**	**Vmax**
Glycogen	10 – 42 mg/mL	6.63 mmole glucose/mg/h	18 mg/mL	5.2	21 mg/mL	5.8		
Maltose	2 – 22 mM	14.3 U/mg					16 mM	0.186 mmol/mg/h
4-MU-Glyc	0.8 – 1.1 mM	0.55 mmole/mg/h	0.95 mM	0.85	0.85 mM	0.65	1.08 – 2.1 mM	0.07 – 0.548 mmole/mg/h
Isomaltose	40 mM		ND		ND			
Starch	15.4 mM	10 U/mg	ND		ND			
Inhibitors IC_{50}								
Acarbose on 4-MU-Glc on glycogen	81 umol/L	54 umole/L	85 umol/L		75 umol/L		59 umol/L	
Maltose on 4-MU-Glc			45 mM		45 mM		ND	
Glycogen on 4-MU-Glc			15 mg/mL		10 mg/mL		ND	
Optimal pH		4 – 5.5	4 – 4.5 for 4-MU-Glc		4 – 4.5 for 4-MU-Glc		4 – 4.25 for glycogen 4 – 5.5 for 4-MU-Glc	
Heat stability		Variable depending on methods used	<5% residual activity after 5 min at 56°C		<5% residual activity after 5 min at 56°C		<5% residual activity after 24 h at 37°C	

Notes: *From literature for various species and tissue sources; **Published data for Myozyme is limited.
Abbreviations: GAA: Acid α-glucosidase; h: Hour; ND: Not found; U: Units; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase.

Table 2. GAA/NAG (mean±standard deviation) assay of mouse tissues after three administrations of tobrhGAA over 7 days through oral gavage (each dose containing 75 μg of tobrhGAA)

	Hindlimb tibialis anterior Skeletal Muscle	Heart	Diaphragm	Liver
Treated GAA KO - Oral tobrhGAA	0.12±0.04	0.10±0.05	0.13±0.07	0.19±0.02
***P* versus mock**	***P*≤0.015**	***P*≤0.15**	***P*≤0.16**	***P*≤0.0001**
Mock GAA KO- PBS	0.043±0.005	0.06±0.008	0.07±0.03	0.05±0.007
Wild-type-Balb/c	1.50±0.2	0.43±0.16	0.77±0.12	1.00±0.11
% of WT	*8*	*23*	*17*	*19*

Notes: Bold values indicate the *P* value versus the mock treated; Italic values indicate the percent of wildtype. Abbreviations: GAA: Acid α-glucosidase; PBS: Phosphate buffered saline; KO: Knockout; WT: Wild-type; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase.

– 95%) to the mock-treated wild-type mice (Figure 4). Both treatment groups improved significantly from the mock-treated group ($P \leq 0.007$). We found an increase in GAA activity in the tissues (heart, hindlimb skeletal muscle, kidney, liver, and diaphragm) from the GAA KO mice treated with tobrhGAA by oral gavage, thereby supporting Oral-ERT with tobrhGAA. The improvement percentage compared to the wild-type ranged from 13% to 38% depending on the tissue (Table 3) (mean ± SD, with statistical significance indicated), with the diaphragm being the highest at 38%. We also measured the tissue glycogen levels[109] after 203 days of treatment and found it ranged from 18% to 48% reduction from the mock-treated mice, with the greatest reduction in the skeletal muscle and diaphragm (43% and 48%, respectively) (Table 4) (mean ± SD, with statistical significance indicated).

Serum antibody titer to oral tobrhGAA at 203 days was undetectable at 4 weeks, barely detected at a 1:10 at 8 weeks for both 1× and 3× treatments, and detected at 1:20, thus demonstrating a low immune response.[100] We simultaneously generated mouse antibodies to human placental GAA by subcutaneous injection of 15 μg over 3 months and found the titer to be 1:1500 (data not shown). Thus, oral tobrhGAA of a similar antigen quantity did not generate a high serum immunoreaction. We determined whether tobrhGAA was taken up by taking a tissue sample from the tail end without sacrificing the mice and assayed for GAA at days 45 and 203 (Figure 5). Wild-type mice had GAA activity of 0.46 ± 0.14, mock-treated GAA KO had 0.064 ± 0.021, and treated GAA KO had 0.10 – 0.2 ± 0.05 for the 1× or 3× treatments ($P \leq 0.006$ treated *vs.* mock-treated).

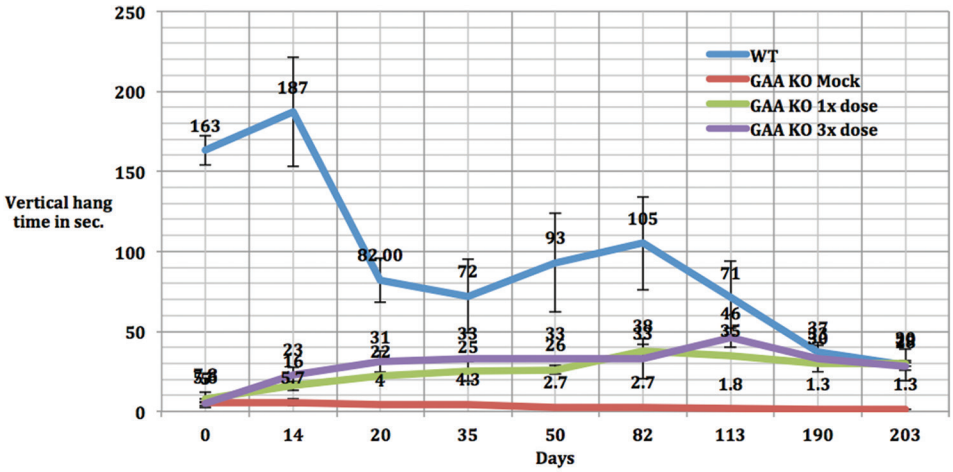

Figure 4. Long-term treatment and vertical hang-time measurements. We treated two groups of GAA KO mice (exon 6^{neo}) (n = 3) 3 times a week with either a 1× or 3× dose (25 or 75 μg of tobrhGAA, respectively) orally and measured the vertical hang-time over 203 days (mean ± standard deviation) Abbreviations: GAA: Acid α-glucosidase; KO: Knockout; sec.: Seconds; WT: Wild-type.

Table 3. GAA/NAG (mean±standard deviation) assay of mouse tissues after administration of tobrhGAA through oral gavage (oral-enzyme replacement therapy) at day 203

	Hindlimb tibialis anterior Skeletal Muscle	Heart	Diaphragm	Liver	Kidney
Treated GAA KO –1×Oral tobrhGAA	0.11±0.04	0.14±0.02	0.24±0.01	0.17±0.03	0.24±0.04
(*P* versus mock)	***P*≤0.05**	***P*≤0.05**	***P*≤0.027**	***P*≤0.06**	***P*≤0.037**
% of WT	***14***	***18***	***37***	***13***	***22***
Treated GAA KO –3×Oral tobrhGAA	0.13±0.01	0.13±0.01	0.25±0.01	0.21±0.05	0.23±0.01
(*P* versus mock)	***P*≤0.007**	***P*≤0.08**	***P*≤0.06**	***P*≤0.09**	***P*≤0.01**
% of WT	***16***	***17***	***38***	***16***	***20***
Mock GAA KO- PBS	0.07±0.044	0.065±0.044	0.08±0.14	0.05±0.009	1.35±0.02
Wild-type	0.8±0.11	0.76±0.23	0.65±0.38	1.33±0.36	1.12±0.08

Notes: bold values indicate the *P* value versus the mock treated. Abbreviations: GAA: Acid α-glucosidase; PBS: Phosphate buffered saline; KO: Knockout; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; WT: Wild-type.

Table 4. Percentage of glycogen reduction (mean±standard deviation) in mouse tissues after administration of tobrhGAA at day 203 (oral-enzyme replacement therapy)

	Hindlimb tibialis anterior skeletal muscle	Heart	Diaphragm	Liver	Kidney
Treated GAA KO–1×Oral tobrhGAA	659±36	616±9	429±62	840±26	680±26
% reduction from mock	39	32	48	18	28
(*P* versus mock)	*P*≤0.05	*P*≤0.001	*P*≤0.012	*P*≤0.014	*P*≤0.18
Treated GAA KO–3×Oral tobrhGAA	615±77	740±38	627±140	762±14	672±37
% reduction from mock	43	18	24	25	29
(*P* versus mock)	*P*≤0.05	*P*≤0.03	*P*≤0.15	*P*≤0.003	*P*≤0.02
Mock GAA KO	1067±201	898±18	825±2	1016±23	939±64

Abbreviations: GAA: Acid α-glucosidase; KO: Knockout; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase.

3.11. Assessment of spontaneous alternation in wild-type and GAA KO mice

Spontaneous alternation is used to assess the cognitive ability of rodents to choose one of the two goal arms of the T-maze. The advantage of a free-choice procedure is that hippocampal or lesioned animals often develop a side preference and score below 50%. Controls generally achieve a 60% – 80% correct alternation. We assessed the spontaneous alternative learning for cognitive ability in the T-maze in both male and female GAA KO mice and wild-type-129/C57 mice from 2 to 9 months of age. We found that a deficiency in spontaneous learning appeared

by 2 – 3 months in males and 3 – 4 months in female GAA KO mice (Figure 6). Spontaneous learning in GAA KO mice was significant *versus* wild-type mice (mean ± SD) ($P \leq 0.05$).

3.12. Oral-ERT with tobrhGAA in GAA KO mice

GAA KO mice were administered 50 mg or 150 mg of tobrhGAA ground sterile seeds daily and tissues, urine, blood, weight, and serum were collected. The mice were tested every 3 weeks for motor activity with a running wheel, forelimb muscle strength using GSM, motor coordination and balance with a rotarod, open-field mobility using a 5-min video to determine distance traveled, and spontaneous learning with a T-maze. Reversal of muscle weakness revealed that young mice responded faster than older mice and older mice required higher levels of tobrhGAA. Both age groups and doses showed improvement in spontaneous alternation by T-maze after 2 – 3 weeks of treatment (Table 5). *Indicates $P \leq 0.05$ when compared to pre-treatment data. CBC differentials of GAA-treated KO mice were identical to pre-treatment GAA KO and wild-type-129/C57 mice.

3.13. Steady-state GAA levels in treated tissues

We measured the steady-state GAA/NAG activity from the mice above. Interestingly, but not unexpectedly, as the tobrhGAA was given orally, the stomach and small intestine sections contained the highest ratio and higher levels than the wild-type-129/C57 mice (Table 6) (mean ± SD, with statistical significance indicated).[117,122-132]

3.14. PK

The plasma and urine clearance rate was measured in the GAA KO mice treated with the 3× dose (75 μg of tobrhGAA) or whole seeds at 0, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h. TobrhGAA activity showed maximum serum GAA concentration at 8 – 10 h and peak urine excretion at 10 – 12 h (Figures 7 and 8) (mean ± SD). Serum and

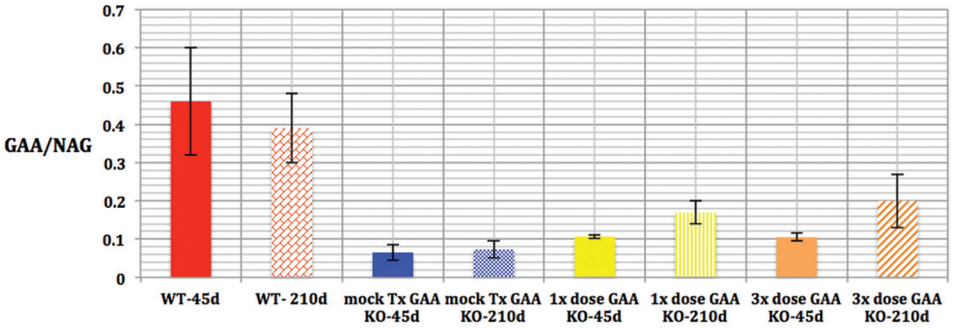

Figure 5. Take up of tobrhGAA by distant tissues. We determined if tobrhGAA was taken up by a distant tail tissue without sacrificing the mice and assayed for GAA at days 45 and 203 (mean ± standard deviation)
Abbreviations: d: Days; GAA: Acid α-glucosidase; NAG: neutral α-glucosidase; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase.

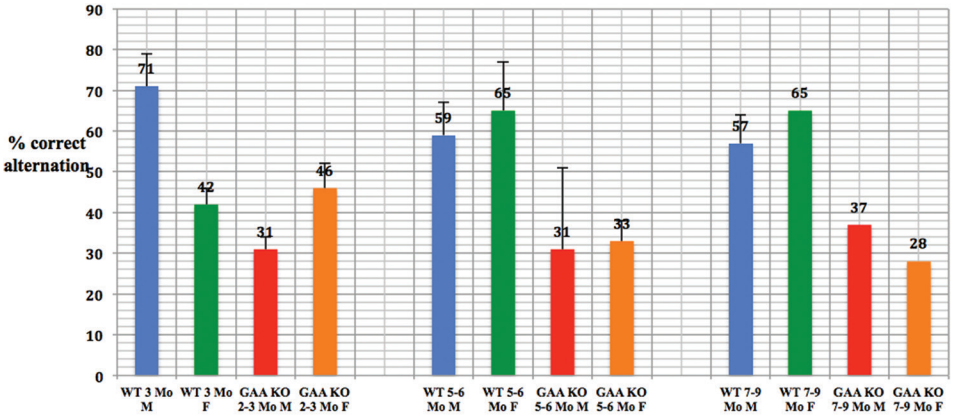

Figure 6. Assessment of spontaneous alternation in wild-type and GAA KO mice. We assessed the spontaneous alternative learning for cognitive ability using the T-maze evaluation in both male and female GAA KO mice and WT-129/C57 mice from the age of 2 – 9 months (mean ± standard deviation)
Abbreviations: GAA: Acid α-glucosidase; KO: Knockout; F: Female; M: Male; Mo: Mouse; WT: Wild-type.

Table 5. Motor activity by RW, forelimb muscle strength by GSM, motor coordination/balance with a rotarod, open-field mobility, and spontaneous learning with a T-maze

	RW	GSM	Rotarod	T-maze	Open-field mobility	Complete blood count diff.
Mean±standard deviation	rev/12 h	lbs at release	Time (s)	% correct alternation	cm/min±standard deviation	
Pre-Tx *n*=7 – 37 M/F	404±128	0.80±59	25±49	32±25	182±79	
GAA KO M/F-50 mg, *n*=4						
3 weeks	1800±80*	0.310±133*	158±138*	82±5*	243±46	Nl
6 weeks	2528±60*	0.394±178*	171±70*	65±9*	449±53*	Nl
12 weeks	2194±57*	0.304±126*	258±103*	70±2*	436±63*	Nl
GAA KO M/F-150 mg, *n*=3						
3 weeks	436±40	0.307±138*	120±73*	86±1*	442±75*	Nl
6 weeks	1248±55*	0.452±147*	323±57*	81±3*	432±49*	Nl
12 weeks	2549±68*	0.397±180*	355±104*	67±15*	344±54*	Nl
WT						
M/F *n*=12 – 15	3200±1423	0.569±296	264±101	60±16	334±96	

Note: *$P \leq 0.05$ when compared to pretreatment data using the Student's *t*-test (1-tail and 2-equal variance).
Abbreviations: GAA: Acid α-glucosidase; KO: Knockout; RW: Running wheel, GSM: Grip-strength meter; WT: Wild-type.

urine peaks showed a significant difference of $P \leq 0.011$ and $P \leq 0.05$, respectively, *versus* the baseline.[97,120,133-136]

3.15. Maximum tolerated dose

We determined the lethal dose in GAA KO mice ($n = 4$) starting at 10 mg seeds/mouse, doubling daily to 320 mg seeds/mouse. No weight loss or deaths were observed, and the CBC remained unchanged (data not shown).

4. Discussion

There is currently no effective treatment or cure for people living with PD. Sanofi-Genzyme, Inc. using a rhGAA (alglucosidase alfa) secreted from a CHO cell line has demonstrated moderate success in patients[28]; however, annual treatment costs are very high. Although alglucosidase alfa has been a wonderful first step in treating PD, it has revealed subtle aspects that must be considered for successful treatment.[19,41,43-50] ERT usually starts when the patients are already symptomatic; however, all secondary problems are already present, which are further compounded by the low uptake of alglucosidase alfa in the muscle (Table 7).[19] Lysosomal enzymes, such as GAA, are targeted to the lysosome by a mannose-6-phosphate (M6P) recognition sequence that is exposed by post-translational modification in the Golgi apparatus that may be the mechanism by which extracellular GAA can be recycled and targeted back to the lysosomes. This mechanism will potentially allow rhGAA to be delivered to the cells or tissues and directed to the lysosome. However, some GAA may be taken up or recycled by endocytosis or through a M6P-independent mechanism.[23-26] In skeletal muscle, there is a low abundance of cation-independent M6P receptors (CI-MPR), which is compounded by low blood flow plus low affinity of the CHO-produced alglucosidase alfa for CI-MPR. Alglucosidase alfa is delivered to the lysosome by receptor-mediated endocytosis after binding to the M6P-glycan of the CI-MPR. Alglucosidase alfa has one M6P per enzyme[137-139] and low affinity[140] with alglucosidase alfa, the high level of infused enzyme is transient, leaving the patients with no therapeutic enzyme for the remaining 10 – 13 days between treatments. In August 2021, the FDA-approved Nexviazyme (avalglucosidase alfa-ngpt) for the biweekly treatment of patients aged 1 year and older with late-onset PD. Nexviazyme is specifically designed to target M6P to improve cellular enzyme uptake and enhance glycogen clearance in the target tissues with an approximately 15-fold increase in M6P content. Nexviazyme has been demonstrated in clinical trials to provide late-onset patients with improvements in respiratory function and walking distance. Treated patients had improved forced vital capacity percent at week 49. However, the statistical superiority of Nexviazyme over alglucosidase alfa was not achieved ($P = 0.06$). A key secondary endpoint measured the functional endurance with a 6-min walk test; treated patients walked 32.2 m farther at week 49. The most frequently reported adverse reactions (>5%) in treated patients were headache, pruritus (itching sensation), nausea, hives, and fatigue. Infusion-associated reactions were reported in 25% of treated patients.[141] Pre-clinical studies in a PD mouse model have shown that avalglucosidase alfa has a 1,000-fold higher binding affinity to M6P receptors[142-144] and greater glycogen clearance from muscle.[142] In GAAKO mice, 4 mg/kg was the minimum pharmacologically active dose

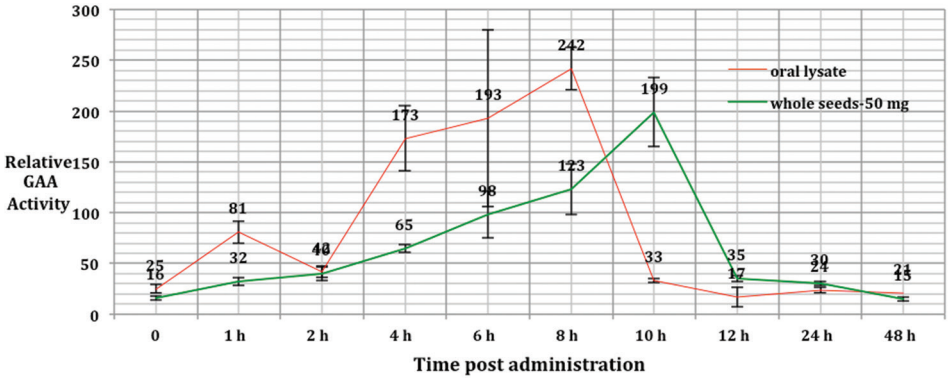

Figure 7. Pharmacokinetics of oral-enzyme replacement therapy in the serum of GAA KO mice. Serum clearance rate in GAA KO mice treated with a 3× dose (75 μg of tobrhGAA) or whole seeds was determined at 0, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h for tobrhGAA activity (mean ± standard deviation) Abbreviations: GAA: Acid α-glucosidase; h: Hours; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; KO: Knockout.

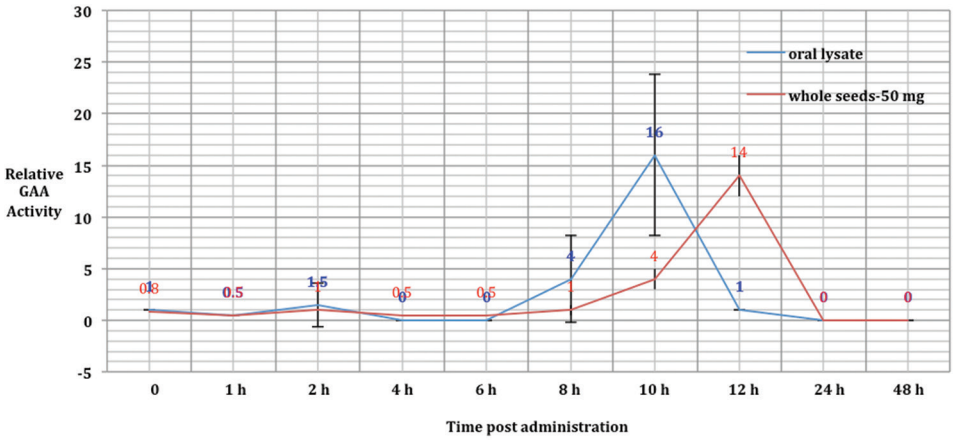

Figure 8. Pharmacokinetics of Oral-enzyme replacement therapy in the urine of GAA KO mice. The urine clearance rate in GAA KO mice treated with a 3× dose (75 mg of tobrhGAA) or whole seeds was determined at 0, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h for tobrhGAA activity (mean ± standard deviation) Abbreviations: GAA: Acid α-glucosidase; h: Hours; tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; KO: Knockout.

for cardiac and 12 mg/kg for skeletal muscle.[144] Therefore, oral formulations of ground tobrhGAA seeds will allow patients to ingest treatments daily in single or multiple doses conveniently at home to sustain a therapeutic dose of enzyme activity and to improve their quality of life.

Investigators are studying the role of autophagy,[19,145-147] an intracellular system for delivering portions of cytoplasm and damaged organelles to lysosomes for degradation and recycling in PD and the reduction of glycogen in skeletal muscle. A GAA and glycogen synthase 1 dual KO mouse model exhibited a profound reduction of glycogen in the heart and skeletal muscles, a decrease in lysosomal autophagic buildup, and correction of cardiomegaly. The abnormalities in glucose metabolism were corrected in the double-KO mice that demonstrated long-term elimination of muscle glycogen synthesis, which resulted in a significant improvement in the structural, metabolic, and functional defects and established a new perspective for the treatment of PD.[18] Lim *et al.*[147] reactivated mTOR in PD mice through TSC knockdown, resulting in the reversal of muscle atrophy and removal of autophagic buildup, as well as revealing that aberrant mTOR signaling can be reversed by arginine alone.[146-148] Jung *et al.*[149,150] produced and characterized a rhGAA in a transgenic rice cell suspension culture with high-mannose glycans, which was similar to the CHO-derived hGAA.

Sariyatun *et al.*[151] produced a human GAA with a paucimannose structure (Man3GlcNAc2M3) in a glycoengineered *Arabidopsis alg3* cell line. Recently, Cohen *et al.*[152,153] reported the safety and efficacy of the *in utero* ERT of a fetus with CRIM-neg IOPD. The family history was positive for IOPD with cardiomyopathy in two previously affected deceased siblings. After receiving six *in utero* ERT through the umbilical vein starting at 24 weeks

Table 6. Steady-state GAA levels in the treated tissues

GAA/NAG (mean±standard deviation) (*P*=Tx versus mock)	Liver	Heart	Hindlimb tibialis anterior muscle	Kidney	Lung	Diaphragm	Brain	SI-D	SI-J	SI-I	Spleen	Pancreas
GAA KO mock	0.073±0.05	0.06±0.03	0.018±0.0039	0.085±0.09	0.094±0.07	0.045±0.03	0.012±0.007	0.4±0.22	0.94±0.7	0.71±0.43	0.015±0.007	0.084±0.05
GAA KO Tx 50 mg	0.52±0.44	0.1±0.014	0.053±0.067	0.3±0.24	0.15±0.036	0.11±0.043	0.023±0.05	1.57±0.58	1.83±1.38	1.54±0.67	0.022±0.014	0.14±0.14
	P≤0.09	*P*≤0.15	*P*≤0.13	*P*≤0.11	*P*≤0.22	*P*≤0.07	*P*≤0.12	*P*≥0.007	*P*≥0.15	*P*≤0.07	*P*≥0.21	*P*≤0.28
GAA KO Tx 150 mg	0.64±1.6	0.2±0.21	0.08±0.29	0.78±3.1	0.3±0.71	0.2±0.21	0.05±0.33	2±6.5	6±47	5.1±22	0.07±0.11	0.45±1.0
	P≤0.038	*P*≤0.16	*P*≤0.05	*P*≤0.01	*P*≤0.1	*P*≤0.05	*P*≤0.1	*P*≤0.09	*P*≤0.12	*P*≤0.0.1	*P*≥0.06	*P*≤0.02
WT-129/C57	6.7±4	0.5±0.25	0.71±0.38	1.67±0.95	0.31±0.2	0.38±0.49	0.73±0.33	2.68±3.6	3.47±6.1	3.3±0.65	1.06±1.4	0.67±0.5
% WT	15	60	10	41	74	40	5	67	112	99	4	44

Abbreviations: GAA: Acid α-glucosidase; KO: Knockout; NAG: Neutral α-glucosidase; Tx: Treated; WT: Wild-type.

Table 7. Comparison of Myozyme and tobacco seeds expressing human acid α-glucosidase

Issues with the current therapy (Myozyme)	Our Oral-ERT with tobrhGAA addresses these issues
• Inefficient uptake	• Efficient uptake in appropriate tissues
• Transient nature every 2 weeks	• Daily Oral-ERT
• Glycogen reduction is a variable	• Glycogen reduction
• Autophagic and lipofuscin accumulation	• Autophagic and lipofuscin accumulation to be determined
• Immune response	• Immune response minimized
• High cost	• Projected lower cost of plant-made pharmaceuticals (1% – 10% of current therapy depending on the level of purification needed)
• Residual effective level	• Sustained daily increased residual GAA level
• Requires refrigeration and a clinic visit	• Extremely stable at room temperature for many years, ERT can be self-administered at home and no refrigeration is required

Abbreviations: tobrhGAA: Tobacco seeds expressing human acid α-glucosidase; ERT: Enzyme replacement therapy.

gestation and standard postnatal therapy, the patient had normal postnatal cardiac and age-appropriate motor functions, was meeting the developmental milestones, had normal biomarker levels, and was feeding and growing well at 13 months.[153,154]

Seeds may be a better vehicle for Oral-ERT of lysosomal diseases, such as PD, compared with an intravenous delivery system. Seeds – but not other plant tissues and organelles – contain the metabolic machinery required for the correct glycosylation, processing, phosphorylation, and synthesis of complex enzymes and proteins, as enzymes or proteins are protected or shielded from digestion in the stomach and small intestine and can be administered daily in single and multiple doses, and seeds provide long-term stable storage of the recombinant enzymes. Several biotechnology companies have tried to mass produce a rhGAA using different platforms. Prompted by studies of oral formulations of edible tissues in broccoli sprouts, corn/maize, pea, rice, tobacco, and tomato for pharmaceutical applications including vaccination to induce effective mucosal immune tolerance and immune reactions, GI, bacterial/viral infections, allergies, asthma, diabetes, endocrine-associated diseases, hypersensitivity, elevated BP, cholera, leucopenia, cancer, and RA,[127,154-173] we hypothesized that rhGAA from edible plant tissues will offer a cost-effective, innovative and safe strategy for Oral-ERT

for PD. We investigated the potential of genetically engineered edible plant tissues as an alternative large-scale production system that overcomes the high cost of producing rhGAA from CHO cells. Oral-ERT can be safer than infusion and can be ingested in a pill/capsule form at frequent intervals daily to maintain therapeutic levels of the enzyme to achieve long-term clinical efficacy. Oral-ERT with tobrhGAA is an innovative approach that overcomes some of the challenges of alglucosidase alfa-ERT and provides an effective, safe, and affordable treatment option as a PMP.[82,87-89,94] Therefore, to provide a less expensive alternative, we generated a rhGAA produced in tobacco seeds for ERT of PD. We found that the tobrhGAA compared favorably and was superior in many aspects to alglucosidase alfa (Table 7). After scaling up, we estimate that the annual cost for an adult living with PD would be $3,000, or <1% of the cost of alglucosidase alfa, which ranges from $250,000 to $650,000 per adult patient per year depending on weight. The successful demonstration of Oral-ERT with tobrhGAA will have significant clinical applications and shift the current clinical practice paradigm by offering a safe, room temperature stable, and affordable lifelong Oral-ERT for PD in the USA and worldwide, especially in areas where access to a clinical setting for biweekly intravenous administration of Myozyme is lacking. We estimate that an adult living with PD will need to take 3 – 4 g/day of tobrhGAA to be equivalent to 0.1% – <1% of Myozyme, depending on how it is branded and marketed to maintain a sustained GAA level of 5% – 10% of normal.

A retrospective study of medical and insurance records indicated that healthcare costs for patients with a rare disease are 3 – 5 times greater than those of patients without a rare disease. Approximately only 10% of rare diseases have an FDA-approved therapy, which emphasizes the urgent need for more research for earlier and more accurate diagnoses of and interventions for rare diseases. Most of the 7,000 – 10,000 known rare diseases disproportionately affect children, adolescents, and young adults. Individually, most rare diseases might affect only a few hundred to a few thousand worldwide. However, collectively rare diseases are common and affect an estimated 25 – 30 million patients in the USA. Many of these diseases are genetic, life-threatening, and challenging to diagnose and treat. Estimates from commercial and insurance payers show costs ranged from $8,812 to $140,044 for patients with a rare disease compared to $5,862 for those without a rare disease. Extrapolating the average cost for the 25 – 30 million patients with rare diseases in the USA results in a total yearly direct medical cost of approximately $400 billion.[174]

5. Conclusion

To provide a more affordable alternative to the current ERT, we generated rhGAA produced in tobacco seeds. We observed that tobrhGAA compared favorably and was superior to alglucosidase alfa in many aspects. After scaling up, we estimate that the annual cost of treatment for an adult living with PD would be $3,000, which is <1% of the cost of alglucosidase alfa ($250,000 – $650,000/adult patient/year). Oral-ERT with tobrhGAA is expected to have significant clinical applications and can lead to a shift in the current clinical practice paradigm by offering a safe, stable, and affordable lifelong Oral-ERT for PD in the USA and globally where access to a clinical setting for biweekly IV administration of Myozyme is lacking.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: All authors
Investigation: All authors
Methodology: Frank Martiniuk, Adra Mack, Justin Martiniuk, Gregory O. Voronin, Shoreh Miller, David Reimer, Nancy Rossi, Leslie Sheppard Bird, Sussan Saleh, Ruby Gupta, Mariel Nigro, Peter Meinke, Benedikt Schoser, Feng Wu, Kam-Meng Tchou-Wong
Writing – original draft: All authors
Writing – review & editing: All authors

Ethics approval and consent to participate

The Institutional Biosafety Committee ratified the project at biosafety level BSL1 containment, and this study was approved by the Institutional Animal Care and Use Committee at Rutgers, The State University of New Jersey (number: PROTO201800168).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

References

1. Slonim A, Bulone L, Ritz S, Goldberg T, Chen A, Martiniuk F. Identification of two subtypes of infantile acid maltase deficiency: Evaluation of twenty-two patients and review of the literature. *J Pediatr*. 2000;137:283-285.

   doi: 10.1067/mpd.2000.107112

2. Engel AG. Acid maltase deficiency. In: Engel AG, Franzini-Armstrong C, editors. *Myology*. New York: McGraw Hill, Inc.; 1994. p. 1533.

3. Reuser AJJ, Kroos M, Willemsen R, Swallow D, Tager JM, Galjaard H. Clinical diversity in glycogenosis type II. *J Clin Invest*. 1987;79:1689-1699.

   doi: 10.1172/JCI113008

4. Beratis N, LaBadie GU, Hirschhorn R. Genetic heterogeneity in acid alpha glucosidase deficiency. *Am J Hum Genet*. 1983;35(1):21-33.

5. Tagers JM, Oude Elferink RPJ, Reuser AJJ, *et al*. Alpha glucosidase deficiency; Pompe's disease. *Enzyme*. 1987;38:280-285.

6. van den Hout HM, Hop W, van Diggelen OP, *et al*. The natural course of infantile Pompe's disease: 20 original cases compared with 133 cases from the literature. *Pediatrics*. 2003;112:332-340.

   doi: 10.1542/peds.112.2.332

7. Winkel LP, Hagemans ML, van Doorn PA, *et al*. The natural course of non-classic Pompe's disease; a review of 225 published cases. *J Neurol*. 2005;252:875-884.

   doi: 10.1007/s00415-005-0922-9

8. Engel AG, Gomez MR, Seybold ME, Lambert EH. The spectrum and diagnosis of acid maltase deficiency. *Neurology*. 1973;23:95-106.

   doi: 10.1212/wnl.23.1.95

9. Mehler M, DiMauro S. Residual acid maltase activity in late-onset acid maltase deficiency. *Neurology*. 1997;27:178-184.

   doi: 10.1212/wnl.27.2.178

10. La Badie GU. *Biochemical and Immunologic Studies of Acid Alpha Glucosidase Deficiency, a Genetically Heterogeneous, Inherited Neuromuscular Disease*. Ph.D. Thesis, City University of New York, Mt. Sinai Hospital; 1986.

11. Raben N, Ralston E, Chien YH, *et al*. Differences in the predominance of lysosomal and autophagic pathologies between infants and adults with Pompe disease: Implications for therapy. *Mol Genet Metab*. 2010;101:324-331.

    doi: 10.1016/j.ymgme.2010.08.001

12. Martiniuk F, Chen A, Mack A, *et al*. Carrier frequency for glycogen storage disease type II in New York and estimates of affected individuals born with the disease. *Am J Med Genet*. 1998;79:69-72.

    doi: 10.1002/(sici)1096-8628(19980827)79:1<69:aid-ajmg16>3.0.co;2-k

13. Park KS. Carrier frequency and predicted genetic prevalence of Pompe disease based on a general population database. *Mol Genet Metab Rep.* 2021;27:100734.

    doi: 10.1016/j.ymgmr.2021.100734

14. Colburn R, Lapidus D. An analysis of Pompe newborn screening data: A new prevalence at birth, insight and discussion. *Front Pediatr*. 2024;11:1221140.

    doi: 10.3389/fped.2023.1221140

15. Reuser AJ, Kroos M, Elferink RP, Tager JM. Defects in synthesis, phosphorylation, and maturation of acid alpha glucosidase. *J Biol Chem*. 1985;260:8336-8341.

16. Strothotte S, Strigl-Pill N, Grunert B, *et al*. Enzyme replacement therapy with alglucosidase alfa in 44 patients with late-onset glycogen storage disease type 2:12-month results of an observational clinical trial. *J Neurol*. 2010;257:91-97.

17. van der Ploeg AT, Clemens PR, Corzo D, *et al*. A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N Engl J Med*. 2010;362:1396-1406.

    doi: 10.1056/NEJMoa0909859

18. Douillard-Guilloux G, Raben N, Takikita S, *et al*. Restoration of muscle functionality by genetic suppression of glycogen synthesis in a murine model of Pompe disease. *Hum Mol Genet*. 2010;19:684-696.

    doi: 10.1093/hmg/ddp535

19. Lim JA, Li L, Raben N. Pompe disease: From pathophysiology to therapy and back again. *Front Aging Neurosci*. 2014;6:177.

    doi: 10.3389/fnagi.2014.00177

20. van Gelder CM, Hoogeveen-Westerveld M, Kroos MA, *et al*. Enzyme therapy and immune response in relation to CRIM status: The Dutch experience in classic infantile Pompe disease. *J Inherit Metab Dis.* 2015;38:305-314.

    doi: 10.1007/s10545-014-9707-6

21. Lacana E, Yao LP, Pariser AR, Rosenberg AS. The role of immune tolerance induction in restoration of the efficacy of ERT in Pompe disease. *Am J Med Genet Part C Semin Med Genet*. 2012;160C:30-39.

    doi: 10.1002/ajmg.c.31316

22. Bali DS, Goldstein JL, Banugaria S, *et al*. Predicting cross-reactive immunological material (CRIM) status in Pompe disease using GAA mutations: Lessons learned from 10 years of clinical laboratory testing experience. *Am J Med Genet Part C Semin*. 2012;160C:40-49.

doi: 10.1002/ajmg.c.31319

23. Joseph A, Munroe K, Housman M, Garman R, Richards S. Immune tolerance induction to enzyme-replacement therapy by co-administration of short-term, low-dose methotrexate in a murine Pompe disease model. *Clin Exp Immunol*. 2013;152:138-146.

doi: 10.1002/ajmg.c.31319

24. Banugaria SG, Prater SN, McGann JK, *et al*. Bortezomib in the rapid reduction of high sustained antibody titers in disorders treated with therapeutic protein: Lessons learned from Pompe disease. *Genet Med*. 2013;15:123-131.

doi: 10.1038/gim.2012.110

25. Berrier KL, Kazi ZB, Prater SN, *et al*. CRIM-negative infantile Pompe disease: Characterization of immune responses in patients treated with ERT monotherapy. *Genet Med*. 2015;17:912-918.

doi: 10.1038/gim.2015.6

26. Banugaria SG, Prater SN, Ng YK, *et al*. The impact of antibodies on clinical outcomes in diseases treated with therapeutic protein: Lessons learned from infantile Pompe disease. *Genet Med*. 2011;13:729-736.

doi: 10.1097/GIM.0b013e3182174703

27. Messinger YH, Mendelsohn NJ, Rhead W, *et al*. Successful immune tolerance induction to enzyme replacement therapy in CRIM–negative infantile Pompe disease. *Genet Med*. 2012;14:135-142.

doi: 10.1038/gim.2011.4

28. Al Khallaf HH, Geffrard J, Botha E, Ali Pervaiz M. CRIM-negative Pompe disease patients with satisfactory clinical outcomes on enzyme replacement therapy. *JIMD Rep*. 2012;192:133-137.

doi: 10.1007/8904_2012_192

29. Brooks DS. Immune response to enzyme replacement therapy in lysosomal storage disorder patients and animal models. *Mol Genet Metab*. 1999;68:268-275.

doi: 10.1006/mgme.1999.2894

30. Kishnani PS, Goldenberg PC, DeArmey SL, *et al*. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab*. 2010;99:26-33.

doi: 10.1016/j.ymgme.2009.08.003

31. de Vries JM, van der Beek N, Kroos MA, *et al*. High antibody titer in an adult with Pompe disease affects treatment with alglucosidase alfa. *Mol Genet Metab*. 2010;101:338-345.

doi: 10.1016/j.ymgme.2010.08.009

32. Patel TT, Banugaria SG, Case LE, Wenninger S, Schoser B, Kishnani PS. The impact of antibodies in late-onset Pompe disease: A case series and literature review. *Mol Genet Metab*. 2012;106:301-309.

doi: 10.1016/j.ymgme.2012.04.027

33. Wang J, Lozier J, Johnson G, *et al*. Neutralizing antibodies to therapeutic enzymes: Considerations for testing, prevention and treatment. *Nat Biotechnol*. 2008;26:901-908.

doi: 10.1038/nbt.1484

34. Mendelsohn NJ, Messinger YH, Rosenberg AS, Kishnani PS. Elimination of antibodies to recombinant enzyme in Pompe's disease. *N Engl J Med*. 2009;360:194-195.

doi: 10.1056/NEJMc0806809

35. Hunley TE, Corzo D, Dudek M, et al. Nephrotic syndrome complicating α-glucosidase replacement therapy for Pompe disease. *Pediatrics*. 2004;114:e532-e535.

doi: 10.1542/peds.2003-0988-L

36. Banugaria SG, Prater N, Patel TT, *et al*. Algorithm for the early diagnosis and treatment of patients with cross reactive immunologic material-negative classic Infantile Pompe disease: A step towards improving the efficacy of ERT. *PLoS One*. 2013;8:e67052-12.

37. Nayak S, Doerfler PA, Porvasnik SL, *et al*. Immune responses and hypercoagulation in ERT for Pompe disease are mutation and rhGAA dose dependent. *PLoS One*. 2014;9:e98336.

doi: 10.1371/journal.pone.0098336

38. de Vries JM, Kuperus E, Hoogeveen-Westerveld M, *et al*. Pompe disease in adulthood: Effects of antibody formation on enzyme replacement therapy. *Genet Med*. 2017;19:90-97.

doi: 10.1038/gim.2016.70

39. Ohashi T, Iizuka S, Shimada Y, *et al*. Oral administration of recombinant human acid α-glucosidase reduces specific antibody formation against enzyme in mouse. *Mol Genet Metab*. 2011;103:98-100.

40. Parini R, De Lorenzo P, Dardis A, *et al*. Long term clinical history of an Italian cohort of infantile onset Pompe disease treated with enzyme replacement therapy. *Orphanet J Rare Dis*. 2018;13:32-44.

doi: 10.1186/s13023-018-0771-0

41. Gutschmidt K, Musumeci O, Díaz-Manera J, *et al*. STIG study: Real-world data of long-term outcomes of adults with Pompe disease under enzyme replacement therapy with alglucosidase alfa. *J Neurol*. 2021;268:2482-2492.

doi: 10.1007/s00415-021-10409-9

42. Korlimarla A, Spiridigliozzib GA, Stefanescua M, Austina SL, Kishnania PS. Behavioral, social and school functioning in children with Pompe disease. *Mol Genet Metab Rep*. 2020;25:100635.

doi: 10.1016/j.ymgmr.2020.100635

43. Raben N, Jatkar T, Lee A, *et al*. Glycogen stored in skeletal but not in cardiac muscle in acid α-glucosidase mutant (Pompe) mice is highly resistant to transgene-encoded

human enzyme. *Mol Ther*. 2002;6:601-608.

44. Neufeld EF. Lysosomal storage diseases. *Annu Rev Biochem*. 1991;60:257-280.

doi: 10.1146/annurev.bi.60.070191.001353

45. van den Hout JM, Reuser AJ, de Klerk JB, Arts WF, Smeitink JA, van der Ploeg AT. Enzyme therapy for Pompe disease with recombinant human α-glucosidase from rabbit milk. *J Inherit Metab Dis*. 2001;24:266-274.

doi: 10.1023/a:1010383421286

46. Reuser AJ, Kroos MA, Hermans MM, *et al*. Glycogenosis type II (acid maltase deficiency). *Muscle Nerve Suppl*. 1995;3S:61-69.

doi: 10.1002/mus.880181414

47. van den Hout H, Reuser AJ, Vulto AG, Loonen MC, Cromme-Dijkhuis A, van der Ploeg AT. Recombinant human α-glucosidase from rabbit milk in Pompe patients. *Lancet*. 2000;356:397-398.

doi: 10.1016/s0140-6736(00)02533-2

48. Amalfitano A, Bengur AR, Morse RP, *et al*. Recombinant human acid α-glucosidase enzyme therapy for infantile glycogen storage disease type II: Results of a phase I/II clinical trial. *Genet Med*. 2001;3:132-138.

49. Corti M, Liberati C, Smith BK, *et al*. Safety of intradiaphragmatic delivery of adeno-associated virus mediated alpha-glucosidase (rAAV1-CMV-hGAA) gene therapy in children affected by Pompe disease. *Hum Gene Ther Clin Dev*. 2017;28:208-218.

doi: 10.1089/humc.2017.146

50. Miyamoto Y, Etoh Y, Joh R, Noda K, Ohya I, Morimatsu M. Adult-onset acid maltase deficiency in siblings. *Acta Pathol Jpn*. 1985;35:1533-1542.

doi: 10.1111/j.1440-1827.1985.tb01450.x

51. Anneser JMH, Pongratz DE, Podskarbi T, Shin YS, Schoser BGH. Mutations in the acid α-glucosidase gene (M. Pompe) in a patient with an unusual phenotype. *Neurology*. 2005;64:368-370.

doi: 10.1212/01.WNL.0000149528.95362.20

52. Refai D, Lev R, Cross DT, Shimony JS, Leonard JR. Thrombotic complications of a basilar artery aneurysm in a young adult with Pompe disease. *Surg Neurol.* 2008;70:518-520.

doi: 10.1016/j.surneu.2007.05.049

53. Kretzschmar HA, Wagner H, Hubner G, Danek A, Witt TN, Mehraein P. Aneurysms and vacuolar degeneration of cerebral arteries in late-onset acid maltase deficiency. *J Neurol Sci*. 1990;98:169-183.

doi: 10.1016/0022-510x(90)90258-o

54. Makos MM, McComb RD, Hart MN, Bennett DR. Alpha-glucosidase deficiency and basilar artery aneurysm: Report of a sibship. *Ann Neurol*. 1987;22:629-633.

doi: 10.1002/ana.410220512

55. Kobayashi H, Shimada Y, Ikegami M, *et al*. Prognostic factors for the late onset Pompe disease with enzyme replacement therapy: From our experience of 4 cases including an autopsy case. *Mol Genet Metab*. 2010;100:14-19.

doi: 10.1016/j.ymgme.2010.01.015

56. Kenney-Jung D, Korlimarla A, Spiridigliozzi GA, *et al*. Severe CNS involvement in a subset of long-term treated children with infantile-onset Pompe disease. *Mol Genet Metab*. 2023;141:108119.

doi: 10.1016/j.ymgme.2023.108119

57. McCall AL, Dhindsa JS, Bailey AM, Pucci LA, Strickland LM, Eimallah MK. Glycogen accumulation in smooth muscle of a Pompe disease mouse model. *J Smooth Muscle Res*. 2021;57:8-18.

doi: 10.1540/jsmr.57.8

58. Hundsberger T, Rohrbach M, Kern L, Rösler KM. Swiss national guideline for reimbursement of enzyme replacement therapy in late-onset Pompe disease. *J Neurol.* 2013;260:2279-2285.

doi: 10.1007/s00415-013-6980-5

59. Kusnadi AR, Evangelista RL, Hood EE, Howard JA, Nikolov ZL. Processing of transgenic corn seed and its effect on the recovery of recombinant beta-glucuronidase. *Biotechnol Bioeng*. 1998;60:44-52.

doi: 10.1002/(sici)1097-0290(19981005)60:1<44:aid-bit5>3.0.co;2-0

60. Reggi S, Marchetti S, Patti T, et al. Recombinant human acid β-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. *Plant Mol Biol*. 2005;57: 101-113.

doi: 10.1007/s11103-004-6832-x

61. Stoeger E, Vaquero C, Torres E, *et al*. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol*. 2000;42:583-590.

doi: 10.1023/a:1006301519427

62. Kermode AR. Plants as factories for production of biopharmaceutical and bioindustrial proteins: Lessons from cell biology. *Can J Bot*. 2006;84:679-694.

63. Kermode AR. Seed expression systems for molecular farming. In: Wang A, Ma S, editors. *Molecular Farming in Plants: Recent Advances and Future Prospects*. New York: Springer; 2012. p. 89-123.

64. Lau OS, Sun SSM. Plant seeds as bioreactors for recombinant protein production. *Biotechnol Adv.* 2009;27:1015-1022.

doi: 10.1016/j.biotechadv.2009.05.005

65. Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J. GMP issues for recombinant plant-derived pharmaceutical

proteins. *Biotechnol Adv*. 2012;30:434-439.

doi: 10.1016/j.biotechadv.2011.08.007

66. Lico C, Santi L, Twyman RM, Pezzotti M, Avesani L. The use of plants for the production of therapeutic human peptides. *Plant Cell Rep.* 2012;31:439-451.

doi: 10.1007/s00299-011-1215-7

67. Twyman RM, Ramessar K, Quemada H, Capell T, Christou P. Plant biotechnology: The importance of being accurate. *Trends Biotechnol*. 2009;27:609-612.

doi: 10.1016/j.tibtech.2009.08.004

68. Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: Host systems and expression technology. *Trends Biotechnol.* 2003;21:570-578.

doi: 10.1016/j.tibtech.2003.10.002

69. Kwon K, Verma D, Singh N, Herzog R, Daniell H. Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells. *Adv Drug Deliv Rev*. 2013;65:782-799.

doi: 10.1016/j.addr.2012.10.005

70. Verma D, Moghimi B, LoDuca PA, *et al*. Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. *Proc Natl Acad Sci U S A*. 2010;107:7101-7106.

doi: 10.1073/pnas.0912181107

71. Sherman A, Su J, Lin S, Wang X, Herzog RW, Daniell H. Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. *Blood*. 2014;124:1659-1668.

doi: 10.1182/blood-2013-10-528737

72. Boothe J, Nykiforuk C, Shen Y, *et al*. Seed-based expression system for plant molecular farming. *Plant Biotechnol J*. 2010;8:588-606.

doi: 10.1111/j.1467-7652.2010.00511.x

73. Kwon KC, Daniell H. Low-cost oral delivery of protein drugs bioencapsulated in plant cells. *Plant Biotechnol J*. 2015;13:1017-1022.

doi: 10.1111/pbi.12462

74. Kaufman J. The economic potential of plant-made pharmaceuticals in the manufacture of biologic pharmaceuticals. *J Commercial Biotechnol*. 2011;17:173-182.

75. Kaiser J. Is the drought over for Pharming? *Science*. 2008;320:473-475.

doi: 10.1126/science.320.5875.473

76. Maxmen A. Drug making plant blooms. *Nature*. 2012;485:160.

doi: 10.1038/485160a

77. Spok A, Karner S. *Plant made Farming-Opportunities and Challenges*. Sevilla (Spain): JRC European Commission; 2008. p. 1-148.

78. Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Lainé AC, Gomord V, Faye L. N-glycosylation of recombinant pharmaceutical glycoproteins produced in transgenic plants: Towards an humanisation of plant N-glycans. *Plant Mol Biol.* 1998;38:31-48.

79. Stoeger E, Ma JK. Sowing the seeds of success: Pharmaceutical proteins from plants. *Curr Opin Biotechnol*. 2005;16:167-173.

doi: 10.1016/j.copbio.2005.01.005

80. Gomord V, Fischette AC, Menu-Bouaouiche L, *et al*. Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol J*. 2010;8:564-587.

81. Saint-Jore-Dupas C, Faye L, Gomord V. From planta to pharma with glycosylation in the toolbox. *Trends Biotechnol*. 2007;25:317-323.

doi: 10.1016/j.tibtech.2007.04.008

82. Gomord V, Faye L. Posttranslational modification of therapeutic proteins in plants. *Curr Opin Plant Biol*. 2004;7:171-181.

doi: 10.1016/j.pbi.2004.01.015

83. Kermode AR. Mechanisms of intracellular protein transport and targeting. *Crit Rev Plant Sci*. 1996;15:285-423.

doi: 10.1038/372055a0

84. He X, Galpin JD, Tropak MB, *et al*. Production of active human glucocerebrosidase in seeds of *Arabidopsis thaliana* complex-glycan-deficient (cgl) plants. *Glycobiology*. 2012;22:492-503.

doi: 10.1093/glycob/cwr157

85. Aviezer D, Almon-Brilla EY, Chertkoffa R, Hashmuelia S, Zimranb A. Novel enzyme replacement therapy for Gaucher disease: Phase III pivotal clinical trial with plant cell expressed recombinant glucocerebrosidase (prGCD)-taliglucerase alpha. *Mol Gen Metab*. 2010;99:S9.

86. Shaaltiel Y, Bartfeld D, Hashmueli S, *et al*. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnol J.* 2007;5:579-590.

doi: 10.1111/j.1467-7652.2007.00263.x

87. Grabowski GA, Golembo M, Shaaltiel Y. Taliglucerase alfa: An enzyme replacement therapy using plant cell expression technology. *Mol Genet Metab.* 2014;112:1-8.

doi: 10.1016/j.ymgme.2014.02.011

88. Tekoah Y, Shulman A, Kizhner T, *et al*. Large-scale production of pharmaceutical proteins in plant cell culture-the Protalix experience. *Plant Biotechnol J*. 2015;13:1199-1208.

89. Shaaltiel Y, Gingis-Velitski S, Tzaban S, Fiks N, Tekoah Y,

Aviezer D. Plant-based oral delivery of β-glucocerebrosidase as an enzyme replacement therapy for Gaucher's disease. *Plant Biotechnol J*. 2015;13:1033-1040.

doi: 10.1111/pbi.12366

90. Martiniuk F, Reggi S, Tchou-Wong KM, Rom WN, Busconi M, Fogher C. Production of a functional human acid maltase in tobacco seeds: Biochemical analysis, uptake by human GSDII cells, and *in vivo* studies in GAA knockout mice. *Appl Biochem Biotechnol*. 2013;171:916-926.

doi: 10.1007/s12010-013-0367-z

91. Martiniuk F, Chen A, Donnabella V, *et al*. Correction of glycogen storage disease type II by enzyme replacement with a recombinant human acid maltase produced by over-expression in a CHO-DHFR (neg) cell line. *Biochem Biophys Res Commun*. 2000;276:917-923.

doi: 10.1006/bbrc.2000.3555

92. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Roger SD, Fraley RT. A simple 344 and general method for transferring genes into plants. *Science*. 1985;227:1229-1231.

doi: 10.1126/science.227.4691.1229

93. Martiniuk F, Honig J, Hirschhorn R. Further studies of the structure of human placental acid alpha-glucosidase. *Arch Biochem Biophys*. 1984;231:454-460.

doi: 10.1016/0003-9861(84)90408-9

94. Paul M, Ma JKC. Plant-made pharmaceuticals: Leading products and production. *Biotech Appl Biochem*. 2011;58:58-67.

doi: 10.1002/bab.6

95. Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: Immediate release dosage forms. *Pharm Res*. 1998;15:11-22.

doi: 10.1023/a:1011984216775

96. Martiniuk F, Hirschhorn R. Characterization of neutral isozymes of human alpha-glucosidase. *Biochim Biophys Acta*. 1981;658:248-261.

doi: 10.1016/0005-2744(81)90295-3

97. Kakkis E, Lester T, Yang R, *et al*. Successful induction of immune tolerance to enzyme replacement therapy in canine mucopolysaccharidosis I. *Proc Natl Acad Sci U S A*. 2004;101:829-834.

doi: 10.1073/pnas.0305480101

98. White RR, Crawley FE, Vellini M, Rovati LA. Bioavailability of 125I bromelain after oral administration to rats. *Biopharm Drug Dispos*. 1988;9:397-403.

doi: 10.1002/bod.2510090408

99. van Hove JK, Yang HW, Wu JY, Brady RO, Chen YT. High-level production of recombinant human lysosomal acid α-glucosidase in Chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. *Proc Natl Acad Sci U S A*. 1996;93:65-70.

doi: 10.1073/pnas.93.1.65

100. Fuller M, van der Ploeg A, Reuser AJJ, Anson DS, Hopwood JJ. Isolation and characterisation of a recombinant, precursor form of lysosomal acid α-glucosidase. *Eur J Biochem*. 1995;234:903-909.

doi: 10.1111/j.1432-1033.1995.903_a.x

101. Hirschhorn R, Reuser AJJ. *Glycogen Storage Disease Type 2 Acid Alpha-Glucosidase Acid Maltase Deficiency*. Ch. 135. United States: Macgraw-Hill; 2001. p. 3389-3420.

102. Chadalavada DM, Sivakami S. Purification and biochemical characterisation of human placental acid α-glucosidase. *Biochem Mol Biol Int*. 1997;42:1051-1061.

doi: 10.1080/15216549700203511

103. Bijvoet AGA, Kroos MA, Pieper FR, *et al*. Recombinant human acid α-glucosidase: High level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice. *Hum Mol Genet*. 1998;7:1815-1824.

doi: 10.1093/hmg/7.11.1815

104. van Diggelen OP, Oemardien LF, van der Beek NAME, *et al*. Enzyme analysis for Pompe disease in leukocytes; superior results with natural substrate compared with artificial substrates. *J Inherit Metab Dis*. 2009;32:416-423.

doi: 10.1007/s10545-009-1082-3

105. Okumiya T, Keulemans JLM, Kroos MA, *et al*. A new diagnostic assay for glycogen storage disease type II in mixed leukocytes. *Mol Genet Metab*. 2006;88:22-28.

doi: 10.1016/j.ymgme.2005.10.016

106. Porto C, Ferrara MC, Meli M, *et al*. Pharmacological enhancement of α-glucosidase by the allosteric chaperone N-acetylcysteine. *Mol Ther*. 2012;20:2201-2211.

doi: 10.1038/mt.2012.152

107. Khanna R, Flanagan JJ, Feng J, *et al*. The pharmacological chaperone AT2220 increases recombinant human acid α-glucosidase uptake and glycogen reduction in a mouse model of Pompe disease. *PLoS One*. 2012;7:e40776.

doi: 10.1371/journal.pone.0040776

108. Hintze S, Limmer S, Dabrowska-Schlepp P, *et al*. Moss-derived human recombinant GAA provides an optimized enzyme uptake in differentiated human muscle cells of Pompe disease. *Int J Mol Sci*. 2020;21:2642-2657.

doi: 10.3390/ijms21072642

109. Raben N, Nagaraju K, Lee E, *et al*. Targeted disruption of the acid alpha glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen

storage disease type II. *J Biol Chem*. 1998;273:19086-19092.

doi: 10.1074/jbc.273.30.19086

110. Wheeler TL, Eppolito AK, Smith LN, Huff TB, Smith RF. A novel method for oral stimulant administration in the neonate rat and similar species. *J Neurosci Methods*. 2007;159:282-285.

doi: 10.1016/j.jneumeth.2006.07.019

111. Martiniuk F, Chen A, Mack A, *et al*. Helios gene gun particle delivery for therapy of acid maltase deficiency. *DNA Cell Biol*. 2002;21:717-725.

doi: 10.1089/104454902760599690

112. Gomez-Lechon MJ, Ponsada X, Castell JV. A microassay for measuring glycogen in 96 well cultured cells. *Anal Biochem*. 1996;236:296-301.

doi: 10.1006/abio.1996.0170

113. Ames BN, Lee FD, Durston WE. An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc Nat Acad Sci U S A*. 1973;70:782-786.

doi: 10.1073/pnas.70.3.782

114. Lin K, Wang A. UV mutagenesis in *Escherichia coli* K-12: Cell survival and mutation frequency of the chromosomal genes *lacZ, rpoB*, *ompF*, and *ampA*. *J Exp Microbiol Immunol*. 2001;1:32-46.

115. Reesora MJ, King IJ, Michael J. Development of a tetracycline resistant strain of *E. coli* sensitive to ultraviolet radiation. *J Stud Res*. 2014;3:63-68.

116. Liu A, Tran L, Becket E, *et al*. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. *Antimicrob Agents Chemother*. 2010;54:1393-1403.

doi: 10.1128/AAC.00906-09

117. *National Toxicology Program*. The *Salmonella*/*E. coli Mutagenicity*. Available from: https://ntp.niehs.nih.gov/go/9407 [Last accessed on 2014 Jun 27].

118. Cheung SCK, Liu LZ, Lan LL, *et al*. Glucose lowering effect of transgenic human insulin-like growth factor-I from rice: *In vitro* and *in vivo* studies. *BMC Biotechnol*. 2011;11:37-47.

doi: 10.1186/1472-6750-11-37

119. Iarmon CS, Phizackerlet PJ. The effects of starvation and re-feeding on glycogen metabolism in mouse tail skin. *Biochem J*. 1983;212:679-683.

doi: 10.1042/bj2120679

120. Inagawa H, Kohchi C, Soma GI. Oral administration of lipopolysaccharides for the prevention of various diseases: Benefit and usefulness. *Anticancer Res*. 2011;31:2431-2436.

121. Martiniuk F, Bodkin M, Tzall S, Hirschhorn R. Identification of the base pair responsible for a α-glucosidase allele with lower affinity for glycogen (GAA 2) and transient gene expression in deficient cells. *Am J Hum Genet*. 1990;47:440-445.

122. Martiniuk F, Ellenbogen A, Hirschhorn K, Hirschhorn R. Further regional localization of the genes for human acid alpha glucosidase (GAA), peptidase D (PEPD) and alpha mannosidase (MANB) by somatic cell hybridization. *Hum Genet*. 1985;69:109-111.

doi: 10.1007/BF00293278

123. Eskelinen EL, Illert AL, Tanaka Y, *et al*. Role of LAMP-2 in lysosome biogenesis and autophagy. *Mol Biol Cell*. 2002;13:3355-3368.

doi: 10.1091/mbc.E02-02-0114

124. Eskelinen EL, Schmidt CK, Neu S, *et al*. Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. *Mol Biol Cell*. 2004;15:3132-3145.

125. Koike M, Shibata M, Waguri S, *et al*. Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). *Am J Pathol*. 2005;167:1713-1728.

doi: 10.1016/S0002-9440(10)61253-9

126. Tanaka Y, Guhde G, Suter A, *et al*. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature*. 2000;406:902-906.

doi: 10.1038/35022595

127. Theeuwes F, Yum SI. Principles of the design and operation of generic osmotic pumps for the delivery of semisolid or liquid drug formulations. *Ann Biomed Eng*. 1976;4:343-353.

doi: 10.1007/BF02584524

128. Cao Y, Espinola JA, Fossale E, *et al*. Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis. *J Biol Chem*. 2006;29:20483-20493.

doi: 10.1074/jbc.M602180200

129. Raben N, Shea L, Hill V, Plotz P. Monitoring autophagy in lysosomal. *Methods Enzymol*. 2009;453:417-449.

doi: 10.1016/S0076-6879(08)04021-4

130. Schmued LC, Stowerts CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res*. 2005;1035:24-31.

doi: 10.1016/j.brainres.2004.11.054

131. Bian GL, Wei LC, Shi M, Wang YQ, Cao R, Chen LW. Fluoro-Jade C can specifically stain the degenerative neurons in the substantia nigra of the 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine-treated C57BL/6 mice. *Brain Res*. 2007;1150:55-61.

doi: 10.1016/j.brainres.2007.02.078

132. Falk DJ, Todd AG, Lee S, *et al*. Peripheral nerve and neuromuscular junction pathology in Pompe disease. *Hum Mol Genet*. 2015;24:625-636.

doi: 10.1093/hmg/ddu476

133. Bourne D. *A First Course in Pharmacokinetics and Biopharmaceutics/Biopharmaceutics*; 2010. Available from: https://www.boomer.org/c/p1 [last accessed on 2024 Dec 17].

134. loannou Y, Zeidner K, Gordon R, Desnick R. Fabry disease: Preclinical studies demonstrate the effectiveness of β-galactosidase a replacement in enzyme-deficient mice. *Am J Hum Genet*. 2001;68:14-25.

doi: 10.1086/316953

135. Schinkel A, Mayer U, Wagnenaar E, *et al*. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci U S A*. 1997;94:4028-4033.

doi: 10.1073/pnas.94.8.4028

136. van Tellingen O, Beijnen J, Verweij J, Scherrenburg E, Nooijen W, Sparreboom A. Rapid esterase-sensitive breakdown of polysorbate 80 and its impact on the plasma pharmacokinetics of docetaxel and metabolites in mice. *Clin Cancer Res*. 1999;5:2918-2924.

137. Maga JA, Zhou J, Kambampati R, *et al*. Glycosylation-independent lysosomal targeting of acid α-glucosidase enhances muscle glycogen clearance in pompe mice. *J Biol Chem*. 2013;288:1428-1434.

doi: 10.1074/jbc.M112.438663

138. Zhu Y, Li X, McVie-Wylie A, *et al*. Carbohydrate-remodelled acid α-glucosidase with higher affinity for the cation-independent mannose 6-phosphate receptor demonstrates improved delivery to muscles of Pompe mice. *Biochem J*. 2005;389:619-628.

doi: 10.1042/BJ20050364

139. McVie-Wylie AJ, Lee KL, Qiu H, *et al*. Biochemical and pharmacological characterization of different recombinant acid α-glucosidase preparations evaluated for the treatment of Pompe disease. *Mol Genet Metab*. 2008;94:448-455.

doi: 10.1016/j.ymgme.2008.04.009

140. Tong PY, Kornfeld S. Ligand interactions of the cation-dependent mannose 6-phosphate receptor. Comparison with the cation-independent mannose 6-phosphate receptor. *J Biol Chem*. 1989;264:7970-7975.

141. Diaz-Manera J, Kishnani PS, Kushlaf H, *et al*. Safety and efficacy of avalglucosidase alfa versus alglucosidase alfa in patients with late-onset Pompe disease (COMET): A phase 3, randomised, multicentre trial. *Lancet Neurol*. 2021;20:1012-1026.

doi: 10.1016/S1474-4422(21)00241-6

142. Zhu Y, Li X, Kyazike J, *et al*. Conjugation of mannose 6-phosphate-containing oligosaccharides to acid α-glucosidase improves the clearance of glycogen in Pompe mice. *J Biol Chem*. 2004;279:50336-50341.

doi: 10.1074/jbc.M409676200

143. Zhu Y, Jiang JL, Gumlaw NK, *et al*. Glycoengineered acid *a*-glucosidase with improved efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of Pompe disease. *Mol Ther*. 2009;17:954-963.

doi: 10.1038/mt.2009.37

144. Zhou Q, Stefano JE, Harrahy J, *et al*. Pan Strategies for neoglycan conjugation to human acid α-glucosidase. *Bioconjug Chem*. 2011;22:741-751.

doi: 10.1021/bc1005416

145. Fukuda T, Ewan L, Bauer M, *et al*. Dysfunction of endocytic and autophagic pathways in a lysosomal storage disease. *Ann Neurol*. 2006;59:700-708.

doi: 10.1002/ana.20807

146. Raben N, Schreiner C, Baum R, *et al*. Suppression of autophagy permits successful enzyme replacement therapy in a lysosomal storage disorder-murine Pompe disease. *Autophagy*. 2010;6:1078-1089.

doi: 10.4161/auto.6.8.13378

147. Lim JA, Li L, Shirihai OS, Trudeau KM, Rosa Puertollano R, Raben N. Modulation of mTOR signaling as a strategy for the treatment of Pompe disease. *EMBO Mol Med*. 2017;9:353-370.

doi: 10.15252/emmm.201606547

148. Carroll B, Maetzel D, Maddocks OD, *et al*. Control of TSC2-Rheb signaling axis by arginine regulates mTORC1 activity. *Elife*. 2016;5:e11058.

doi: 10.7554/eLife.11058

149. Jung JW, Kim NS, Jang SH, Shin YJ, Yang MS. Production and characterization of recombinant human acid alpha-glucosidase in transgenic rice cell suspension culture. *J Biotechnol*. 2016;226:44-53.

doi: 10.1016/j.jbiotec.2016.03.031

150. Jung JW, Huy NX, Kim HB, Kim NS, Giap DV, Yanga MS. Production of recombinant human acid α-glucosidase with high-mannose glycans in gnt1 rice for the treatment of Pompe disease. *J Biotechnol*. 2017;249:42-50.

doi: 10.1016/j.jbiotec.2017.03.033

151. Sariyatun R, Florence, Kajiura H, Ohashi T, Misaki R, Fujiyama K. Production of human acid-alpha glucosidase with a paucimannose structure by glycoengineered *Arabidopsis* cell culture. *Front Plant Sci*. 2021;12:703020.

doi: 10.3389/fpls.2021.703020

152. Cohen JL, Chakraborty P, Fung-Kee-Fung K, *et al*. In utero enzyme-replacement therapy for infantile-onset Pompe's disease. *N Engl J Med*. 2022;387:2150-2158.

doi: 10.1056/NEJMoa2200587

153. van der Ploeg AT. Science behind the study-prenatal enzyme-replacement therapy. *N Eng J Med*. 2022;387:2189-2193.

doi: 10.1056/NEJMe2211515

154. Hashizume F, Hino S, Kakehashi M, *et al*. Development and evaluation of transgenic rice seeds accumulating a type II-collagen tolerogenic peptide. *Transgenic Res*. 2008;17:1117-1129.

doi: 10.1007/s11248-008-9187-2

155. Keum YS, Khor TO, Lin W, *et al*. Pharmacokinetics and pharmacodynamics of broccoli sprouts on the suppression of prostate cancer in transgenic adenocarcinoma of mouse prostate (TRAMP) mice: Implication of induction of Nrf2, H0-1 and apoptosis and the suppression of Akt-dependent kinase pathway. *Pharm Res*. 2009;26:2324-2331.

doi: 10.1007/s11095-009-9948-5

156. Lamphear BJ, Streatfield SJ, Jilka JM, *et al*. Delivery of subunit vaccines in maize seed. *J Control Release*. 2002;85:169-180.

doi: 10.1016/s0168-3659(02)00282-1

157. Ning T, Xie T, Qiu Q, *et al*. Oral administration of recombinant human granulocyte macrophage colony stimulating factor expressed in rice endosperm can increase leukocytes in mice. *Biotech Lett*. 2008;30:1679-1686.

doi: 10.1007/s10529-008-9717-2

158. Pena Ramırez YJ, Tasciotti E, Gutierrez-Ortega A, *et al*. Fruit-specific expression of the human immunodeficiency virus type 1 Tat gene in tomato plants and its immunogenic potential in mice. *Clin Vaccine Immunol*. 2007;14:685-692.

doi: 10.1128/CVI.00028-07

159. Sardana RK, Alli Z, Dudani A, *et al*. Biological activity of human granulocyte-macrophage colony stimulating factor is maintained in a fusion with seed glutelin peptide. *Transgenic Res*. 2002;11:521-531.

doi: 10.1023/a:1020343501475

160. Streatfield SJ, Lane JR, Brooks CA, *et al*. Corn as a production system for human and animal vaccines. *Vaccine*. 2003;21:812-815.

doi: 10.1016/s0264-410x(02)00605-9

161. Suzuki K, Kaminuma O, Yang L, *et al*. Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: Induction of allergen-specific oral tolerance without bystander suppression. *Plant Biotechnol J*. 2011;9:982-990.

162. Tackaberry ES, Dudani AK, Prior F, *et al*. Development of biopharmaceuticals in plant expression systems: Cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. *Vaccine*. 1990;17:3020-3029.

doi: 10.1016/s0264-410x(99)00150-4

163. Takagi H, Hiroi T, Yang I, *et al*. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proc Natl Acad Sci U S A*. 2005;102:17525-17530.

doi: 10.1073/pnas.0503428102

164. Takagi H, Hiroi T, Yang L, *et al*. Efficient induction of oral tolerance by fusing cholera toxin B subunit with allergen-specific T-cell epitopes accumulated in rice seed. *Vaccine*. 2008;26:6027-6030.

doi: 10.1016/j.vaccine.2008.09.019

165. Takagia H, Hiroi T, Hirosea S, Yanga L, Takaiwaa F. Rice seed ER-derived protein body as an efficient delivery vehicle for oral tolerogenic peptides. *Peptides*. 2009;31:1421-1425.

doi: 10.1016/j.peptides.2010.04.032

166. Takaiwa F. Update on the use of transgenic rice seeds in oral immunotherapy. *Immunotherapy*. 2013;5:301-312.

doi: 10.2217/imt.13.4

167. Wu J, Yu L, Li L, Hu J, Zhou J, Zhou X. Oral immunization with transgenic rice seeds expressing VP2 protein of infectious bursal disease virus induces protective immune responses in chickens. *Plant Biotechnol. J*. 2007;5:570-578.

doi: 10.1111/j.1467-7652.2007.00270.x

168. Xie T, Qiu Q, Zhang W, *et al*. A biologically active rhIGF-1 fusion accumulated in transgenic rice seeds can reduce blood glucose in diabetic mice via oral delivery. *Peptides*. 2008;29:1862-1870.

doi: 10.1016/j.peptides.2008.07.014

169. Yamada Y, Nishizawa K, Yokoo M, *et al*. Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin. *Peptides*. 2008;29:331-337.

170. Yang L, Kajiura H, Suzuki H, Hirose S, Fujiyama K, Takaiwa F. Generation of a transgenic rice seed-based edible vaccine against house dust mite allergy. *Bioch Biophys Res Commun*. 2008;365:334-339.

doi: 10.1016/j.bbrc.2007.10.186

171. Yang L, Tada Y, Yamamoto MP, Zhao H, Yoshikawa M, Takaiw F. A transgenic rice seed accumulating an anti-hypertensive peptide reduces the blood pressure of spontaneously hypertensive rats. *FEBS Lett*. 2006;580:3315-3320.

doi: 10.1016/j.febslet.2006.04.092

172. Yang L, Wakasa Y, Takaiwa F. Biopharming to increase bioactive peptides in rice seed. *JAOAC Int*. 2008;91:957-966.

173. Zimmermann J, Saalbach I, Jahn D, *et al*. Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. *BMC Biotechnol*. 2009;9:79.

doi: 10.1186/1472-6750-9-79

174. Tisdale A, Cutillo CM, Nathan R, *et al*. The IDeaS initiative: Pilot study to assess the impact of rare diseases on patients and healthcare systems. *Orphanet J Rare Dis*. 2021;16:429.

doi: 10.1186/s13023-021-02061-3

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Correlation between SIRT1 expression and overall survival across various cancers: A meta-analysis

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Abstract

Sirtuin 1 (SIRT1) plays a complex role in cancer, significantly influencing cancer prognosis. However, its true impact remains unclear, necessitating a comprehensive and unified analysis. This study investigates the prognostic value of SIRT1 expression across different cancers worldwide. Relevant studies were retrieved from Google Scholar, PubMed, and Web of Science. Only human studies exploring the prognostic role of SIRT1 in various cancers were included in the study. Data on patient demographics, SIRT1 expression levels, and overall survival (OS) rates were extracted, followed by subsequent analyses, including meta-regression for heterogeneity and Egger's regression for publication bias. A total of 15 studies (pooled hazard ratio = 1.483 ± 0.2974 [95% CI: 0.900 – 2.065]), predominantly from Asia ($n = 10$), were selected for the meta-analysis. The analysis demonstrated a robust and consistent association between elevated SIRT1 expression and poorer OS across diverse variables ($P < 0.001$), with minimal heterogeneity ($I^2 = 0.0001$). Subgroup analyses confirmed significant effects of SIRT1 on OS rates based on regions (Asia: $P < 0.001$; non-Asia: $P < 0.001$), study size (small: $P = 0.001$, medium: $P = 0.002$, and large cohorts: $P = 0.021$), and specific cancer types (ovarian: $P = 0.041$, lung: $P = 0.025$, and gastric cancer: $P = 0.025$). However, no significant impact was observed in breast ($P = 0.136$) and colorectal cancers ($P = 0.221$). Overall, SIRT1 demonstrates consistent prognostic significance across various cancers and populations. However, the assessment of its long-term prognostic effects remains elusive, highlighting the need for further mechanistic research to guide targeted interventions and precision medicine in cancer therapy.

Keywords: Sirtuin; Prognosis; Cancer; Precision medicine; Oncology research

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1. Introduction

Sirtuin 1 (SIRT1), a member of the sirtuin family, has attracted considerable attention due to its regulatory impact on various cellular pathways. It is the most well-studied isoform among the sirtuin family members,[1] which are involved in regulating a wide range of diseases, including stress responses,[2] apoptosis,[3] and gene stability.[4] This extensive influence positions SIRT1 as a key player in cancer progression and development.

Although the sirtuin family comprises several members, the focus on SIRT1 stems from its prevalence and prominent role in maintaining cellular homeostasis.[5,6] Its unique enzymatic activity and significant involvement in crucial cellular processes distinguish it from other family members, warranting dedicated exploration. Studies have highlighted both tumor-suppressive and tumor-promoting roles for SIRT1. For instance, SIRT1 can promote tumorigenesis by deacetylating p53, which leads to the downregulation of p53 and the upregulation of karyopherin subunit alpha 3 (KPNA3) through modulation of microRNA (miR)-101. This cascade enhances tumor cell survival, migration, chemoresistance, and proliferation in colorectal cancer.[7] In addition, SIRT1 supports epithelial-to-mesenchymal transition, which facilitates metastasis in various tumor types.[8] However, SIRT1 also exhibits tumor-suppressive effects by maintaining genomic stability and regulating oxidative stress responses.[9] The balance between these roles depends on the specific context. For example, in lung cancer, SIRT1 inhibits autophagy in cancer cells, thereby reducing cell proliferation, invasion, and migration.[10]

The current body of research on SIRT1 in cancer prognosis, spanning from 2012 to 2023, reveals inconsistencies. SIRT1 is elevated in ovarian cancer,[11] gastric cancer,[12] liver cancer,[13] and breast cancer.[14] While previous meta-analyses have examined the prognostic significance of SIRT1, they have largely focused on Asian populations, specific cancer types, or older publications.[15-17] For instance, SIRT1 expression has been associated with poor overall survival (OS) and disease-free survival in breast cancer.[14] In esophageal squamous cell carcinoma (ESCC), high SIRT1 expression correlates with advanced tumor stages and poor prognosis.[18,19] A broader meta-analysis encompassing multiple cancer types has shown that elevated SIRT1 levels consistently predict worse survival outcomes and are associated with tumor progression.[17] However, a study by Jung *et al.*[20] reached a different conclusion, suggesting that higher SIRT1 expression may improve survival. This ongoing controversy remains about whether SIRT1 overexpression predicts good or poor prognosis highlights the need for a more robust analysis. Most evidence supports that view that overexpressed SIRT1 negatively affects cancer prognosis.[21-23] These conflicting results underscore the need for a comprehensive investigation to better understand the true role of SIRT1 in cancer progression.

Therefore, this study aims to assess the prognostic significance of SIRT1 expression across various cancer types, synthesizing existing findings to provide a clearer understanding of its impact on cancer prognosis in different malignancies and geographical regions. Furthermore, this paper seeks to offer insights into the potential clinical implications of SIRT1 expression for cancer patients.

2. Methods

2.1. Search strategy

Our meta-analysis follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. The authors – MI, ST, and SA – conducted a comprehensive independent search for relevant studies and retrieved references from Google Scholar, PubMed, and Web of Science using a range of keywords, such as "SIRT1," "cancer," "prognosis," and "survival" (Supplementary File for detailed search strategy). The time window spanned from January 2015 to November 2023. All retrieved references were processed in EndNote v21.2 (Clarivate) for screening.

2.2. Inclusion and exclusion criteria

The retrieved studies were deduplicated and screened by reviewing titles, abstracts, types of articles, and the language of publication independently by three authors – UAKS, FI, and KM. Original research studies that specifically reported the prognostic role of SIRT1 in various human cancers, published in English, and included all necessary details, were included by MI and ST. Studies were excluded if they were not original research articles (e.g., reviews, commentaries, conference abstracts, and protocols), focused on the role of SIRT1 in areas unrelated to cancer, or were conducted using animal models. In addition, studies published in non-English languages or those lacking essential data were excluded from the study. Any discrepancies during the screening process were discussed and resolved by the corresponding authors, AA and MBK.

2.3. Data extraction

The included studies were independently reviewed by three authors – MI, ST, and SA – for data extraction. Various variables were extracted using Google Forms, which were later exported into an Excel spreadsheet in MS Office 2021 v2310 (Microsoft, United States). The extracted variables included: (i) Study characteristics (author, year, country, sample size, cancer type, etc.), (ii) patient characteristics (age, sex, tumor stage, etc.), (iii) SIRT1 expression levels, and (iv) OS rates. Standardized categories were used for tumor stage and sample size to ensure consistency across studies. Studies with incomplete data, such as missing key prognostic details (e.g., tumor stage or survival rates), were excluded from the final analysis. The values for all variables were then standardized and formatted for further analysis by the authors AA and MBK.

2.4. Quality appraisal of studies

Two authors, UAKS and FI, independently assessed the quality of the included studies following the guidelines of the Joanna Briggs Institute. The evaluation involved checking the relevance of each study against specific criteria (Supplementary File for detailed quality appraisal criteria). The focus was on studies examining how SIRT1 influences the prognosis of various human cancers. This thorough review process included comprehensive assessments of the following: sample frame appropriateness, participant sampling, adequacy of sample size, detailed descriptions of subjects and settings, coverage of data analysis, validity of methods for condition identification, standardization and reliability of condition measurement, appropriateness of statistical analysis, and adequacy of response rate. Any disagreements were resolved through discussion, and the corresponding authors, AA and MBK, were consulted.

2.5. Statistical analysis

Meta-analysis was conducted using SPSS Statistics v28.0.1.1 (IBM, United States) under a subscription-based license, with pre-calculated effect sizes, primarily hazard ratios (HRs) for OS rates, and the selection of a random effects model. A forest plot was generated for the overall analysis. Subgroup analyses, based on each variable, were performed using the same protocol. Meta-regression was conducted to investigate sources of heterogeneity, with variables such as study location, sample size categories, cancer types, age groups, tumor stage, and SIRT1 expression included as moderators. The criteria for assessing heterogeneity included Cochran's Q-statistics and the I^2 statistic. Small-study effects were evaluated using Egger's regression-based test, where the intercept of the regression line was used to assess asymmetry in the funnel plot, which could indicate potential bias. Tests of residual homogeneity and residual heterogeneity were also conducted. Bubble plots were generated to visually represent the relationship between moderators and effect sizes. Publication bias was assessed through Egger's regression-based test, including intercept regression and statistical estimation based on *t*-statistics, examining the effect size and standard errors of individual studies. Finally, bubble and funnel plots were drawn to further assess the results.

3. Results

Upon evaluation, 15 studies (total number of cases = 3059) out of 158 eligible studies met the inclusion criteria (Figure 1) and were selected for analysis (Table 1). The majority of studies were conducted in Asia (n = 10), specifically China (n = 5), while only five studies were conducted in non-Asian regions. The included studies covered seven cancer types: ovarian (n = 3), uterine (n = 2), renal cell (n = 2), lung (n = 3), gastric (n = 2), breast (n = 1), and colorectal cancer (n = 2). Most studies employed immunohistochemistry to analyze SIRT1 expression, with the exception of Tan *et al.*,[35] who used gene set enrichment analysis. The majority of studies (n = 8) had smaller sample sizes (<100), while five studies had medium sample sizes (≈100 – 500), and only two studies had larger sample sizes (>500).

3.1. Overall findings

The current meta-analysis, using a random-effects model to account for potential variability between studies, revealed a significant prognostic effect for SIRT1 (pooled HR = 1.483 ± 0.2974, 95% confidence interval (CI): 0.900 – 2.065), as depicted in Figure 2. This pooled effect size demonstrated a strong, statistically significant, and positive prognostic value for SIRT1. The statistical significance was underscored by a Z-score of 4.98 (P < 0.001), indicating that SIRT1 has a substantial impact in the context of the analyzed studies. However, the heterogeneity statistics (I^2 = 0.00, specifically, H^2 = 1.00 and Tau^2 = 0.0001) in our meta-analysis indicated a remarkably low level of variability among the included studies. In addition, the homogeneity analysis showed high homogeneity (I^2 = 0.0001), further supporting the robustness and consistency of the findings. These results underscore the significance of SIRT1, demonstrating its substantial impact as a prognostic factor in the analyzed studies.

3.2. Confirmatory analysis for heterogeneity

The presence of heterogeneity in the meta-analysis was extensively examined for various potential moderators, as shown in Table 2, including country (Figure 3A), sample size categories (Figure 3B), cancer type (Figure 3C), age group (Figure 3D), tumor stage (Figure 3E), and SIRT1

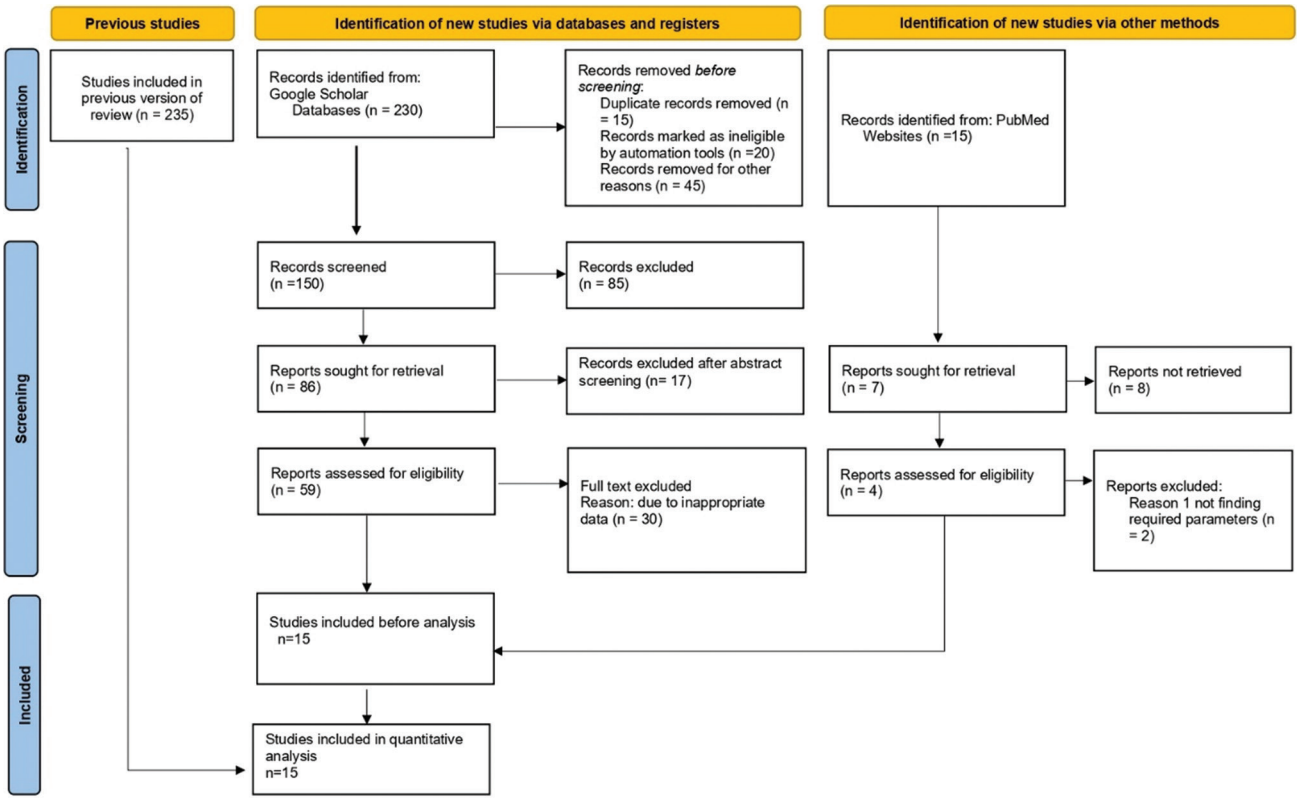

Figure 1. Flowchart illustrating the systematic selection process. The screening resulted in 15 eligible records after excluding duplicates, non-English literature, studies that were not original research, and those with missing details.

Table 1. Characteristics of the studies selected for the meta-analysis

S. No.	Authors	Year	Country	Sample size	Male	Female	Cancer type	Mean age	Tumor stage	Detection method	Expression level
1	Benard *et al.*[24]	2015	Netherlands	254	128	126	CRC	50	I–III	Immunohistochemistry	Low
2	Li *et al.*[25]	2015	China	75	39	36	Lung cancer	59	I–III & IV	Immunohistochemistry	High
3	Grbesa *et al.*[26]	2015	Spain	105	93	12	Lung cancer	63	I–IV	Immunohistochemistry	High
4	Zhang *et al.*[27]	2015	China	50	32	18	CRC	60	I–IV	Immunohistochemistry	High
5	Teramae *et al.*[28]	2015	Japan	62	N/A	62	Uterine cancer	70	III	Immunohistochemistry	High
6	Shuang *et al.*[11]	2015	China	63	N/A	63	Ovarian cancer	63	I–IV	Immunohistochemistry	Low
7	Qiu *et al.*[29]	2016	China	96	75	21	Gastric cancer	60	I–III & IV	Immunohistochemistry	High
8	Szász *et al.*[30]	2016	Hungary	1065	566	244	Gastric cancer	N/A	I–IV	Immunohistochemistry	High
9	Chung *et al.*[31]	2016	South Korea	344	N/A	344	Breast cancer	N/A	N/A	Immunohistochemistry	Low
10	Jeh *et al.*[32]	2017	South Korea	119	74	28	Renal cell carcinoma	59	I	Immunohistochemistry	Low
11	Mvunta *et al.*[33]	2017	Japan	68	N/A	68	Ovarian cancer	50	III–IV	Immunohistochemistry	High
12	Gharabaghi *et al.*[34]	2018	Iran	40	N/A	N/A	Lung cancer	N/A	I–III	Immunohistochemistry	High
13	Tan *et al.*[35]	2020	China	530	344	186	Renal cell carcinoma	60	I–III & IV	Gene set enrichment analysis	Low
14	Beyer *et al.*[36]	2020	Germany	65	N/A	65	Uterine cancer	64	I–IV	Immunohistochemistry	High
15	Chen *et al.*[37]	2022	Germany	123	N/A	123	Ovarian cancer	59	I–IV	Immunohistochemistry	High

Abbreviations: CRC: Colorectal cancer; N/A: Not available.

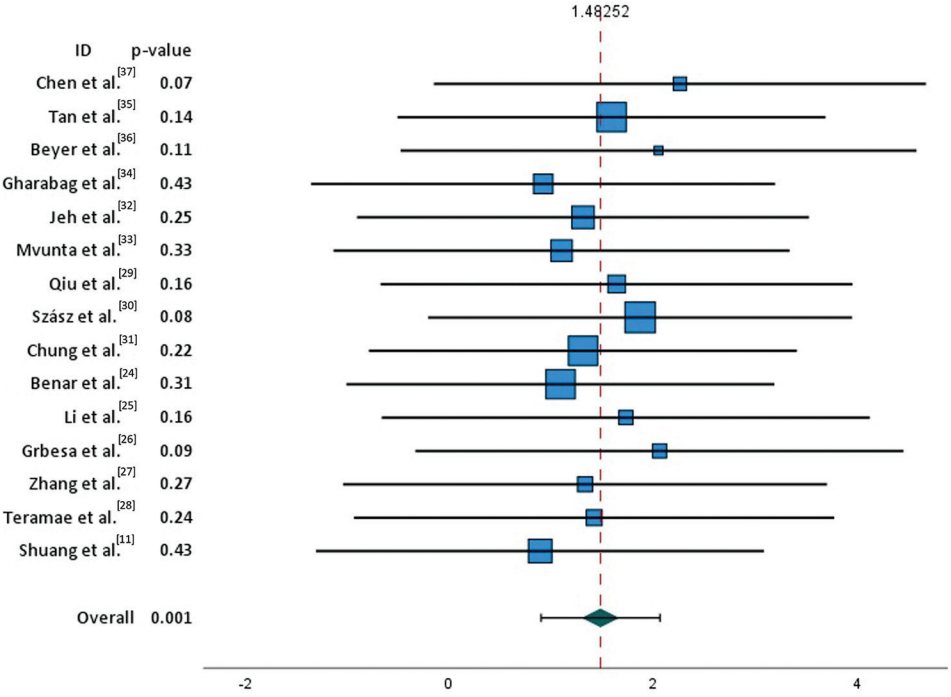

Figure 2. Forest plot illustrating the individual effect of each study. Among the 15 studies, Shuang *et al.*[11] ($P = 0.43$) show a notably distinct effect with a higher *P*-value compared to others. The meta-analysis, as a whole, indicates the overall significance of the collective findings.

Table 2. Meta-regression analysis across various moderators to further confirm the absence of heterogeneity

Moderators	Test of residual homogeneity			Residual heterogeneity			
	Q-stat	df	Sig.	Tau^2	I^2	H^2	R^2
Country/location	1.225	13	0.999	0	0	1	0
Sample size	1.637	13	0.999	0	0	1	0
Cancer type	1.836	13	0.999	0	0	1	0
Age groups	1.756	13	0.999	0	0	1	0
Sex	1.852	13	0.999	0	0	1	0
Tumor stage	1.547	13	0.999	0	0	1	0
SIRT1 expression level	1.475	13	0.999	0	0	1	0

Abbreviations: df: Degrees of freedom; Sig: Significance; SIRT1: Sirtuin 1; stat: Statistics.

expression (Figure 3F). However, the results consistently demonstrated no statistically significant heterogeneity for any of these moderators ($P \approx 1.000$), indicating homogeneity. In each case, the Q-statistics showed no heterogeneity ($P = 1.000$).

The absence of heterogeneity across these moderators implies that the prognostic value of SIRT1 remains stable and unaffected by the specific countries, sample sizes, cancer types, age groups, tumor stages, or SIRT1 expression levels considered. The findings suggest that the selected moderators do not significantly impact the overall consistency of the meta-analysis results, thereby providing confidence in the robustness of the observed prognostic value of SIRT1 expression.

3.3. Subgroup analyses

Further subgroup analyses revealed that the prognostic value of SIRT1 in cancer was significant (Table 3) across different geographical regions, with consistent effect sizes in both Asian (overall effect = 1.32, 95% CI [0.608 – 2.029], $P < 0.001$) and non-Asian (overall effect = 1.82, 95% CI [0.8 – 2.839], $P < 0.001$) populations. Specifically, the subgroup analysis for China (overall effect = 1.43, 95% CI [0.415 – 2.441]) indicated a significant effect, although with slightly higher heterogeneity ($P = 0.006$) compared to the overall Asia and non-Asia subgroups. The prognostic value of SIRT1 appeared significant across different study sizes. Small, medium, and large studies all showed statistically significant results ($P = 0.001$, $P = 0.002$, $P = 0.021$, respectively), with minimal to no observed heterogeneity ($I^2 = 0.0001$, $I^2 = 0.0001$, $I^2 = 0.000$, respectively). These consistent effect sizes across various study sizes indicate robust findings.

Studies on ovarian cancer ($n = 3$) showed a statistically significant effect ($P = 0.041$) and a moderate level of observed heterogeneity ($I^2 = 0.04$). Similarly, studies on

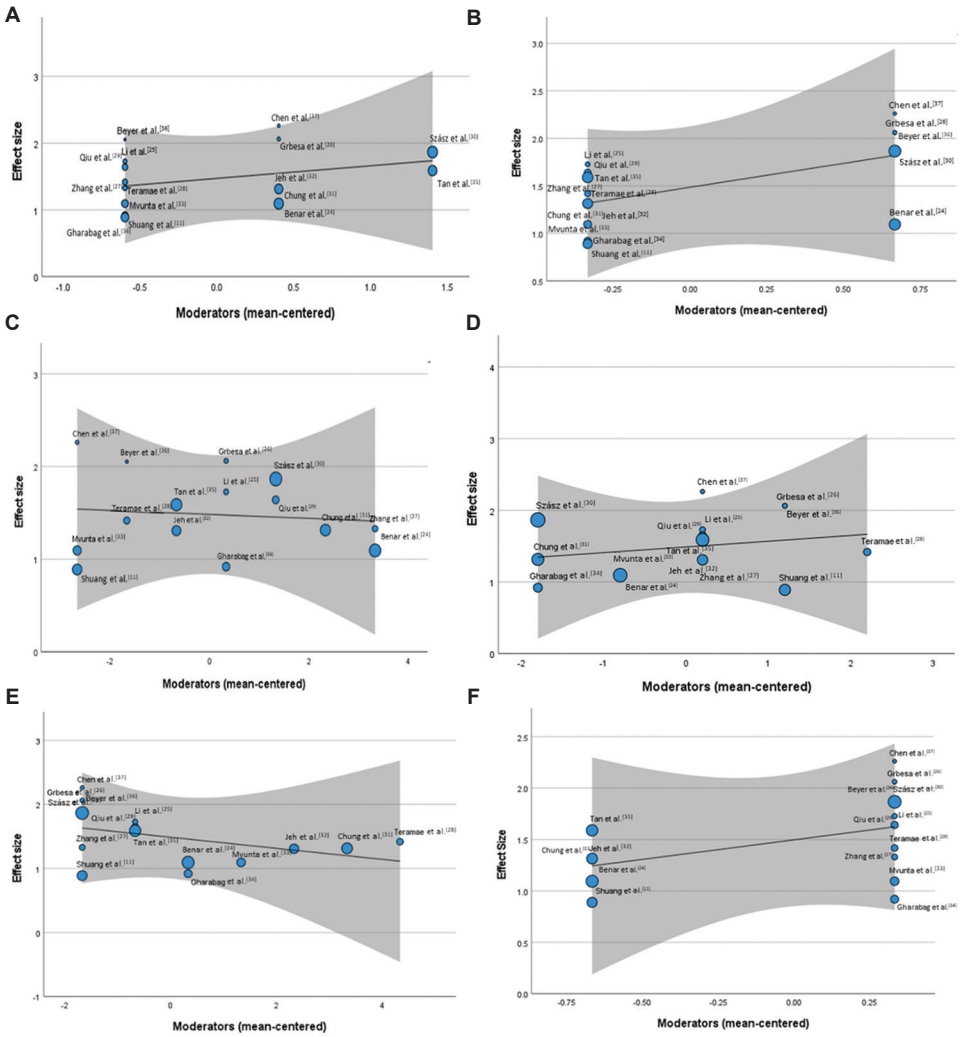

Figure 3. Bubble plots analyzing various moderators of the studies. Our analysis showed no statistically significant heterogeneity (P = 0.9899) across (A) Asian versus non-Asian countries, (B) different sample sizes, (C) cancer types included in the current analysis, (D) age groups, (E) tumor stages, and (F) SIRT1 expression levels.

lung cancer (n = 3, P = 0.025) and gastric cancer (n = 2, P = 0.025) also showed significant effects with low heterogeneity (I^2 = 0.03). In contrast, studies on uterine cancer (n = 2, P = 0.051) and renal cell carcinoma (n = 2, P = 0.061) showed trends toward significance, with low heterogeneity (I^2 = 0.05 and I^2 = 0.06, respectively). However, studies on breast cancer and colorectal cancer did not show statistically significant results (P = 0.136 and P = 0.221, respectively). The prognostic value of SIRT1 also varied across different age groups. Significant effects were observed in the age groups 56 – 60 with no heterogeneity (I^2 = 0.00) and 61 – 65 with low heterogeneity (I^2 = 0.02). However, the age groups 50 – 55 and 66 – 70 did not show statistically significant results (P = 0.161 and P = 0.237, respectively). The prognostic effect of SIRT1 remained significant and consistent across both male (overall effect = 1.5, 95% CI [0.752 – 2.244], $P \leq 0.001$) and female (overall effect = 1.46, 95% CI [0.524 – 2.393], P = 0.002) cohorts, with no observed heterogeneity (I^2 = 0.00). Various tumor stages also showed significant prognostic value for SIRT1, including stages I–IV ($P < 0.001$) and I–III & IV (P = 0.013), with low to no heterogeneity.

Importantly, the prognostic value of SIRT1 appeared to vary based on SIRT1 expression levels in a relatively unique manner. The analysis revealed that studies (n = 10) reporting high SIRT1 expression showed highly statistically significant effects (P < 0.001), with no observed heterogeneity (I^2 = 0.00%). Conversely, five studies focusing on cancers with low SIRT1 expression showed statistically significant results (P = 0.011), with a low level of heterogeneity (I^2 = 0.01%). Overall, the subgroup analyses revealed consistent

Table 3. Subgroup analyses of various variables included in the study

Subgroup	No. of studies	Summary effect size	95% confidence interval		Sig. (2-tailed)	Overall *P*-value	*Z*-value	I^2 statistics
			Lower	Upper				
Geographical region								
Asia	10	1.319	0.608	2.029	<0.001	0.00	3.638	0.0001
Non-Asia	5	1.819	0.8	2.839	<0.001	0.00	3.498	0.0001
China	5	1.428	0.415	2.441	0.006	0.01	2.763	0.0000
Sample size								
Small (<100)	8	1.357	0.535	2.18	0.001	0.00	3.234	0.0001
Medium (101 – 500)	5	1.553	0.556	2.55	0.002	0.00	3.053	0.0001
Large (>500)	2	1.731	0.255	3.207	0.021	0.02	2.299	0.0000
Cancer type								
Ovarian cancer	3	1.369	0.056	2.682	0.041	0.04	2.043	0.0001
Uterine cancer	2	1.712	−0.01	3.434	0.051	0.05	1.949	0.0001
Renal cell carcinoma	2	1.458	−0.065	2.98	0.061	0.06	1.876	0.0000
Lung cancer	3	1.548	0.191	2.905	0.025	0.03	2.236	0.0001
Gastric cancer	2	1.767	0.222	3.313	0.025	0.03	2.241	0.0001
Breast cancer	1	1.31	−0.787	3.407	0.221	N/A	1.224	N/A
Colorectal cancer	2	1.195	−0.376	2.766	0.136	0.14	1.491	0.0001
Age group								
50 – 55=1	2	1.095	−0.434	2.624	0.161	0.16	1.403	0.0000
56 – 60=2	6	1.63	0.695	2.565	<0.001	0.00	3.416	0.0001
61 – 65=3	3	1.607	0.244	2.969	0.021	0.02	2.311	0.0001
66 – 70=4	1	1.42	−0.932	3.772	0.237	N/A	1.183	N/A
Sex								
Male	9	1.498	0.752	2.244	<0.001	0.00	3.937	0.0001
Female	6	1.458	0.524	2.393	0.002	0.00	3.058	0.0001
Tumor stage								
I–IV	6	1.717	0.773	2.662	<0.001	0.00	3.563	0.0000
I–III & IV	3	1.647	0.345	2.95	0.013	0.01	2.479	0.0001
I–III	2	1.012	−0.53	2.553	0.198	0.2	1.287	0.0001
III–IV	1	1.1	−1.134	3.334	0.335	N/A	0.965	N/A
I	1	1.31	−0.905	3.525	0.246	N/A	1.159	N/A
III	1	1.42	−0.932	3.772	0.237	N/A	1.183	N/A
SIRT1 expression level								
Low	5	1.243	0.286	2.199	0.011	0.01	2.547	0.0001
High	10	1.624	0.889	2.36	<0.001	0.00	4.329	0.0000

Note: N/A: Not applicable for single-study analysis.
Abbreviations: No.: Number; Sig.: Significance.

results within each subgroup, indicating robust findings across different variables in the current study.

3.4. Publication bias

Despite concerns raised by the absence of heterogeneity in the overall meta-analysis, both Egger's test and the trim-and-fill analysis provided no significant evidence of publication bias in the present study (Egger's test: $P = 0.752$; trim-and-fill analysis: $P < 0.001$). However, the aggregation of primary studies at the base of the funnel plot and the asymmetry in the bubble plot (Figure 4A and B) may raise concerns. Consequently, the non-significant

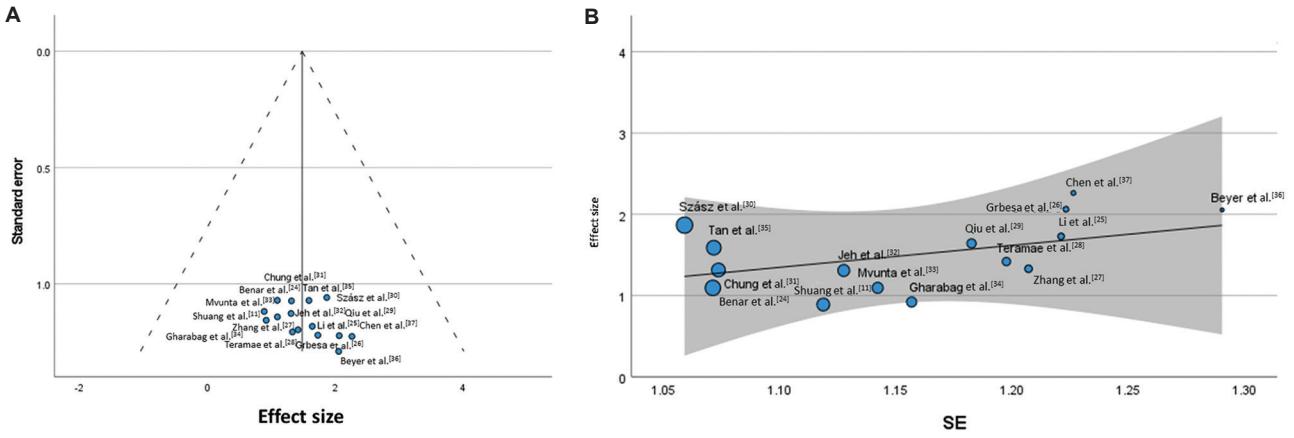

Figure 4. Depiction of publication bias. (A) The funnel plot shows the concentration of individual studies with larger SE near the base around the midline symmetrically. (B) The bubble plot illustrates the asymmetrical distribution of studies relative to the middle regression line, with respect to the standard error of the effect size for each study.
Abbreviation: SE: Standard errors.

intercept (coefficient of the intercept = −1.634, 95% CI [−12.579 – 9.310], $P = 0.752$) in Egger's test suggested that the observed asymmetry in the funnel plot may be due to factors other than publication bias. Further analysis using trim-and-fill, with no imputed studies, showed no change in the effect size (effect size = 1.483 ± 0.2974, 95% CI [0.900 – 2.066], $P < 0.001$), further supporting the robustness of the observed effect size. Overall, these analyses contribute to a more nuanced understanding of potential biases in the study, while reinforcing the reliability of the reported results.

3.5. Quality assessment

During quality appraisal process, discrepancies were addressed through discussion, with the involvement of corresponding authors when necessary. The results, summarized in Table 4, consistently show high assessment scores ranging from 7 to 9 across all studies, indicating the overall methodological robustness in addressing the research objectives.

4. Discussion

Our meta-analysis focused on the prognostic role of SIRT1 in various cancers, predominantly from Asia, suggesting a strong positive prognostic value for SIRT1 (pooled HR = 1.483, 95% CI: 0.900 – 2.065). Despite low variability ($I^2 = 0.00$), indicating a consistent impact, meta-regression across various variables confirmed no significant heterogeneity, reinforcing the stability of SIRT1's prognostic value. The subgroup analyses support consistent and significant effects in both Asian and non-Asian populations, varying by cancer type, sample size, age, sex, tumor stage, and SIRT1 expression levels, with minimal heterogeneity. Publication bias tests revealed no significant evidence of bias, supporting the robustness of the findings, despite some visual asymmetry in the data presentation.

The findings of our meta-analysis are consistent with several studies. For instance, Jiang *et al.*[38] investigated the clinical and prognostic value of SIRT1 in gastric cancer and discovered a notable association between high SIRT1 expression and poorer 3-year OS. This finding is parallel with our meta-analysis, which also demonstrated a significant prognostic effect for SIRT1 across various cancers. Both studies found correlations between SIRT1 expression and clinical features, such as patient age, tumor stage, lymph node involvement, and tumor differentiation, suggesting a potential role of SIRT1 in influencing cancer progression.[38] However, the difference in the association with 5-year OS warrants further exploration, as it highlights the complex long-term prognostic role of SIRT1 and necessitates additional comprehensive research to understand its varying effects over time. Moreover, Sun *et al.*[17] conducted an extensive meta-analysis and established a clear link between increased SIRT1 expression and adverse cancer outcomes. Their results showed higher HRs across various survival metrics associated with elevated SIRT1 expression. Similar to our findings, they correlated elevated SIRT1 levels with advanced tumor stages and metastasis. Interestingly, Sun *et al.*[17] emphasized the influence of ethnic background on the predictive role of SIRT1 in cancer outcomes, particularly in the Asian population, with a stronger impact observed in China, and supported the significance of regional variations in the prognostic impact of SIRT1. Despite differences in the specific survival metrics assessed, both studies converge on the conclusion that SIRT1 plays a crucial role in cancer progression and metastasis, suggesting that targeting SIRT1

Table 4. Quality appraisal of the included studies and their respective scores

Study	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Assessment score*
Benard *et al.*[24]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Li *et al.*[25]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Grbesa *et al.*[26]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Zhang *et al.*[27]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Teramae *et al.*[28]	✓	✓	×	✓	×	✓	✓	✓	✓	07
Shuang *et al.*[11]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Qiu *et al.*[29]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Szász *et al.*[30]	✓	✓	✓	✓	×	✓	✓	✓	✓	08
Chung *et al.*[31]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Jeh *et al.*[32]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Mvunta *et al.*[33]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Gharabaghi *et al.*[34]	✓	✓	×	✓	×	✓	✓	✓	✓	07
Tan *et al.*[35]	✓	✓	✓	✓	✓	✓	✓	✓	✓	09
Beyer *et al.*[36]	✓	✓	×	✓	✓	✓	✓	✓	✓	08
Chen *et al.*[37]	✓	✓	×	✓	✓	✓	✓	✓	✓	08

Note: *See full criteria for the assessment score in the Supplementary File.

could potentially improve patient outcomes. Furthermore, the findings from Wu *et al.*[39] align with our meta-analysis, particularly regarding the association between high SIRT1 expression and poor OS in gastrointestinal cancers. Their research focused on non-colorectal gastrointestinal cancers, such as gastric cancer and hepatocellular carcinoma, reflecting trends consistent with our analysis.[39] Their study also demonstrated a lack of association between SIRT1 expression and colorectal cancer outcomes, reinforcing the distinct behavior of SIRT1 in colorectal cancer compared to other gastrointestinal cancers. The comprehensive approach and subgroup analyses by Wu *et al.*[39] further support our understanding of SIRT1's varying prognostic value across different gastrointestinal cancer types. Similarly, our results are consistent with those of Wang *et al.*,[15] who found that SIRT1 overexpression significantly correlates with OS (HR: 1.483, 95% CI: 0.900 – 2.065), supporting its prognostic relevance in solid malignancies. Our analysis, which included 3059 cases from 15 studies, reflects a consistent association with a strong positive prognostic value, despite low variability ($I^2 = 0.00$). While Wang *et al.*[15] emphasized the negative prognosis associated with SIRT1 overexpression, our meta-analysis highlights the consistent and impactful association of SIRT1 without significant variability, signifying its substantial prognostic role in various cancers. Moreover, our meta-analysis aligns with the findings of Özcan *et al.*,[40] who observed an association between SIRT1 overexpression and unfavorable prognosis in gastric adenocarcinoma. They reported SIRT1 upregulation in all tumor stages except stage I, correlating it with an unfavorable prognosis. Similarly, our pooled analysis indicates a strong positive prognostic value for SIRT1 across various cancer types, underscoring its potential role in cancer prognosis, despite low variability across different study characteristics. This consistency in the prognostic impact of SIRT1 across various cancers further supports the conclusions drawn by Özcan *et al.*[40] regarding gastric adenocarcinoma. In addition, the findings from Feng *et al.*[41] support our results, demonstrating a significant correlation between high SIRT1 expression and poor prognosis in gastric cardiac carcinoma. Their study highlighted elevated SIRT1 expression in malignant tumor tissues, associating it with lymphatic metastasis, TNM stage, and survival rates. This association between increased SIRT1 expression and adverse clinicopathologic features aligns with our meta-analysis, emphasizing the potential of SIRT1 as a crucial marker for assessing malignancy and prognosis in gastric cardiac carcinoma.[41] Furthermore, the observed association between SIRT1 overexpression and advanced-stage tumors mirrors our meta-analysis findings across various cancers, suggesting the consistent prognostic significance of SIRT1 expression. Jiang *et al.*[42] specifically noted that SIRT1 overexpression was correlated with poor overall and disease-free survival in colorectal cancer patients. This observation is consistent with our meta-analysis, where the pooled analysis indicated a strong positive prognostic value for SIRT1 across different cancer types, despite low variability among the studies

included in the study. These collective findings emphasize the potential role for SIRT1 in cancer progression and prognosis, reinforcing the importance of understanding its impact on various cancers. Finally, our findings align with Zhou *et al.*,[43] who examined the distinct associations between specific cancers and SIRT1 expression. While our analysis focused on SIRT1, their research emphasized SIRT3, exhibiting its diverse impact across different cancer types. They found SIRT3 to be an unfavorable prognostic factor in breast, colon, and non-small-cell lung cancers, but a favorable factor in chronic lymphocytic leukemia, hepatocellular carcinoma, pancreatic cancer, and renal cell carcinoma. These varying prognostic roles of SIRT3 across cancers echo the specific associations that we observed between SIRT1 and different cancer types in our study.

The findings from all these studies confirm the significant prognostic role of SIRT1 across various cancers, despite some discrepancies in specific cancer types and regional differences. For example, our analysis shows non-significant correlations in colorectal and breast cancers. The lack of significant correlations in colorectal cancer may stem from the complexity of tumorigenesis, where multiple genetic and epigenetic pathways overshadow the impact of SIRT1 expression. SIRT1 regulation in colorectal cancer involves genetic interactions with the Vitamin D receptor, which enhances SIRT1 expression in response to 1,25-dihydroxyvitamin D3,[44] as well as epigenetic modifications like acetylation at lysine 610, which boosts its deacetylase activity.[7] In addition, microRNA regulation[45] and the role of E3 ubiquitin ligases[46] have notable impact on SIRT1 expression in colorectal cancer. Previous studies have suggested that factors such as microsatellite instability,[47] chromosomal instability,[48] and mutations in key oncogenes and tumor suppressor genes (e.g., adenomatous polyposis coli [*APC*], Kirsten rat sarcoma virus [*KRAS*], and p53 tumor suppressor protein [*TP53*])[49] might dominate the prognosis in colorectal cancer, potentially diminishing the prognostic relevance of SIRT1. Similarly, breast cancer encompasses various subtypes (e.g., hormone receptor-positive, human epidermal growth factor receptor 2 [HER2]-positive, and triple-negative), each with distinct molecular profiles. The role of SIRT1 may vary across these subtypes, and the single breast cancer study included in our analysis may not have had sufficient statistical power to detect the subtype-specific effects of SIRT1.

An important aspect revealed by our subgroup analyses is the variation in the prognostic value of SIRT1 between Asian and non-Asian populations. Studies conducted in Asian populations, particularly in China, consistently demonstrated significant prognostic effects of SIRT1, while the results in non-Asian populations, though significant, exhibited different effect sizes. This regional difference may be attributed to genetic and environmental factors that influence cancer biology across different ethnic groups, as previously defined by Sun *et al.*[17]

The overrepresentation of Asian studies in our meta-analysis could affect the generalizability of the findings. In Asian populations, dietary, lifestyle, and genetic predispositions may affect tumorigenesis, and the role of SIRT1 as a regulator of cancer progression could be more pronounced. Moreover, environmental and lifestyle factors prevalent in Asian populations may differ from those in non-Asian populations, which may limit the applicability of our results to other regions. This imbalance potentially skews the global prognostic significance of SIRT1. To enhance generalizability, future studies should include a more diverse set of populations to account for regional variations in cancer biology and SIRT1 expression. The inclusion of a higher number of studies from Asia, particularly China, in our meta-analysis, may have contributed to the observed differences in effect sizes. This limitation highlights the need for further research in diverse populations, especially in non-Asian cohorts, to better understand how SIRT1 expression correlates with cancer prognosis globally.

While Egger's test and trim-and-fill analysis indicated no significant publication bias, the observed funnel plot asymmetry and bubble plot suggested the potential influence of underlying factors. Specifically, the aggregation of studies with larger standard errors near the base of the funnel plot reflects selective reporting of smaller studies with more positive findings. Although the non-significant intercept from Egger's test and the absence of imputed studies in trim-and-fill analysis reinforce the robustness of the effect size, this asymmetry warrants further scrutiny. Factors such as study design, regional differences, or selective reporting of positive results may contribute to this asymmetry, necessitating caution in interpreting the overall findings.

Mechanistically, SIRT1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase that influences cancer progression through various pathways. Specifically, SIRT1 undergoes post-translational modifications, including covalent linking to interferon-stimulated genes (ISGs). SIRT1 and ISGs have been found to colocalize in cellular compartments such as the nucleus and mitochondria, promoting physical interactions between various proteins. The ISGylation of SIRT1 can enhance its deacetylase enzymatic function by disassociating it from inhibitory proteins.[50] This regulatory mechanism has been implicated in lung cancer progression and may diminish the efficacy of deoxyribonucleic acid-damaging treatments.[51]

Elevated levels of SIRT1 have also been linked to resistance to chemotherapy and radiotherapy in ESCC.[19] Morishita *et al.*[52] demonstrated the potential of SIRT1 as a predictive marker for therapeutic response, showing that reducing SIRT1 levels through knockdown enhances sensitivity to treatment in ESCC. Further, Mvunta *et al.*[33] showed that increased levels of SIRT1 are associated with elevated tumor aggressiveness. This relationship involves SIRT1 downregulating p53 and miR-101, while upregulating the KPNA3 axis, thereby promoting proliferation and invasive metastasis in colorectal cancer.[7] In addition, in renal clear cell carcinoma, elevated SIRT1 expression is associated with poor prognosis and is linked to lipid metabolism and immune infiltration.[7] These findings highlight the role of SIRT1 in modulating the tumor microenvironment and its potential as a target for therapeutic strategies.

In a clinical context, targeting SIRT1 in cancer therapy shows promise. Inhibition of SIRT1 enhances p53 acetylation, thereby increasing apoptosis and reducing cell proliferation, while potential inhibitors such as Doxercalciferol and Timiperone have demonstrated efficacy in various cancer models. However, the context-dependent role of SIRT1 complicates its therapeutic targeting, as it may also promote tumor growth at certain stages of cancer development.

While this meta-analysis provides valuable insights, several limitations warrant consideration. The reliance on existing studies, predominantly from Asia, may introduce inherent biases or exclusions that potentially affect the overall analysis. This imbalance between Asian and non-Asian populations limits the generalizability of our findings, highlighting the potential bias in the prognostic significance of SIRT1 across different regions. Future studies should focus on non-Asian populations with varying genetic, environmental, and lifestyle factors. Further investigation into SIRT1 expression and its role as a prognostic marker is particularly warranted in breast and colorectal cancers, which are influenced by multiple genetic, epigenetic, and environmental factors, as discussed earlier. Although Egger's test and trim-and-fill analysis indicated no significant publication bias, the observed asymmetry in the funnel plots suggests a higher likelihood of publication bias in smaller studies with positive findings. Therefore, study design, regional differences, and other moderators should be assessed for further scrutiny. In addition, the duration of patient follow-up in individual studies may impact the evaluation of long-term prognostic effects, underscoring the need for more extensive investigations to comprehensively understand SIRT1's role over varying time frames.

The clinical significance of SIRT1 lies in its role in cancer progression, influencing tumor growth, metastasis, and patient survival. Our meta-analysis, along with other studies, demonstrates that elevated SIRT1 expression is associated with poorer outcomes across various cancer types, making it a potential prognostic biomarker. A key contribution of this study is its application in clinical settings, where SIRT1 could help predict patient outcomes and guide treatment decisions. Identifying SIRT1 as a modulator of tumorigenesis suggests its potential utility in precision medicine, enabling personalized strategies aimed at inhibiting SIRT1 activity to enhance patient survival. Understanding the molecular mechanisms of SIRT1, particularly its interactions with pathways like p53 and epigenetic modifications, provides valuable insights for therapeutic intervention. Future research should focus on developing SIRT1 inhibitors or modulators to improve cancer treatments by sensitizing tumors to chemotherapy and radiotherapy or by targeting SIRT1-specific pathways. Integrating SIRT1-based biomarkers into precision medicine could enable tailored therapies, optimizing treatment plans based on individual tumor profiles and SIRT1 expression levels, ultimately improving patient outcomes.

5. Conclusion

The comprehensive meta-analysis, which pooled results from 15 studies involving 3059 cases, predominantly from Asian populations, underscores the strong and consistent positive prognostic value of SIRT1 across various cancers. The absence of significant variability in our analysis ($I^2 = 0.00$) further supports the stable impact of SIRT1 across different study variables, reinforcing its prognostic significance. Our findings align with previous research, demonstrating that the influence of SIRT1 transcends geographical and demographic boundaries, emphasizing its critical role in cancer prognosis. By synthesizing data from multiple studies, our meta-analysis highlights the consistent association between elevated SIRT1 expression and poorer outcomes, reinforcing its relevance as a prognostic factor. These insights pave the way for future research on SIRT1 as a therapeutic target, particularly in aggressive cancers such as gastrointestinal cancers and hepatocellular carcinoma.

Future investigations should focus on the mechanistic role of SIRT1 in cancer progression and its interaction with other prognostic factors to enhance targeted interventions. Specifically, developing SIRT1 inhibitors or modulators as therapeutic agents could significantly improve treatment strategies for certain cancer types. In addition, research should aim to identify biomarkers that predict the efficacy of SIRT1-targeted therapies, facilitating precision medicine approaches tailored to individual patient profiles. These efforts would not only deepen our understanding of the

mechanistic roles of SIRT1 in cancer progression but also pave the way for more effective cancer management strategies.

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Conflict of interest

Umair Ali Khan Saddozai and Xin-Ying Ji are members of the Editorial Board of this journal but were not involved in the editorial and peer-review processes for this paper, either directly or indirectly. Separately, the other authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

References

1. Kumar A, Chauhan S. How much successful are the medicinal chemists in modulation of SIRT1: A critical review. *Eur J Med Chem*. 2016;119:45-69.

   doi: 10.1016/j.ejmech.2016.04.063

2. McBurney MW, Clark-Knowles KV, Caron AZ, Gray DA. SIRT1 is a highly networked protein that mediates the adaptation to chronic physiological stress. *Genes Cancer*. 2013;4(3-4):125-134.

   doi: 10.1177/1947601912474893

3. Luo J, Nikolaev AY, Imai SI, *et al*. Negative control of p53 by Sir2α promotes cell survival under stress. *Cell*. 2001;107(2):137-148.

   doi: 10.1016/s0092-8674(01)00524-4

4. Wang RH, Sengupta K, Li C, *et al*. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. *Cancer Cell*. 2008;14(4):312-323.

   doi: 10.1016/j.ccr.2008.09.001

5. Khawar MB, Sohail AM, Li W. SIRT1: A key player in male reproduction. *Life*. 2022;12(2):318.

   doi: 10.3390/life12020318

6. Khawar MB, Liu C, Gao F, *et al*. Sirt1 regulates testosterone biosynthesis in Leydig cells via modulating autophagy. *Protein Cell*. 2021;12(1):67-75.

   doi: 10.1007/s13238-020-00771-1

7. Wang XW, Jiang YH, Ye W, Shao CF, Xie JJ, Li X. SIRT1 promotes the progression and chemoresistance of colorectal cancer through the p53/miR-101/KPNA3 axis. *Cancer Biol Ther*. 2023;24(1):2235770.

   doi: 10.1080/15384047.2023.2235770

8. Carafa V, Altucci L, Nebbioso A. Dual tumor suppressor and tumor promoter action of sirtuins in determining malignant phenotype. *Front Pharmacol*. 2019;10:38.

   doi: 10.3389/fphar.2019.00038

9. Alves-Fernandes DK, Jasiulionis MG. The role of SIRT1 on DNA damage response and epigenetic alterations in cancer. *Int J Mol Sci*. 2019;20(13):3153.

   doi: 10.3390/ijms20133153

10. Karbasforooshan H, Roohbakhsh A, Karimi G. SIRT1 and microRNAs: The role in breast, lung and prostate cancers. *Exp Cell Res*. 2018;367(1):1-6.

    doi: 10.1016/j.yexcr.2018.03.023

11. Shuang T, Wang M, Zhou Y, Shi C. Over-expression of Sirt1 contributes to chemoresistance and indicates poor prognosis in serous epithelial ovarian cancer (EOC). *Med Oncol*. 2015;32:260.

    doi: 10.1007/s12032-015-0706-8

12. Noguchi A, Kikuchi K, Zheng H, *et al*. SIRT1 expression is associated with a poor prognosis, whereas DBC1 is associated with favorable outcomes in gastric cancer. *Cancer Med*. 2014;3(6):1553-1561.

doi: 10.1002/cam4.310

13. Lee CW, Wong LL, Tse EY, *et al*. AMPK Promotes p53 acetylation via phosphorylation and inactivation of SIRT1 in liver cancer cells. *Cancer Res*. 2012;72(17):4394-4404.

doi: 10.1158/0008-5472.CAN-12-0429

14. Zhang H, Ma C, Peng M, Lv X, Xie X, Huang R. The prognostic implications of SIRTs expression in breast cancer: A systematic review and meta-analysis. *Disc Oncol*. 2022;13(1):69.

doi: 10.1007/s12672-022-00529-7

15. Wang C, Yang W, Dong F, *et al*. The prognostic role of Sirt1 expression in solid malignancies: A meta-analysis. *Oncotarget*. 2017;8(39):66343-66351.

doi: 10.18632/oncotarget.18494

16. Cao YW, Li YC, Wan GX, Du XM, Li F. Clinicopathological and prognostic role of SIRT1 in breast cancer patients: A meta-analysis. *Int J Clin Exp Med*. 2015;8(1):616-624.

17. Sun M, Du M, Gong X. Survival and clinicopathological significance of SIRT1 expression in cancers: A meta-analysis. *Front Endocrinol*. 2019;10:442007.

18. Otsuka R, Sakata H, Murakami K, *et al*. SIRT1 expression is a promising prognostic biomarker in esophageal squamous cell carcinoma: A systematic review and meta-analysis. *Cancer Diagn Prog*. 2022;2(2):126-133.

doi: 10.21873/cdp.10086

19. Zhang YL, Chen P, Guo Y, Zhang YJ. Clinical value of SIRT1 as a prognostic biomarker in esophageal squamous cell carcinoma, a systematic meta-analysis. *Open Med*. 2022;17(1):527-535.

doi: 10.1515/med-2022-0454

20. Jung W, Hong KD, Jung WY, *et al*. SIRT1 expression is associated with good prognosis in colorectal cancer. *Korean J Pathol*. 2013;47(4):332-339.

doi: 10.4132/KoreanJPathol.2013.47.4.332

21. Zhou J, Xu M, Le K, *et al*. SRC promotes tamoxifen resistance in breast cancer via up-regulating SIRT1. *Onco Targets Ther*. 2020;13:4635-4647.

doi: 10.2147/OTT.S245749

22. Tan J, Liu Y, Maimaiti Y, *et al*. Combination of SIRT1 and Src overexpression suggests poor prognosis in luminal breast cancer. *Onco Targets Ther*. 2018;11:2051-2061.

doi: 10.2147/OTT.S162503

23. Ryota O, Hiroki M, Keiko I, *et al*. Serum versus tissue SIRT1 as potentially valuable biomarkers in gastric cancer. *Anticancer Res*. 2023;43(4):1485.

doi: 10.21873/anticanres.16297

24. Benard A, Goossens-Beumer IJ, van Hoesel AQ, *et al*. Nuclear expression of histone deacetylases and their histone modifications predicts clinical outcome in colorectal cancer. *Histopathology*. 2015;66(2):270-282.

doi: 10.1111/his.12534

25. Li C, Wang L, Zheng L, *et al*. SIRT1 expression is associated with poor prognosis of lung adenocarcinoma. *Onco Targets Ther*. 2015;8:977-984.

doi: 10.2147/OTT.S82378

26. Grbesa I, Pajares MJ, Martinez-Terroba E, *et al*. Expression of sirtuin 1 and 2 is associated with poor prognosis in non-small cell lung cancer patients. *PLoS One*. 2015;10(4):e0124670.

doi: 10.1371/journal.pone.0124670

27. Zhang X, Chen S, Cheng M, Cao F, Cheng Y. The expression and correlation of SIRT1 and Phospho-SIRT1 in colorectal cancer. *Int J Clin Exp Med*. 2015;8(1):809-817.

28. Teramae M, Fukuda T, Wada T, *et al*. Sirtuin1 expression predicts the efficacy of neoadjuvant chemotherapy for locally advanced uterine cervical cancer. *Mol Clin Oncol*. 2015;3(1):73-78.

doi: 10.3892/mco.2014.427

29. Qiu G, Li X, Wei C, *et al*. The prognostic role of SIRT1-autophagy axis in gastric cancer. *Dis Markers*. 2016;2016(1):6869415.

doi: 10.1155/2016/6869415

30. Szász AM, Lánczky A, Nagy Á, et al. Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget*. 2016;7(31):49322.

doi: 10.18632/oncotarget.10337

31. Chung SY, Jung YY, Park IA, *et al*. Oncogenic role of SIRT1 associated with tumor invasion, lymph node metastasis, and poor disease-free survival in triple negative breast cancer. *Clin Exp Metastasis*. 2016;33:179-185.

doi: 10.1007/s10585-015-9767-5

32. Jeh SU, Park JJ, Lee JS, *et al*. Differential expression of the sirtuin family in renal cell carcinoma: aspects of carcinogenesis and prognostic significance. *Urol Oncol*. 2017;35:675-e79.

doi: 10.1016/j.urolonc.2017.08.016

33. Mvunta DH, Miyamoto T, Asaka R, *et al*. Overexpression of SIRT1 is associated with poor outcomes in patients with ovarian carcinoma. *Appl Immunohistochem Mol Morphol*. 2017;25(6):415-421.

doi: 10.1097/PAI.0000000000000316

34. Gharabaghi MA. Diagnostic investigation of BIRC 6 and SIRT 1 protein expression level as potential prognostic biomarkers in patients with non-small cell lung cancer. *Clin Respir J*. 2018;12(2):633-638.

doi: 10.1111/crj.12572

35. Tan Y, Li B, Peng F, Gong G, Li N. Integrative analysis of sirtuins and their prognostic significance in clear cell renal cell carcinoma. *Front Oncol*. 2020;10:218.

doi: 10.3389/fonc.2020.00218

36. Beyer S, Chen F, Meister S, *et al*. Sirtuin1 expression and survival in endometrial and clear-cell uterine cancer. *Histochem Cell Biol*. 2020;154:189-195.

doi: 10.1007/s00418-020-01873-x

37. Chen F. *The Expression of Sirtuin1 and Its Role in Ovarian Cancer*. Germany: Ludwig Maximilian University of Munich; 2022.

38. Jiang B, Chen JH, Yuan WZ, *et al*. Prognostic and clinical value of Sirt1 expression in gastric cancer: A systematic meta-analysis. *J Huazhong Univ Sci Technol*. 2016;36(2):278-284.

doi: 10.1007/s11596-016-1580-0

39. Wu S, Jiang J, Liu J, Wang X, Gan Y, Tang Y. Meta-analysis of SIRT1 expression as a prognostic marker for overall survival in gastrointestinal cancer. *Oncotarget*. 2017;8(37):62589-62599.

doi: 10.18632/oncotarget.19880

40. Ozcan O, Belli AK, Cetin ES, *et al*. Upregulation of SIRT1 gene in gastric adenocarcinoma. *Turk J Gastroenterol*. 2019;30(4):326-330.

doi: 10.5152/tjg.2019.18550

41. Feng AN, Zhang LH, Fan XS, *et al*. Expression of SIRT1 in gastric cardiac cancer and its clinicopathologic significance. *Int J Surg Pathol*. 2011;19(6):743-750.

doi: 10.1177/1066896911412181

42. Jiang K, Lyu L, Shen Z, *et al*. Overexpression of SIRT1 is a poor prognostic factor for advanced colorectal cancer. *Chin Med J*. 2014;127(11):2021-2024.

43. Zhou Y, Cheng S, Chen S, Zhao Y. Prognostic and clinicopathological value of SIRT3 expression in various cancers: A systematic review and meta-analysis. *Onco Targets Ther*. 2018;11:2157-2167.

doi: 10.2147/OTT.S157836

44. García-Martínez JM, Chocarro-Calvo A, Martínez-Useros J, *et al*. Vitamin D induces SIRT1 activation through K610 deacetylation in colon cancer. *eLife*. 2023;12:RP86913.

doi: 10.7554/eLife.86913

45. Wang TW, Chern E, Hsu CW, Tseng KC, Chao HM. SIRT1-Mediated expression of CD24 and epigenetic suppression of novel tumor suppressor miR-1185-1 increases colorectal cancer stemness. *Cancer Res*. 2020;80(23):5257-5269.

doi: 10.1158/0008-5472.CAN-19-3188

46. Yu L, Dong L, Li H, *et al*. Ubiquitination-mediated degradation of SIRT1 by SMURF2 suppresses CRC cell proliferation and tumorigenesis. *Oncogene*. 2020;39(22):4450-4464.

doi: 10.1038/s41388-020-1298-0

47. Lin EI, Tseng LH, Gocke CD, *et al*. Mutational profiling of colorectal cancers with microsatellite instability. *Oncotarget*. 2015;6(39):42334-42344.

doi: 10.18632/oncotarget.5997

48. Dong W, Lu J, Li Y, *et al*. SIRT1: A novel regulator in colorectal cancer. *Biomed Pharmacother*. 2024;178:117176.

doi: 10.1016/j.biopha.2024.117176

49. Parmar S, Easwaran H. Genetic and epigenetic dependencies in colorectal cancer development. *Gastroenterol Rep*. 2022;10:goac035.

doi: 10.1093/gastro/goac035

50. Deng Y, Wang Y, Li L, Miao EA, Liu P. Post-translational modifications of proteins in cytosolic nucleic acid sensing signaling pathways. *Front Immunol*. 2022;13:898724.

doi: 10.3389/fimmu.2022.898724

51. Kang JA, Kim YJ, Jang KY, *et al*. SIRT1 ISGylation accelerates tumor progression by unleashing SIRT1 from the inactive state to promote its deacetylase activity. *Exp Mol Med*. 2024;56(3):656-673.

doi: 10.1038/s12276-024-01194-2

52. Morishita H, Otsuka R, Murakami K, *et al*. SIRT1 Promotes chemoradiotherapy resistance in esophageal squamous cell carcinoma. *Oncology*. 2024;102:960-968.

doi: 10.1159/000540247

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Antidepressant effects of fisetin: Identifying molecular mechanisms by network pharmacology and molecular docking

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Abstract

Major depressive disorder (MDD) is a heterogeneous condition influenced by a complex interplay of social, psychological, and biological factors. Fisetin (FT), a flavonoid polyphenol found in various plants, has demonstrated neuroprotective properties that may be beneficial in treating MDD. This research aims to evaluate the potential molecular mechanisms of FT in treating MDD using network pharmacology analysis, with validation through molecular docking methods. We assessed the drug-like properties of FT using the TCMSP and SwissADME platforms. Potential drug targets for FT were identified through SuperPred and SwissTargetPrediction. We compiled MDD-associated targets from established databases and identified common genes shared between FT and MDD. The common targets were analyzed for protein-protein interactions using the STRING database to identify essential targets. Consequently, these key targets were further investigated through Kyoto Encyclopedia of Genes and Genomes and Gene Ontology (GO) enrichment analyses with the help of ShinyGO software. The results indicated that FT targets are linked to specific pathways involved in the pathogenesis of MDD, with the IL-17 signaling pathway emerging as a significant pathway of interest. Strong binding affinities were found between FT and key proteins, including glycogen synthase kinase 3 beta, monoamine oxidase A, acetylcholinesterase, matrix metalloproteinase 9, and myeloperoxidase, suggesting that FT may serve as a promising therapeutic agent for MDD by targeting components of the IL-17 pathway. In conclusion, this research successfully employed computational methods to elucidate the potential effectiveness of FT in managing MDD. It offered important perspectives on the regulatory mechanisms involved and emphasized the IL-17 signaling pathway as a possible target for MDD therapy.

Keywords: Major depressive disorder; Fisetin; Traditional Chinese medicine; Network pharmacology; Molecular docking

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1. Introduction

Modern society is witnessing a variety of psychiatric disorders, with major depressive disorder (MDD) being a highly prevalent form that leads to significant disability and

increased risk of suicide.[1] As a major mental illness, MDD has a profound impact on both individuals and society as a whole.[2] The World Health Organization has raised grave concerns over the alarming increase in the prevalence of MDD and other mental disorders worldwide.[3] In addition, the prevalence of MDD has increased in the wake of the COVID-19 pandemic.[4] MDD is marked by intense and common emotional symptoms such as feelings of guilt and a lack of pleasure; cognitive symptoms like low self-esteem; and physical symptoms including loss of energy, insomnia, irritability, and fatigue.[5] MDD remains one of the most prevalent mental health conditions, impacting cognitive abilities, quality of life, and overall health.[6] Nonetheless, the etiology of MDD is not fully understood.

Extensive research conducted over the course of several decades has centered on the development and evaluation of pharmacological interventions for the management of MDD.[7] Although selective serotonin reuptake inhibitors are often prescribed for treating MDD, many patients find them ineffective and experience various adverse effects and tolerance. This underscores the need for antidepressants that provide a rapid onset of action, improved tolerability, greater efficacy, and a reduced side effect profile.[8] Consequently, there has been growing interest in multi-target drugs, which offer the advantage of additive and synergistic effects.[9]

Chinese herbal medicine is recognized for its multi-pathway and multi-target approach to treating diseases.[10] Fisetin (FT, 3,7,3′,4′-tetrahydroxyflavone), a well-known flavonoid with antioxidant properties, is abundantly found in vegetables and fruits[11] and is an active component of traditional Chinese medicine (TCM) known as *Cotinus coggygria*.[12] Scientific evidence has demonstrated the diverse pharmacological effects of FT, including its anti-angiogenic, anti-cancer, anti-aging, and neuroprotective properties.[13] Due to these characteristics, FT has been proposed as a potentially effective treatment for several neuropsychological disorders, including Huntington's disease, stroke, and Alzheimer's disease (AD).[14,15] However, its antidepressant effects have yet to be investigated. In theory, FT's anti-inflammatory properties could help reduce the chronic, low-grade inflammation seen in individuals with MDD.[16] Additional research has shown that FT supports the protection and survival of neurons and enhances memory in mice.[17,18]

TCM requires the utilization of big data to identify all relevant targets and pathways associated with active components and diseases. Network pharmacology, an interdisciplinary field combining bioinformatics and pharmacology, offers a systematic approach toward a comprehensive exploration of the complex pharmacological effects of drugs.[19] This approach is gaining traction in drug discovery, particularly in the development of plant-based herbal medicines for complex diseases.[20,21]

Building on the importance of traditional pharmacology, this study aims to explore the possible target genes and proteins involved, as well as the specific molecular cascades, associated with the TCM compound FT in the treatment of MDD. This investigation utilized integrated network pharmacology and *in silico* molecular docking techniques. After identifying the common targets between FT and MDD, we used protein-protein interaction (PPI) analysis to find the hub genes within the network. Subsequently, we utilized the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment methods to clarify the possible molecular pathways by which FT could deliver its therapeutic benefits in treating MDD.

2. Materials and methods

2.1. Pharmacology and toxicological properties

After obtaining the structure of FT from PubChem database, we analyzed the physicochemical properties and drug-likeness (DL) of the compound using two platforms: TCMSP (https://old.tcmsp-e.com/tcmsp.php) and SwissADME (http://www.swissadme.ch/). These tools were employed to assess the compound's absorption, distribution, metabolism, and excretion characteristics. Furthermore, the toxicity of FT was evaluated using the ProTox-II platform (accessible at: https://tox-new.charite.de/).

2.2. Target prediction for ingredient

We utilized prediction tools, such as the Super Pred and Swiss Target Prediction platforms, to identify potential targets of FT. The Canonical SMILES of FT was used with the Swiss Target Prediction platform (accessible at: http://www.swisstargetprediction.ch/), a web-based platform specifically designed for the target identification of small molecules.[22] This platform maintains a high predictive performance level, listing the correct target within the top 15 for over 70% of tested external compounds.[22] We also used another platform, SuperPred (accessible at: https://prediction.charite.de/), with a prediction probability threshold >70%, to find the targets of FT.[23] The gene symbols of the potential targets were obtained by searching the UniProt databases (accessible at: http://www.uniprot.org/uniprot/). Finally, we removed any duplicate entries and prepared the finalized list of targets for further analysis.

2.3. Target prediction for disease

To identify genes associated with MDD, we employed the keyword "major depressive disorder or depression" in our search across the following three distinct databases: GeneCard (accessible at: https://www.genecards.org/),[24]

MalaCard (accessible at: https://www.malacards.org/), and the Human Phenotype Ontology (HPO) database (accessible at: https://hpo.jax.org/). After the initial search, we utilized the UniProt database (http://www.uniprot.org/uniprot/) to verify and standardize the protein names, ensuring they reflect the official nomenclature. In addition, we specified the source species for each identified protein, providing clarity and context to our findings.

2.4. Identification of common targets and disease-target network construction

To elucidate the interactions between MDD-associated targets and FT's potential targets, we conducted an intersection analysis. This was visualized using Venn diagrams created with an online platform (accessible at http://bioinformatics.psb.ugent.be/webtools/Venn/). Subsequently, we utilized the Panther Classification System to categorize the proteins linked to the anti-MDD effects of FT (accessible at http://www.pantherdb.org/). Moreover, we employed Cytoscape software (Ver. 3.9.0, accessible at https://cytoscape.org/) for the development of a network illustrating the relationship between diseases, ingredients, targets, and pathways. After constructing this network, we carried out a network topology analysis using the "Analyze Network" feature. This comprehensive approach allowed for a detailed understanding of the connections between the various components involved.[25]

2.5. PPI network

The PPI network was constructed by submitting the identified potential therapeutic targets of MDD to the STRING database (accessible at https://string-db.org), with the species filter was set to "Homo sapiens." All interactions within the resulting network were retained if they had confidence scores of 0.4 or higher, indicating medium to high confidence levels. Subsequently, this interaction network was imported into Cytoscape (accessible at http://apps.cytoscape.org/apps/networkanalyzer, Version 3.9.0) for further visualization. The CytoNCA plug-in was employed to compute key network metrics, including "Degree," "Betweenness," and "Closeness." From these analyses, the top ten targets were identified based on their degree, marking them as the core targets of interest.

2.6. GO and KEGG

The enrichment analysis for GO and the KEGG pathways was performed using the ShinyGO 0.80 platform (accessible at: https://bioinformatics.sdstate.edu/go80/).[26] A significance threshold of <0.05 was established to select the ten most significant GO terms for further analysis. The results were visually represented using bar plots, highlighting the categories of molecular function (MF), cellular component (CC), and biological process (BP). For the KEGG cascade analysis, we focused on the 10 key genes, applying a false discovery rate (FDR) and a *P*-value cutoff of <0.05. This FDR cutoff ensures that a maximum of 5% of the results considered significant are false positives, enhancing the reliability of our analysis.[27] The enrichment results for KEGG pathways were illustrated using dot plots, which were generated through the ShinyGO 0.80 platform. This comprehensive approach allowed us to effectively visualize and interpret the biological significance of the identified pathways and gene functions.

2.7. Molecular docking

We selected five top targets obtained from PPI to conduct molecular docking. The 3D structures of these proteins were retrieved from the Protein Data Bank (PDB; accessible at: https://www.rcsb.org/) using their respective "PDB" file suffixes. For FT, its 3D structure was sourced from the PubChem database in the "SDF" format. To visualize interactions between core targets and the FT, we employed CB-Dock2 (accessible at: https://cadd.labshare.cn/cb-dock2/php/index.php/). This tool allowed us to analyze the docking results, offering important insights into the interactions between the key targets and the active compound. Notably, CB-Dock2 surpasses other leading methods due to its precision in identifying binding sites and predicting binding poses, thanks to its advanced knowledge-based docking engine.[28] In our visualizations, the ligand and receptor were represented using the "spacefill" and "cartoon" styles, respectively.[29]

3. Results

3.1. Pharmacology and toxicity of FT

The pharmacological and toxicological profile of FT (PubChem ID: 5281614, Molecular ID: MOL013179) offers important insights into its potential therapeutic applications and safety considerations. FT ($C_{15}H_{10}O_6$; molecular weight, MW: 286.24 g/mol; Figure 1A), exhibits an oral bioavailability of 52.6%, indicating good absorption following oral administration. However, it has a blood-brain barrier (BBB) permeability score of −0.69, suggesting limited ability to cross the BBB. The DL score is reported at 0.24. The compound has a high gastrointestinal absorption rating and a topological polar surface area of 111.13 Å. Moreover, FT has 6 hydrogen bond acceptors, 4 hydrogen bond donors, and 1 rotatable bond, contributing to its overall molecular flexibility. Toxicological predictions indicate that FT is active in hepatotoxicity, neurotoxicity, respiratory toxicity, and immunotoxicity, while being inactive for nephrotoxicity, cardiotoxicity, carcinogenicity, mutagenicity, cytotoxicity, and clinical toxicity. The predicted median lethal dose

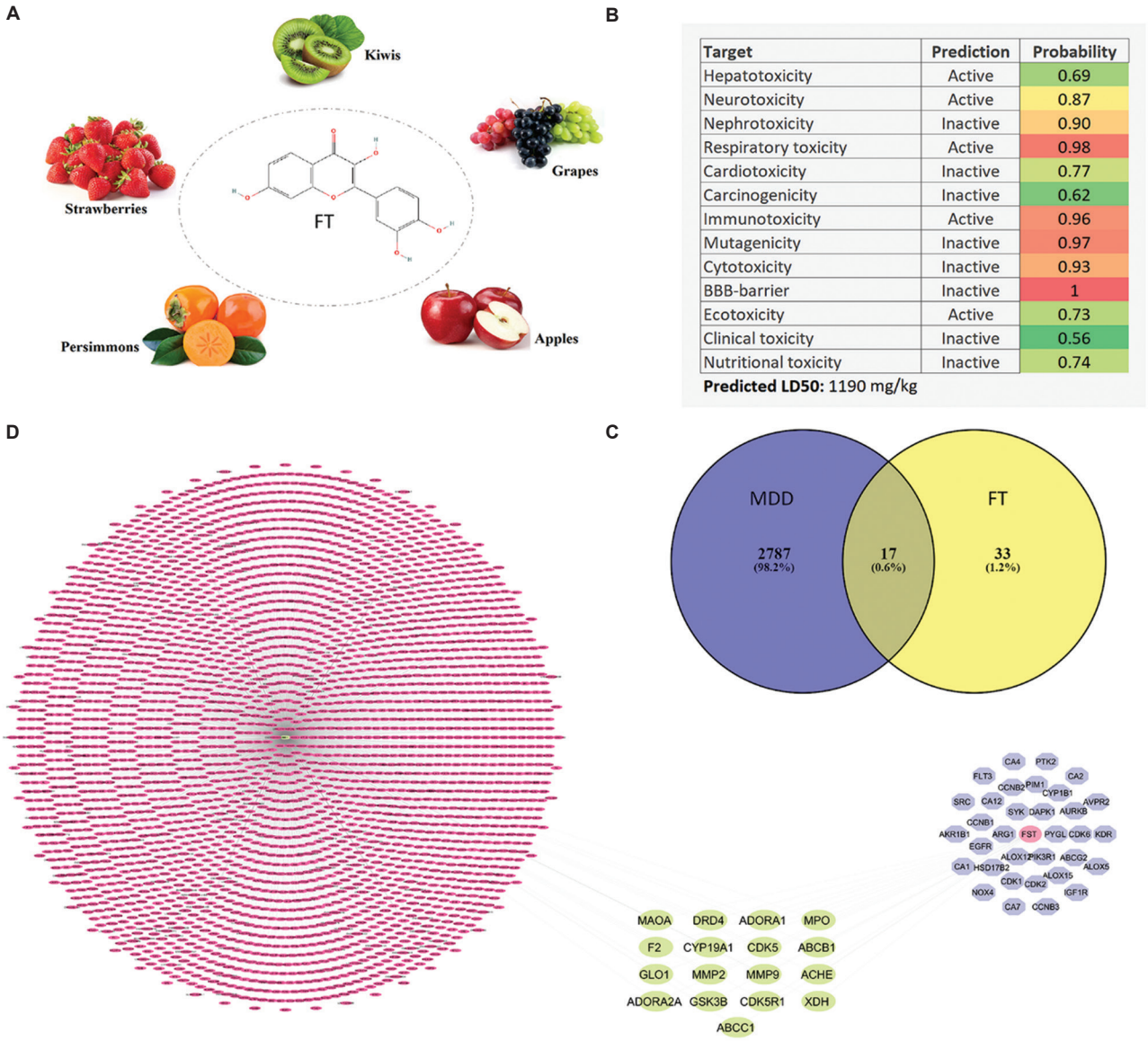

Figure 1. The pharmacological, toxicological profile, common genes, and network reconstruction of FT. (A) Molecular structure of FT; (B) toxicological information of FT; (C) Venn diagram illustrating the relationship between FT and MDD; and (D) ingredients-target network of FT and MDD.
Abbreviations: BBB: Blood-brain barrier; FT: Fisetin; LD50: Median lethal dose; MDD: Major depressive disorder.

(LD_{50}) for FT is 1190 mg/kg, indicating the amount of the substance required to kill 50% of a tested population (Figure 1B and Table 1). The analysis indicates that while FT possesses beneficial pharmacological properties, its active toxicological effects in certain domains warrant careful consideration in therapeutic contexts.

3.2. Common genes and network reconstruction between the MDD and FT targets

We found 1,805 and 50 genes associated with MDD and FT, respectively. The Venn diagram detected 17 common genes between MDD and FT (Figure 1C). The network reconstruction between the MDD and FT targets reveals an intricate relationship between the genes implicated in both conditions. This analysis identified a total of 2,839 nodes representing the genes and 2,854 edges indicating interactions between them. The network's structure suggests relatively sparse connectivity, with an average of 2.011 neighbors per node. The network's diameter of 4 units and radius of 2 units show that the maximum distance between any two nodes is four edges. Furthermore, the high centralization score of 0.988 indicates that only a handful of key genes may be instrumental in mediating interactions between FT and MDD (Figure 1D).

Table 1. Physicochemical, pharmacokinetics, and drug-likeness properties of FT

PubChemID	5281614
Mol ID	MOL013179
Molecule Name	Fisetin
Formula	C15H10O6
OB (%)	52.6
BBB	−0.69
DL	0.24
MW (g/mol)	286.24
Hacc	6
Hdon	4
Rbon	1
TPSA (Å)	111.13
Lipinski	Yes
GIA	High
Log Kp (cm/s)	−6.65 cm/s
BBB Permeant	No

Abbreviations: OB: Oral availability; BBB: Blood-brain barrier; DL: Drug-likeness; MW: Molecular weight; Hacc: Number of H-bond acceptors; Hdon: Number of H-bond donors; Rbon: Number of rotatable bonds; TPSA: Topological polar surface area; Log Kp: Logarithm of permeability coefficient; GIA: Gastrointestinal absorption.

3.3. PPI of FT

Utilizing the STRING database, PPI analysis was carried out to identify the FT targets in the context of treating MDD, (Figure 2A). A total of 17 target proteins were examined, and the resulting data were input into Cytoscape for topological analysis of the constructed PPI network. To measure the significance of nodes within this network, we utilized the CytoNCA application to select the top genes (Figures 2B and C). Through this comprehensive analysis, we successfully identified 10 critical target proteins as follows: glycogen synthase kinase 3 beta (GSK3B), monoamine oxidase A (MAOA), acetylcholinesterase (ACHE), matrix metalloproteinase 9 (MMP9), myeloperoxidase (MPO), xanthine dehydrogenase (XDH), matrix metalloproteinase 2 (MMP2), adenosine triphosphate-binding cassette sub-family B member 1 (ABCB1), cyclin-dependent kinase 5 (CDK5), and CDK5 regulatory subunit 1 (CDK5R1) (Figure 2C and detailed in Table 2).

3.4. Molecular mechanisms of FT

We performed GO and KEGG analyses to identify the possible molecular mechanisms underlying FT's effects in the treatment of MDD. The BP analysis revealed a total of 1,000 items, highlighting the top five components, namely, response to amyloid-beta, excitatory post-synaptic potential, positive regulation of neuron death, chemical synaptic transmission post-synaptic, and regulation of neurotransmitter levels (Figure 3A). MF analysis showed 163 items, with the top five items being G protein-coupled adenosine receptor activity, cholinesterase activity, sphingolipid floppase activity, monoamine oxidase activity, and tau-protein kinase activity (Figure 3B). The CC analysis identified 103 items, with the leading five being the protein kinase 5 complex, post-synaptic density, axon, dendrite, and axolemma (Figure 3C). The results of the KEGG enrichment analysis detected a total of 101 signaling pathways with a significance level of $P < 0.05$, among which the 10 most important pathways were as follows: pathways in cancer, bladder cancer, diabetic cardiomyopathy, cocaine addiction, drug metabolism, endocrine resistance, neuroactive ligand-receptor interaction, interleukin (IL)-17 signaling pathway, AD, and Prostate cancer (Figure 3D).

3.5. Molecular docking

The docking analysis performed using CB-Dock2 demonstrated significant interactions between FT and five target proteins, underscoring their crucial role in drug-disease interactions for the treatment of MDD. The updated results reveal that FT exhibits high binding affinities with the following proteins: GSK3B, with a Vina score of −8.4; MAOA, with a Vina score of −10.2; ACHE, with a Vina score of −9.2; MMP9, with a Vina score of −8.3; and MPO, with a Vina score of −10.2. These findings highlight FT's potential as a therapeutic agent in MDD treatment through its interactions with these key targets. In comparison, the established antidepressant fluoxetine (FLX) shows varying binding affinities across the same targets, with Vina scores of −7.7 for GSK3B, −9.0 for MAOA, −10.0 for ACHE, −8.5 for MMP9, and −8.0 for MPO. Notably, FT demonstrates superior binding affinities, particularly with MAOA and MPO, suggesting that it may offer a competitive advantage in targeting these proteins compared to FLX. This comparative analysis not only emphasizes FT's therapeutic potential but also provides a clearer evaluation of its efficacy in the context of existing treatments for MDD (Figure 4 and Table A1).

4. Discussion

MDD is a complex condition linked to numerous physiological disturbances, including heightened neuroinflammation. Growing research points to inflammatory pathways, especially the nuclear factor kappa B (NF-κB) pathway, as key contributors to the development of MDD.[15,30] In individuals with MDD, increased levels of proinflammatory cytokines, such as interleukin-1 beta, interleukin-6 (IL-6), and tumor necrosis factor α (TNFα),

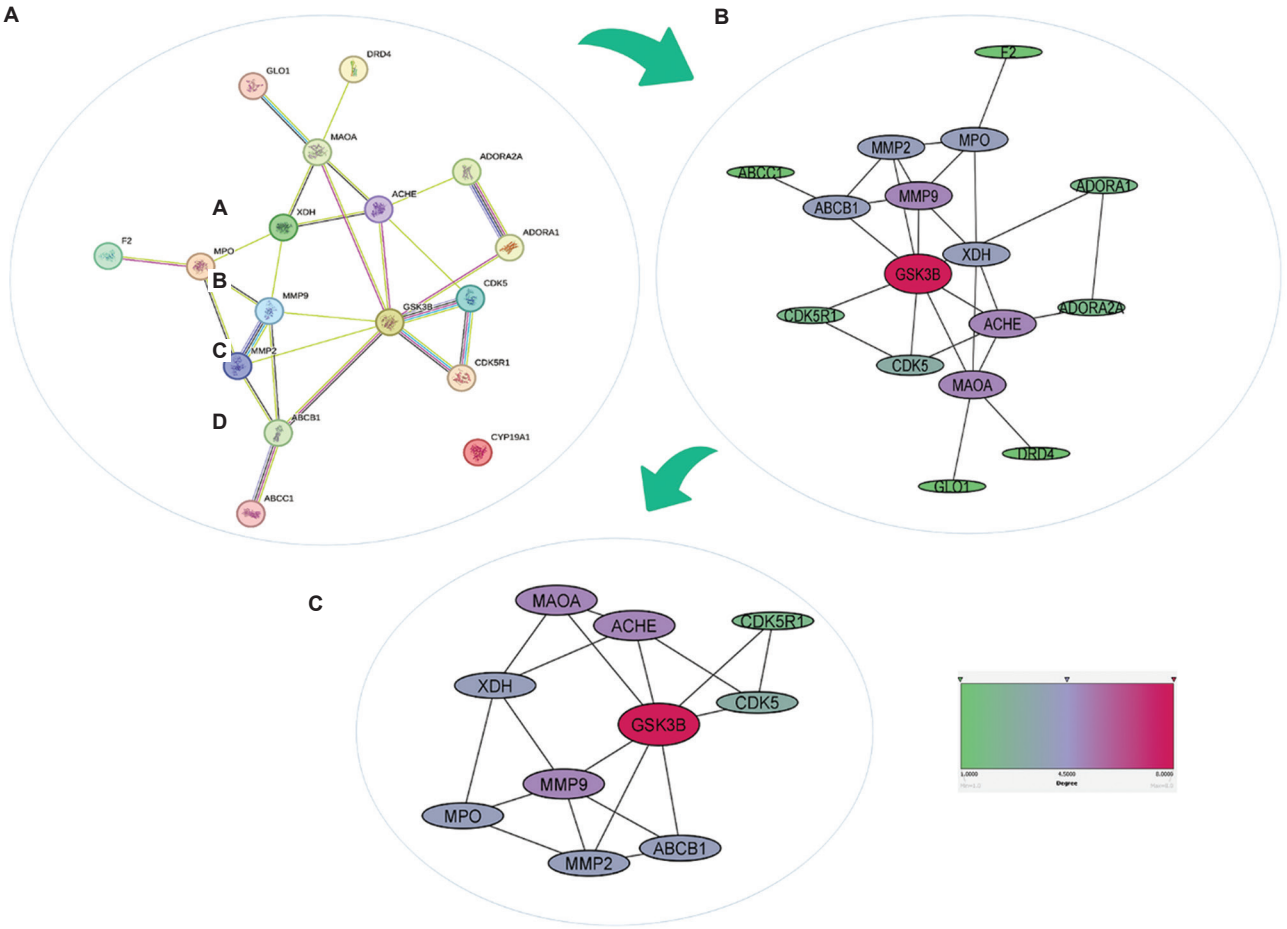

Figure 2. PPI network and key targets of FT in the treatment of MDD. (A) STRING data; (B) data visualization through Cytoscape; and (C) identification of the top target proteins.
Abbreviations: FT: Fisetin; MDD: Major depressive disorder; PPI: Protein-protein interaction.

Table 2. Top 10 targets of PPI network

	Targets	Degree	Betweenness	Closeness
1	GSK3B	8.0	95.33	0.65
2	MAOA	5.0	55.66	0.55
3	ACHE	5.0	29.66	0.55
4	MMP9	5.0	18.0	0.53
5	MPO	4.0	29.0	0.45
6	XDH	4.0	26.0	0.51
7	MMP2	4.0	11.33	0.51
8	ABCB1	4.0	28.0	0.5
9	CDK5	3.0	2.16	0.457
10	CDK5R1	2.0	0.0	0.416

Abbreviations: ABCB1: Adenosine triphosphate-binding cassette sub-family B member 1; ACHE: Acetylcholinesterase; CDK: Cyclin-dependent kinase; CDK5R1: CDK 5 regulatory subunit 1 FLX: Fluoxetine; GSK3B: Glycogen synthase kinase 3 beta; MAOA: Monoamine oxidase A; MMP: Matrix metalloproteinase; MPO: Myeloperoxidase; XDH: Xanthine dehydrogenase.

have been observed in both their blood and cerebrospinal fluid.[15] As a result, reducing neuroinflammation has emerged as a promising approach for treating MDD.

FT functions as a potential anti-inflammatory agent by inhibiting the upstream phosphorylation of IκBα and NF-κB. It also acts as an antioxidant by enhancing the expression of antioxidant factors through extracellular signal-regulated kinase phosphorylation.[31] This compound is likely to serve as a therapeutic agent for various neuroinflammatory conditions, including mood disorders and AD.[32] This comprehensive study employed a multifaceted approach to thoroughly investigate the antidepressant therapeutic potential of the TCM compound FT. Using the power of network pharmacology and *in silico* molecular docking techniques, the researchers set out to elucidate the underlying molecular mechanisms and pathways through which FT may exert its beneficial effects in the context of MDD. The study began by employing a variety of

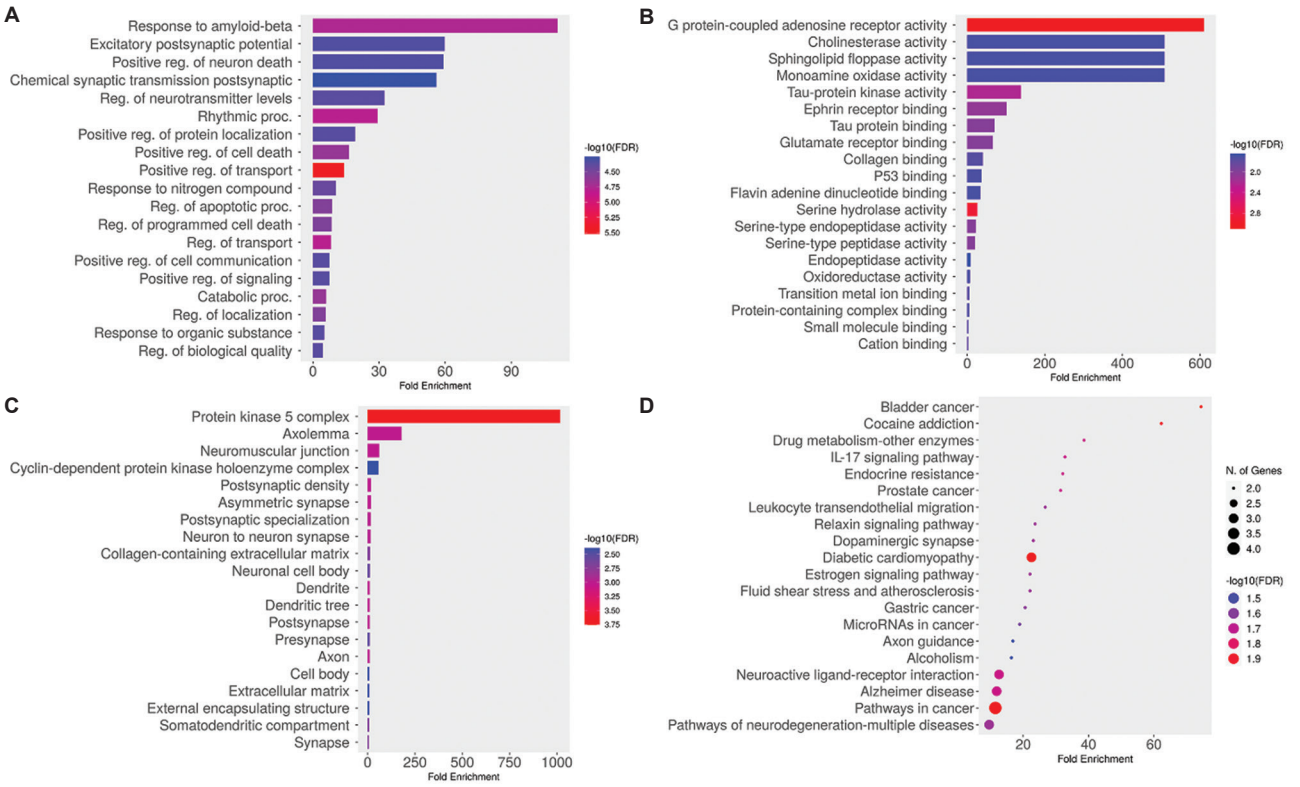

Figure 3. GO and KEGG enrichment analysis for FT targets in the treatment of MDD. Bar plots for GO enrichment include (A) biological process, (B) cellular component, and (C) molecular function, along with (D) a dot plot for KEGG enrichment.
Abbreviations: FT: Fisetin; GO: Gene Ontology; IL: Interleukin; KEGG: Kyoto Encyclopedia of Genes and Genomes; MDD: Major depressive disorder; Proc: Process; Reg: Regulation.

specialized databases and computational tools to evaluate the pharmacokinetic profile, DL attributes, permeability across the BBB, detailed compound information, and potential toxicological risks associated with FT.

Our findings indicated that FT has beneficial medicinal properties with a DL score of 0.24 and good absorption following oral administration. However, it has a negative BBB permeability score of −0.69, suggesting a limited ability to cross the BBB. Therefore, its delivery likely requires the use of transporting nanoparticles. The compound exhibited neurotoxicity and immunotoxicity, suggesting its potential as a therapeutic agent for neuroinflammatory diseases such as MDD, AD, traumatic brain injury, Parkinson's disease (PD), schizophrenia, stroke, vascular dementia, and diabetic neuropathy.[33] According to the study by Currais *et al.*,[34] the compound FT has been found to have several beneficial effects in rapidly aging mice, including decreasing inflammation, reducing the number of senescent cells, and lowering oxidative stress.[34] In addition, the research has shown that FT can regulate key signaling pathways induced by neurotrophic factors, suggesting its potential as a promising neuroprotective compound capable of preventing cell death mediated by oxytosis (oxidative stress-induced cell death) and ferroptosis (iron-dependent cell death); this positions FT as a potential therapeutic target for mitigating various age-related pathological processes.[32,33] The analysis of GO and KEGG pathways identified multiple signaling pathways that FT may regulate in the treatment of MDD. A notable discovery from the research was the identification of the IL-17 signaling pathway as a significant common target related to MDD. The cytokine IL-17 plays a significant role in the central nervous system (CNS) and its associated pathological processes. IL-17 acts on multiple resident cells within the CNS, including neurons, astrocytes, and microglia, to enhance the neuroinflammatory response.[35] Furthermore, Ling *et al.* (2023) conducted comprehensive research aimed at predicting FT targets for reversing cisplatin resistance using bioinformatics methodologies. They found that FT might inhibit the chemoresistance of head and neck cancer cells to cisplatin by targeting the HSP90AA1/IL-17 pathway.[36] FT may also alleviate MDD by inhibiting IL-17 as a proinflammatory cytokine and enhancing the release and activation of macrophages.[37] The study suggests that by modulating the IL-17 signaling pathway, FT may exert neuroprotective and

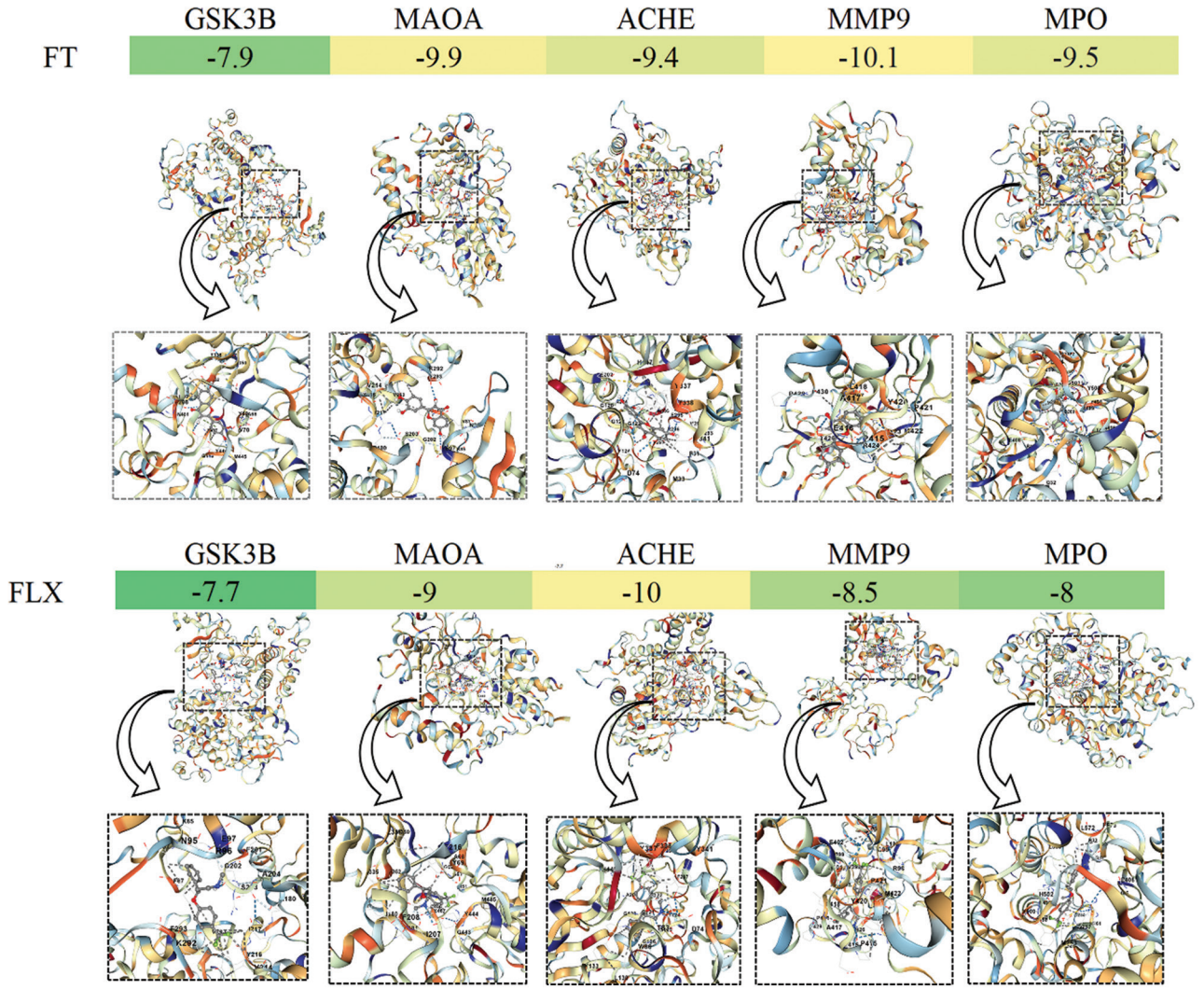

Figure 4. Docking analysis of FT with five key targets implicated in MDD, compared to the established antidepressant FLX. The values display the affinity scores between ligands and receptors, illustrating the strength of their interactions.
Abbreviations: ACHE: Acetylcholinesterase; FLX: Fluoxetine; FT: Fisetin; GSK3B: Glycogen synthase kinase 3 beta; MAOA: Monoamine oxidase A; MDD: Major depressive disorder; MMP9: Matrix metalloproteinase 9; MPO: Myeloperoxidase.

anti-inflammatory actions, which could be beneficial in the treatment of MDD and other neurodegenerative diseases.

To delve deeper into the mechanism by which the bioactive compound FT operates, we performed an extensive analysis aimed at identifying the target genes linked to its effects. This investigation revealed a total of 1,805 genes associated with MDD and 50 genes related to FT. Significantly, 17 of these genes were found to overlap between MDD and FT, suggesting the possibility of shared pathways and mechanisms in MDD treatment. To identify the crucial genes that contribute to FT's therapeutic effects, the researchers employed network pharmacology and PPI analyses. As a result, they pinpointed 10 key target proteins: GSK3B, MAOA, ACHE, MMP9, MPO, XDH, MMP2, ABCB1, CDK5, and CDK5R1. These identified target genes are involved in various cellular functions and signaling pathways, offering important insights into the molecular targets and mechanisms through which FT may provide therapeutic benefits in MDD and potentially other neurological conditions. For instance, one of the key target proteins identified in the analysis was GSK3B. GSK3B is a versatile protein that modulates a diverse array of cellular processes, including apoptosis (programmed cell death) and cell survival. It plays a crucial role in the signal transduction cascades that regulate neuronal cell development and energy metabolism within the CNS.[38] Moreover, the MAOA gene, as an important candidate gene for depressive symptoms, interacts with

environmental factors and is involved in the regulation of serotonin, noradrenaline, and dopamine.[39,40] In addition, increased ACHE activity may decrease dopamine activity and therefore increase depressive symptoms.[41]

The analysis also identified two other critical target proteins – MMP9 and MPO – as potential targets for FT's therapeutic effects in the context of MDD. During inflammatory states, excessive levels of MMP-9 and MPO are believed to be involved in the process of demyelination. MMP-9 and MPO can disrupt the BBB through their effects on tight junction proteins or by degrading the basement membrane. This disruption of the BBB can lead to increased neuroinflammation, which may contribute to the development and progression of MDD and other neuropsychiatric disorders like bipolar disorder, especially when accompanied by cognitive decline.[41,42] On the other hand, a sustained reduction of MPO and MMP9 activity reduces inflammation and increases cell protection.[42] Thus, FT may alleviate the MPO and MMP9 function while improving the MDD condition.

The docking analysis revealed that FT exhibited promising interactions with five key proteins associated with MDD: GSK3B, MAOA, ACHE, MMP9, and MPO. Notably, the highest binding affinities were observed for MAOA and MPO, both with a Vina score of −10.2. These findings suggest that FT may have significant therapeutic potential in MDD treatment through its interactions with these critical targets. In comparison, FLX, a well-established antidepressant, displayed varying binding affinities across the same targets. The Vina scores for FLX were −7.7 for GSK3B, −9.0 for MAOA, −10.0 for ACHE, −8.5 for MMP9, and −8.0 for MPO. While FLX demonstrated strong interactions, FT's superior binding affinities, particularly with MAOA and MPO, indicate that FT may be more effective in targeting these proteins. This suggests that FT could potentially offer enhanced therapeutic effects compared to FLX, especially in modulating the pathways associated with MDD. The implications of these results are significant. The strong binding of FT to MAOA, an enzyme involved in the metabolism of neurotransmitters such as serotonin, norepinephrine, and dopamine, highlights its potential role in enhancing mood regulation. Similarly, the interaction with MPO, which is implicated in inflammatory processes, suggests that FT may also exert anti-inflammatory effects that could further benefit MDD treatment.

Overall, the comparative analysis of FT and FLX underscores the potential of FT as a novel therapeutic agent for MDD. The enhanced binding affinities observed in this study warrant further investigation into FT's mechanisms of action and its efficacy in clinical settings, paving the way for future research into its role in treating MDD. In the CNS, MMPs play crucial roles in various physiological processes, such as myelin formation, axonal growth, angiogenesis, and regeneration.[43] However, the overproduction or dysregulation of MMPs has been linked to the pathogenesis of several neurological disorders, including AD, PD, ischemia, and MDD.[44,45] Interestingly, the bioactive compound FT has been shown to possess the ability to suppress the expression of specific MMPs, including MMP-1, MMP-3, MMP-7, and MMP-9, in tumor cells.[46] Furthermore, FT has been found to inhibit the metastatic potential of PC-3 prostate cancer cells by reducing the expression of MMP-2 and MMP-9, potentially through the suppression of the c-Jun N-terminal kinase and phosphoinositide 3-kinase/Akt signaling pathways.[47]

In addition to its effects on MMPs, FT has also been reported to inhibit the activity of the MAOA isoform in C6 glial cells.[48] MAOA plays a crucial role in the regulation of catecholamine and serotonin (5-HT) levels within the brain. Dysregulation of MAOA has been implicated in the pathogenesis of various neuropsychiatric psychiatric and age-related disorders, such as MDD and PD.[49]

Furthermore, FT has been shown to exert anti-inflammatory effects by inhibiting the production of proinflammatory mediators, including TNF, IL-1, IL-6, and IL-17, and the suppression of their signaling pathways in macrophages.[44,50] In addition, FT has been shown to decrease MPO activity and alleviate symptoms associated with neurocognitive impairments, such as AD.[51] Interestingly, FT administration has also been observed to ameliorate alterations in the levels of neurotransmitters, such as dopamine, GABA, and glutamate, as well as ACHE in the hippocampus and cortex of kindled mice.[52] Thus, FT counteracts the pathological processes involved in MDD. On the other hand, FT has previously been reported to inhibit GSK3B,[53] a regulator that suppresses mitochondrial biogenesis. Moreover, it has been reported that GSK3B inhibition protects against dopaminergic neurodegeneration[54] while increasing the brain's energy metabolism.[55] Therefore, by affecting the IL-17 signaling pathway, FT could reduce the expression of targets such as GSK3B, MAOA, ACHE, MMP9, and MPO, potentially improving neurological disorders like MDD.

5. Conclusion

This comprehensive investigation utilized docking analyses to explore a novel therapeutic approach for managing MDD. The primary goal was to assess the potential of the bioactive compound FT and analyze its interactions with key molecular targets associated with MDD. Insights gained from GO and KEGG pathway analyses illuminated the

mechanisms through which FT may exert its therapeutic effects on MDD. In addition, molecular docking studies reinforced the therapeutic relevance of FT's active components and their interactions with the identified targets. The findings unveiled a complex web of interactions between FT and its respective targets. We identified five specific bioactive components of FT – GSK3B, MAOA, ACHE, MMP9, and MPO – that demonstrated promising efficacy in treating MDD by modulating pathways related to the proinflammatory cytokine IL-17. Importantly, our comparative analysis with FLX highlighted FT's superior binding affinities, particularly with MAOA and MPO, suggesting that FT may offer enhanced therapeutic effects. This investigation underscores the potential of FT as a multi-target therapeutic agent for the management of MDD. The study's findings provide a solid foundation for further exploration of FT's therapeutic applications and the underlying mechanisms responsible for its beneficial effects in MDD and related neuropsychiatric disorders. Although FT has a limited ability to cross the BBB, it can be potentially delivered using nanoparticles, enhancing its therapeutic potential. However, it is crucial to note that studies of this nature require validations from animal models to confirm the efficacy and safety of FT *in vivo*. This represents a limitation of our current study, as *in vitro* tests and follow-up studies are necessary to validate our findings and further elucidate the mechanisms by which FT improves MDD by interfering with the IL-17 signaling pathway and reducing the expression of proinflammatory targets. Overall, while our research highlights FT's promising potential, the lack of experimental validation limits its translational relevance. Future experimental studies are essential to validate our *in silico* findings and explore FT's role as a novel therapeutic agent in treating depression.

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Conflict of interest

Tahmineh Mokhtari is the Editorial Board Member of the journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Other authors declare no conflicts of interest.

Author contributions

Conceptualization: Tahmineh Mokhtari
Formal analysis: Tahmineh Mokhtari
Investigation: All authors
Methodology: All authors
Writing–original draft: All authors
Writing–review & editing: Tahmineh Mokhtari

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author on reasonable request.

References

1. Otte C, Gold SM, Penninx BW, *et al*. Major depressive disorder. *Nat Rev Dis Primers*. 2016;2(1):16065.

   doi: 10.1038/nrdp.2016.65

2. Liu L, Liu C, Wang Y, Wang P, Li Y, Li B. Herbal medicine for anxiety, depression and insomnia. *Curr Neuropharmacol*. 2015;13(4):481-493.

   doi: 10.2174/1570159X1304150831122734

3. World Health Organization. *Depression and Other Common Mental Disorders*: *Global Health Estimates*. Geneva, Switzerland: World Health Organization; 2017.

4. Santomauro DF, Herrera AMM, Shadid J, *et al*. Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. *Lancet*. 2021;398(10312):1700-1712.

   doi: 10.1016/S0140-6736(21)02143-7

5. Elzamzamy, K., & Khan, Y. S. (2022). Major depressive and dysthymic disorders. J Alternative Med Research, 14(3): 309-324.

6. Bhatt S, Nagappa AN, Patil CR. Role of oxidative stress in depression. *Drug Discov Today*. 2020;25(7):1270-1276.

   doi: 10.1016/j.drudis.2020.05.001

7. Beck AT, Alford BA. *Depression: Causes and Treatment*. Philadelphia, PA: University of Pennsylvania Press; 2009.

8. Seifert J, Maier HB, Führmann F, *et al*. Pharmacological treatment of major depressive disorder according to severity in psychiatric inpatients: Results from the AMSP pharmacovigilance program from 2001-2017. *J Neural Transm* (*Vienna*). 2022;129(7):925-944.

   doi: 10.1007/s00702-022-02504-6

9. Mokhtari T. Targeting autophagy and neuroinflammation pathways with plant-derived natural compounds as potential antidepressant agents. *Phytother Res*. 2022;36(9):3470-3489.

doi: 10.1002/ptr.7551

10. Zhang W, Huai Y, Miao Z, Qian A, Wang Y. Systems pharmacology for investigation of the mechanisms of action of traditional Chinese medicine in drug discovery. *Front Pharmacol*. 2019;10:743.

    doi: 10.3389/fphar.2019.00743

11. Qi Z, Xie P, Yang C, *et al*. Developing fisetin-AgNPs incorporated in reinforced chitosan/pullulan composite-film and its application of post-harvest storage in litchi fruit. *Food Chem*. 2023;407:135122.

    doi: 10.1016/j.foodchem.2022.135122

12. Matić S, Stanić S, Mihailović M, Bogojević D. Cotinus coggygria Scop.: An overview of its chemical constituents, pharmacological and toxicological potential. *Saudi J Biol Sci*. 2016;23(4):452-461.

    doi: 10.1016/j.sjbs.2015.05.012

13. Ding H, Li Y, Chen S, *et al*. Fisetin ameliorates cognitive impairment by activating mitophagy and suppressing neuroinflammation in rats with sepsis-associated encephalopathy. *CNS Neurosci Ther*. 2022;28(2):247-258.

    doi: 10.1111/cns.13765

14. Wang Y, Wang B, Lu J, *et al*. Fisetin provides antidepressant effects by activating the tropomyosin receptor kinase B signal pathway in mice. *J Neurochem*. 2017;143(5):561-568.

    doi: 10.1111/jnc.14226

15. Yu X, Jiang X, Zhang X, *et al*. The effects of fisetin on lipopolysaccharide-induced depressive-like behavior in mice. *Metab Brain Dis*. 2016;31:1011-1021.

    doi: 10.1007/s11011-016-9839-5

16. Kohler O, Krogh J, Mors O, Benros ME. Inflammation in depression and the potential for anti-inflammatory treatment. *Curr Neuropharmacol*. 2016;14(7):732-742.

    doi: 10.2174/1570159X14666151208113700

17. Maher P, Akaishi T, Abe K. Flavonoid fisetin promotes ERK-dependent long-term potentiation and enhances memory. *Proc Natl Acad Sci U S A*. 2006;103(44):16568-16573.

    doi: 10.1073/pnas.0607822103

18. Ishige K, Schubert D, Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radic Biol Med*. 2001;30(4):433-446.

    doi: 10.1016/S0891-5849(00)00498-6

19. Han X, Yang C, Guo C, *et al*. Bioinformatics analysis to screen key targets of curcumin against colorectal cancer and the correlation with tumor-infiltrating immune cells. *Evid Based Complement Alternat Med*. 2021;2021(1):9132608.

    doi: 10.1155/2021/9132608

20. Nogales C, Mamdouh ZM, List M, Kiel C, Casas AI, Schmidt HH. Network pharmacology: Curing causal mechanisms instead of treating symptoms. *Trends Pharmacol Sci*. 2022;43(2):136-150.

    doi: 10.1016/j.tips.2021.11.004

21. Noor F, Tahir ul Qamar M, Ashfaq UA, Albutti A, Alwashmi ASS, Aljasir MA. Network pharmacology approach for medicinal plants: Review and assessment. *Pharmaceuticals* (*Basel*). 2022;15(5):572.

    doi: 10.3390/ph15050572

22. Daina A, Michielin O, Zoete V. SwissTargetPrediction: Updated data and new features for efficient prediction of protein targets of small molecules. *Nucleic Acids Res*. 2019;47(W1):W357-W364.

    doi: 10.1093/nar/gkz382

23. Nickel J, Gohlke BO, Erehman J, *et al*. SuperPred: Update on drug classification and target prediction. *Nucleic Acids Res*. 2014;42(W1):W26-W31.

    doi: 10.1093/nar/gku477

24. Safran M, Dalah I, Alexander J, *et al*. GeneCards Version 3: The human gene integrator. *Database* (*Oxford*). 2010;2010:baq020.

    doi: 10.1093/database/baq020

25. Franz M, Lopes CT, Fong D, *et al*. Cytoscape.js 2023 update: A graph theory library for visualization and analysis. *Bioinformatics*. 2023;39(1):btad031.

    doi: 10.1093/bioinformatics/btad031

26. Ge SX, Jung D, Yao R. ShinyGO: A graphical gene-set enrichment tool for animals and plants. *Bioinformatics*. 2020;36(8):2628-2629.

    doi: 10.1093/bioinformatics/btz931

27. Karakuş F, Kuzu B. Predicting the molecular mechanisms of cardiovascular toxicity induced by per-and polyfluoroalkyl substances: An *in silico* network toxicology perspective. *Toxicol Res* (*Camb*). 2024;13(6):tfae206.

    doi: 10.1093/toxres/tfae206

28. Liu Y, Cao Y. Protein-ligand blind docking using CB-Dock2. *Methods Mol Biol*. 2024;2714:113-125.

    doi: 10.1007/978-1-0716-3441-7_6

29. Liu Y, Yang X, Gan J, Chen S, Xiao ZX, Cao Y. CB-Dock2: Improved protein-ligand blind docking by integrating cavity detection, docking and homologous template fitting. *Nucleic Acids Res*. 2022;50(W1):W159-W164.

    doi: 10.1093/nar/gkac394

30. Arora V, Chopra K. Possible involvement of oxido-nitrosative stress induced neuro-inflammatory cascade and monoaminergic pathway: Underpinning the correlation between nociceptive and depressive behaviour in a rodent model. *J Affect Disord*. 2013;151(3):1041-1052.

    doi: 10.1016/j.jad.2013.08.032

31. Ravula AR, Teegala SB, Kalakotla S, Pasangulapati JP, Perumal V, Boyina HK. Fisetin, potential flavonoid with multifarious targets for treating neurological disorders: An updated review. *Eur J Pharmacol*. 2021;910:174492.

doi: 10.1016/j.ejphar.2021.174492

32. Hassan SSu, Samanta S, Dash R, *et al*. The neuroprotective effects of fisetin, a natural flavonoid in neurodegenerative diseases: Focus on the role of oxidative stress. *Front Pharmacol*. 2022;13:1015835.

doi: 10.3389/fphar.2022.1095648

33. Maher P. Preventing and treating neurological disorders with the flavonol fisetin. *Brain Plast*. 2020;6(2):155-166.

doi: 10.3233/BPL-200104

34. Currais A, Farrokhi C, Dargusch R, *et al*. Fisetin reduces the impact of aging on behavior and physiology in the rapidly aging SAMP8 mouse. *J Gerontol A Biol Sci Med Sci*. 22018;73(3):299-307.

doi: 10.1093/gerona/glx104

35. Fu J, Huang Y, Bao T, Liu C, Liu X, Chen X. The role of Th17 cells/IL-17A in AD, PD, ALS and the strategic therapy targeting on IL-17A. *J Neuroinflammation*. 2022;19(1):98.

doi: 10.1186/s12974-022-02446-6

36. Ling J, Zhang L, Wang Y, *et al*. Fisetin, a dietary flavonoid, increases the sensitivity of chemoresistant head and neck carcinoma cells to cisplatin possibly through HSP90AA1/IL-17 pathway. *Phytother Res*. 2023;37(5):1997-2011.

doi: 10.1002/ptr.7723

37. Shukla R, Pandey V, Vadnere GP, Lodhi S. Role of flavonoids in management of inflammatory disorders. In: *Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases*. Amsterdam, Netherlands: Elsevier; 2019. p. 293-322.

doi: 10.1016/B978-0-12-813820-5.00018-0

38. Chen J, Wang M, Khan RAW, *et al*. The GSK3B gene confers risk for both major depressive disorder and schizophrenia in the Han Chinese population. *J Affect Disord*. 2015;185:149-155.

doi: 10.1016/j.jad.2015.06.040

39. Williams LM, Gatt JM, Kuan SA, *et al*. A polymorphism of the MAOA gene is associated with emotional brain markers and personality traits on an antisocial index. *Neuropsychopharmacology*. 2009;34(7):1797-1809.

doi: 10.1038/npp.2009.1

40. Cao C, Wang M, Cao Y, Ji L, Zhang W. The interactive effects of monoamine oxidase A (MAOA) gene and peer victimization on depressive symptoms in early adolescent boys: The moderating role of catechol-O-methyltransferase (COMT) gene. *Acta Psychol Sin*. 2017;49(2):206-218.

doi: 10.3724/SP.J.1041.2017.00206

41. Walczak-Nowicka ŁJ, Herbet M. Acetylcholinesterase inhibitors in the treatment of neurodegenerative diseases and the role of acetylcholinesterase in their pathogenesis. *Int J Mol Sci*. 2021;22(17):9290.

doi: 10.3390/ijms22179290

42. Bliźniewska-Kowalska K, Gałecki P, Su KP, Halaris A, Szemraj J, Gałecka M. Expression of PON1, PON2, PON3 and MPO genes in patients with depressive disorders. *J Clin Med*. 2022;11(12):3321.

doi: 10.3390/jcm11123321

43. Coughlin JM, Wang Y, Minn I, *et al*. Imaging of glial cell activation and white matter integrity in brains of active and recently retired national football league players. *JAMA Neurol*. 2017;74(1):67-74.

doi: 10.1001/jamaneurol.2016.3764

44. Caraffa A. The neuroprotective role of flavonoids. *Eur J Neurodegener Dis*. 2021;10:25-29.

45. Gupta J, Nebreda AR. Roles of p38α mitogen-activated protein kinase in mouse models of inflammatory diseases and cancer. *FEBS J*. 2015;282(10):1841-1857.

doi: 10.1111/febs.13250

46. Chen JK, Peng SF, Lai KC, *et al*. Fistein suppresses human osteosarcoma U-2 OS cell migration and invasion via affecting FAK, uPA and NF-κB signaling pathway *in vitro*. *In Vivo*. 2019;33(3):801-810.

doi: 10.21873/invivo.11542

47. Chien CS, Shen K-H, Huang JS, Ko SC, Shih YW. Antimetastatic potential of fisetin involves inactivation of the PI3K/Akt and JNK signaling pathways with downregulation of MMP-2/9 expressions in prostate cancer PC-3 cells. *Mol Cell Biochem*. 2010;333:169-180.

doi: 10.1007/s11010-009-0217-z

48. Zhen L, Zhu J, Zhao X, *et al*. The antidepressant-like effect of fisetin involves the serotonergic and noradrenergic system. *Behav Brain Res*. 2012;228(2):359-366.

doi: 10.1016/j.bbr.2011.12.017

49. Naoi M, Maruyama W, Shamoto-Nagai M. Type A monoamine oxidase and serotonin are coordinately involved in depressive disorders: From neurotransmitter imbalance to impaired neurogenesis. *J Neural Transm* (*Vienna*). 2018;125(1):53-66.

doi: 10.1007/s00702-017-1709-8

50. Li C, Tang Y, Ye Y, Zuo M, Lu Q. Potential of natural flavonols and flavanones in the treatment of ulcerative colitis. *Front Pharmacol*. 2023;14:1120616.

doi: 10.3389/fphar.2023.1120616

51. Deghiedy NM, Abdel-Naby DH, Aziz MM, El-Sheikh MM.

Fisetin-loaded pluronic-based nanogel: Radiation synthesis for alleviating neurocognitive impairments in a rat model of Alzheimer's disease via modulation of the apoptotic cascade. *Int J Biol Macromol*. 2024;274:133472.

doi: 10.1016/j.ijbiomac.2024.133472

52. Khatoon S, Samim M, Dahalia M, Nidhi. Fisetin provides neuroprotection in pentylenetetrazole-induced cognition impairment by upregulating CREB/BDNF. *Eur J Pharmacol*. 2023;944:175583.

doi: 10.1016/j.ejphar.2023.175583

53. Molagoda IMN, Karunarathne WAH, Park SR, *et al*. GSK-3β-targeting fisetin promotes melanogenesis in B16F10 melanoma cells and zebrafish larvae through β-catenin activation. *Int J Mol Sci*. 2020;21(1):312.

doi: 10.3390/ijms21010312

54. Li J, Ma S, Chen J, *et al*. GSK-3β contributes to parkinsonian dopaminergic neuron death: Evidence from conditional knockout mice and tideglusib. *Front Mol Neurosci*. 2020;13:81.

doi: 10.3389/fnmol.2020.00081

55. Martin SA, Souder DC, Miller KN, *et al*. GSK3β regulates brain energy metabolism. *Cell Rep*. 2018;23(7):1922-1931.e4.

doi: 10.1016/j.celrep.2018.04.045

Appendix

Table A1. Docking analysis

Ligands	Targets	VS	CV (Å^3)	Center	Contact residues
FT	GSK3B	−8.4	2588	29, 27, 62	Chain A: ASN64 GLY65 SER66 PHE67 GLY68 VAL70 LYS85 VAL87 LEU88 GLN89 ASP90 LYS94 ASN95 ARG96 GLU97 ARG180 ASP181 LYS183 ASP200 PHE201 GLY202 SER203 ALA204 LYS205 VAL214 TYR216 ILE217 SER219 Chain B: SER261 VAL263 ASP264 PHE291 LYS292 PHE293 PRO294
	MAOA	−10.2	10294	28, 0, 2	Chain A: ILE23 ARG51 THR52 VAL65 GLY66 GLY67 ALA68 TYR69 VAL70 GLY110 ASN125 TRP128 ASP132 PHE173 PHE177 ILE180 ASN181 THR204 THR205 ARG206 ILE207 PHE208 SER209 VAL210 GLY214 GLU216 VAL303 ILE304 LYS305 CYS323 MET324 ILE325 ILE335 THR336 LEU337 MET350 PHE352 TRP397 CYS406 TYR407 THR435 GLY443 TYR444 MET445 ALA448
	ACHE	−9.2	1094	121, 109, −135	Chain A: ASP74 GLY82 THR83 GLU84 MET85 TRP86 ASN87 PRO88 GLY120 GLY121 GLY122 TYR124 SER125 LEU130 TYR133 GLU202 ALA204 GLY205 SER229 TRP236 TRP286 SER293 VAL294 PHE295 ARG296 PHE297 TYR337 PHE338 TYR341 HIS447 Chain B: PRO31 LYS32 MET33
	MMP9	−8.3	419	43, 53, 39	Chain A: ASP74 GLY82 THR83 GLU84 MET85 TRP86 ASN87 PRO88 GLY120 GLY121 GLY122 TYR124 SER125 LEU130 TYR133 GLU202 ALA204 GLY205 SER229 TRP236 TRP286 SER293 VAL294 PHE295 ARG296 PHE297 TYR337 PHE338 TYR341 HIS447 Chain B: PRO31 LYS32 MET33
	MPO	−10.2	1192	12, 31, 133	Chain A: PHE252 MET253 GLN254 GLY256 GLN257 ASP260 HIS261 ASP264 PHE265 THR266 ARG405 GLU408 MET409 TYR462 THR495 ASN496 PHE498 ARG499 TYR500 GLY501 HIS502 LEU504 ILE505 LEU572 PHE573 LEU583 LEU586 ASN587 Chain B: GLN32 ASN33 LEU37
FLX	GSK3B	−7.7	2588	29, 27, 62	Chain A: GLY65 SER66 PHE67 GLY68 VAL69 VAL70 ALA83 LYS85 VAL87 LEU88 PHE93 LYS94 ASN95 ARG96 GLU97 VAL110 LEU132 ASP133 THR138 TYR140 ARG141 ARG180 ASP181 LYS183 GLN185 ASN186 LEU188 CYS199 ASP200 PHE201 GLY202 SER203 ALA204 VAL214 TYR216 ILE217 Chain B: VAL263 ASP264 LYS292 PHE293 PRO294
	MAOA	−9	10294	28, 0, 2	Chain A: ARG51 GLY66 GLY67 ALA68 TYR69 VAL70 GLY71 GLN74 ILE180 ASN181 VAL182 TYR197 ARG206 ILE207 PHE208 SER209 GLY214 GLU216 ILE335 THR336 LEU337 MET350 PHE352 CYS406 TYR407 TRP441 GLY443 TYR444 MET445
	ACHE	−10	1094	121, 109, −135	Chain A: GLN71 TYR72 VAL73 ASP74 GLY82 THR83 TRP86 ASN87 PRO88 TYR119 GLY120 GLY121 GLY122 PHE123 TYR124 SER125 GLY126 LEU130 TYR133 GLU202 ALA204 SER229 TRP236 TRP286 VAL294 PHE295 ARG296 PHE297 TYR337 PHE338 TYR341 TRP439 HIS447 GLY448 TYR449 ILE451 Chain B: PRO31 MET33
	MMP9	−8.5	419	43, 53, 39	Chain A: PRO97 ARG98 CYS99 LEU188 ALA189 LEU397 VAL398 ALA399 HIS401 GLU402 VAL414 PRO415 GLU416 ALA417 LEU418 MET419 TYR420 PRO421 MET422 TYR423 ARG424 PHE425 THR426 GLU427 GLY428 PRO429 PRO430 LEU431 HIS432 ASP435
	MPO	−8	1192	12, 31, 133	Chain A: MET253 GLY256 GLN257 ASP260 HIS261 ASP264 PHE265 THR266 GLU268 ARG405 GLU408 MET409 THR495 PHE498 ARG499 TYR500 GLY501 HIS502 ILE505 LEU527 PHE531 PHE532 ILE568 LEU572 PHE573 LEU583 LEU586 ASN587 ARG590 Chain B: GLN32 ASN33 ILE35 LEU37

Abbreviations: ACHE: Acetylcholinesterase; CV: Cavity volume; FLX: Fluoxetine; FT: Fisetin; GSK3B: Glycogen synthase kinase 3 beta; MAOA: Monoamine oxidase A; MDD: Major depressive disorder; MMP9: Matrix metalloproteinase 9; MPO: Myeloperoxidase; VS: Vina score.

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Genotypic diversity of human and porcine group A rotaviruses in Uttar Pradesh, India

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Abstract

Diarrheal diseases, particularly those caused by rotaviruses, pose a significant health threat, especially among children, and cause huge economic losses to the pig industry in the form of high morbidity, mortality, and stunted growth. *Rotavirus* A (RVA) remains the predominant viral agent for severe diarrheal episodes, contributing to high hospitalization and mortality rates in India. RVA's high genetic diversity is attributed to frequent reassortment and mutations. This study aims to characterize the *VP4*, *VP6*, *VP7*, and *NSP4* genes of RVA in stool samples collected from children and piglets in and around Bareilly, Uttar Pradesh, India. A total of 300 samples, including 100 from children and 200 from piglets, were screened for the detection of double-stranded RNA of RVA using ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) and reverse transcription polymerase chain reaction (RT-PCR). Results revealed the RVA incidence, particularly in winter (end of November to beginning of February), aligning with observed seasonal trends. Among the 32 *Rotavirus* (RV)-positive samples from children, 21 (65.63%) were detected by RNA-PAGE, whereas 28 (87.5%) were identified by RT-PCR. Whereas, of the 80 RT-PCR positive samples from piglets, only 51 (63.75%) were detected by RNA-PAGE, indicating the superiority of RT-PCR. Molecular analysis identified the prevalent genotypes in human strains as G1, G2, G3, and P[8], whereas G9P[13]-I5-E1 dominated among piglets in a single farm outbreak. The findings underscore the critical need for continuous surveillance to monitor evolving RV genotypes from both humans and piglets, enabling the identification of new strains of RVA and subsequent modification of vaccination strategies to reduce RVA's impact in India.

Keywords: Diarrheal diseases; Epidemiology; Genotypic diversity; India; RNA-PAGE; *Rotavirus* A; Reverse transcription polymerase chain reaction

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1. Introduction

According to the Foodborne Disease Burden Epidemiology Reference Group, diarrheal diseases result in 420,000 fatalities globally, with rotaviruses identified as the predominant cause of diarrhea among young children in India, accounting for an estimated 21,357 deaths in 2010.[1] There are nine *Rotavirus* (RV) species referred to as A, B, C, D, F, G, H, I, and J, and among these, RVA is the predominant viral agent responsible for diarrhea, resulting in around 100,000 fatalities and 400,000 – 800,000 hospitalizations among children in India.[2] It has significant genetic diversity attributed to frequent genetic reassortment and/or mutations.[3,4] All 11 segments of the RV genome, which include *VP1*, *VP2*, *VP3*, *VP4*, *VP6*, *VP7*, *NSP1*, *NSP2*, *NSP3, NSP4,* and *NSP5* genes showing nucleotide variations, result in genotypes R28, C20, L24, P[58], I32, G42, A32, N28, T28, E32, and H28, respectively (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg, accessed on 06.11.2024). The majority of human rotaviruses exhibit Wa-like (Gx-P[x]-I1-R1- C1-M1-A1-N1-T1-E1-H1) or DS-1-like (Gx-P[x]-I2-R2-C2-M2-A2-N2-T2-E2-H2) constellations, which originate from pigs or cattle.[3] In addition, a few strains derived from felines belong to the AU-1-like (Gx-P[x]-I3-R3-C3-M3-A3-N3-T3-E3-H3) constellation.[5] This genetic variation in RV constellations observed globally in both animals and humans is a result of distinct evolutionary processes.[6] Moreover, the Indian RVA strains from both animals and humans exhibit diverse genotypic traits. In general, the common genotypes observed among Indian RV strains are G1, G2, G3, G4, G9, and G12, often associated with P[6], P[8], and P[4]. While 89% of strains (92% from Wa-like and 86% from DS-1-like) showed classical constellations, reassortant constellations were identified in 11% of the strains (8% from Wa-like and 14% from DS-1-like).[7]

In India, the introduction of the RV vaccine to the Union Government's Universal Immunization Programme in March 2016 has led to a significant reduction in the prevalence of cases among children under five. Reports indicate a decrease of approximately 33.7% in cases, 38.3% in deaths, and about 21.8% in overall antibiotic misuse related to RV infections.[8] However, despite the vaccine's availability and its impressive preventive results, there are still frequent reports of diarrheal episodes caused by RVA in both children and animals, particularly during the winter months across the country. This ongoing issue may be attributed to several contributing factors, with genotypic diversity and reassortment of the virus being significant. Therefore, conducting studies on the molecular epidemiology and antigenic diversity of circulating RVA, particularly in animals, is crucial for the development and potential modification of effective indigenous vaccines to address RV episodes. Our previous experiment identified the circulating piglet strain G9P[13] in the Bareilly region. While characterizing this strain for multiple sequence alignment and phylogenetic analysis of each gene (*VP6*, *VP4*, *VP7*, *NSP*, and *NSP4*), it was observed that the *VP7*, *NSP3*, and *NSP4* genes are closely related to human strains, whereas the *VP4* gene was closely related to porcine strain.[9] However, reports on the circulation of the same G9P[13] strain in humans/children from the same area are not available. Further, some of the pig farms located in this region also need to be investigated for the presence/absence of this RVA strain. Therefore, in this study, we determined the incidence of RVA in piglets from different farms and children from five different hospitals and characterized the *VP4*, *VP6*, *VP7*, and *NSP4* genes of RVA to identify the circulating genotypes in humans and piglets from in and around Bareilly, Uttar Pradesh, India.

2. Materials and methods

2.1. Specimen collection

Institute Biosafety Committee (IBSC) approval was obtained from ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India, for the research project on "Genotypic diversity of human, bovine and porcine group A rotaviruses," under which the present study was carried out (proceedings of the IBSC meeting held on July 06, 2018). Children under 3 years old who exhibited acute gastroenteritis with at least five episodes of watery stools per day (with or without vomiting and fever) were included in a study conducted between 2017 and 2019. Informed consent was obtained from each child's guardian and from the administrators of five childcare hospitals in the Bareilly district of Uttar Pradesh. Two to ten mL/g stool samples were collected from each child experiencing diarrhea in sterile containers. In addition, a total of 136 diarrheal and 64 non-diarrheal fecal samples were obtained (after proper consent from the owners) from three pig farms in the Bareilly district: Izatnagar (n = 14), Rupapur (n = 171), and Ismilepur (n = 15). During previous analyses of some samples from the Rupapur farms, it was noted that many piglets were affected during that period. Thus, an outbreak of diarrhea in piglets was reported at the Rupapur farm, leading to extensive sampling in this farm. In the end, a total of 300 stool samples were collected: 100 samples from children and 200 samples from piglets, and they were subsequently screened for RV, followed by genotypic analysis of positive samples to characterize the *VP4*, *VP6*, *VP7*, and *NSP4* genes.

2.2. Extraction of RV double-stranded RNA (ds-RNA) from stool samples

All plasticware and glassware were decontaminated using 0.05% (v/v) diethyl pyrocarbonate to remove RNase/DNase activity before the extraction of RV dsRNA from stool samples. The RNA extraction was performed utilizing either TRIzol LS reagent (Invitrogen, USA) or RNAsure Virus kit (Nucleopore, Genetix Biotech Asia Ltd., India), adhering to the manufacturers' instructions. The extracted dsRNA was kept in 20 µL of RNA storage solution (Riboreserve, Amresco, USA) and stored at −20°C for future use. A positive control stool sample containing rotaviruses maintained by the Division of Veterinary Public Health at IVRI, Bareilly, Uttar Pradesh, was incorporated at every stage of the experiments, from dsRNA extraction to RV detection through ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) and reverse transcription polymerase chain reaction (RT-PCR).

2.3. Detection of RV by RNA-PAGE

The dsRNA extracted from each sample was analyzed using RNA-PAGE (comprising a 7.5% resolving gel and a 5.0% stacking gel), followed by silver staining.[10,11]

2.4. Detection of RV by RT-PCR

A two-step RT-PCR assay was optimized using positive control samples by fine-tuning the concentrations of various reagents and cycling conditions. To enhance coverage of the 3' end, reverse transcription utilizing the random primer "FR26RV-N" paired with the poly T-tagged primer "FR40RV-T" was also optimized, employing the M-MuLV reverse transcriptase (RT) enzyme.[12] To start the RT-PCR, the dsRNA denaturation and elimination of secondary structures were performed in a final volume of 14 µL reaction mix, comprising 3.0 µL of FR26RV-N (10 pmol), 1.0 µL of FR40RV-T (10 pmol), 1.5 µL of dimethyl sulfoxide, 3.5 µL of nuclease-free water (NFW), and 5.0 µL of dsRNA. The reaction mix was spun and incubated at 95°C for 5 min in a thermocycler (Eppendorf, Germany), followed by snap chilling on ice for 1 – 5 min. Next, the reaction mix was added with 11 µL of other reagents, including 2.5 µL of ×5 RT buffer, 2.0 µL of 10 mM dithiothreitol, 0.5 µL of 10 mM dNTPs, 0.25 µL of RNase inhibitor (40 U/µL), 0.25 µL of M-MuLV RT (20 U/µL), and 5.5 µL of NFW. First-strand cDNA synthesis was performed at 37°C for 60 min, followed by 65°C for 10 min. The resulting first-strand cDNA was either used for PCR immediately or stored at −20°C for later use.

The PCR assay was set up in a total reaction volume of 25 µL consisting of 1× PCR buffer with $MgCl_2$, 1.25 U of Dream Taq DNA polymerase (Thermo Scientific, Lithuania), 0.2 mM dNTPs, 0.6 pmol of forward primer, 0.6 pmol of reverse primer, 5.0 µL of first-strand cDNA, and NFW (Amresco, USA). The cycling parameters included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at temperatures ranging from 48°C to 55°C for 30 s, and extension at 72°C for 1 to 2 min, concluding with a final extension at 72°C for 10 min. The gene-specific primer pairs used in the present study are detailed in Table 1, with their respective annealing temperatures and product sizes. To increase the genetic diversity among RV strains, two or more primer pairs were used to amplify each gene segment. The amplified PCR products were subsequently resolved through electrophoresis on a 1.5% (w/v) agarose gel in Tris-borate buffer. The DNA bands were visualized with an ultraviolet transilluminator and the image was captured using a gel documentation system (Vilber, Germany). To purify the amplicons, the QIAquick Gel Extraction Kit (Qiagen, Germany) was employed according to the manufacturer's instructions. The eluted products were stored at -20°C for future use and nucleotide sequencing. Furthermore, samples whose *VP7* gene failed to be amplified using 9Con1/9Con2 primers were subsequently amplified with primer pairs Vp7-F59/Vp7-R995 and TNG C1- F/TNG C2-R.

2.5. RVA genotyping

The G genotype of RV was determined through nucleotide sequencing of the *VP7* gene amplified products resulting from all three primer pairs. The nucleotide sequencing was conducted by Eurofins Genomics India Pvt. Ltd. in Bangalore, India. The DNA sequences obtained were analyzed using the Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and classified using RotaC (http://rotac.regatools.be), a freely available web-based tool that can be used for fast RV genotype differentiation of all 11 group A RV gene segments according to the new guidelines proposed by the RV Classification Working Group.[13] To identify the P genotypes, a partial length of the *VP4* gene segment was amplified using the previously mentioned protocol, but specific primers (con3F/con2R and Wang F20/Wang 1185R) and different annealing conditions were applied (Table 1). Similarly, for I and E genotyping, full-length *VP6 (*1340 bp), and *NSP4* genes (722 bp) were amplified accordingly (Table 1).[3,14,15] All amplified products were subjected to agarose gel electrophoresis, visualization, and image acquisition as mentioned in Section 2.4. The identification of other genotypes was carried out similarly to the method used for G genotypes.

Table 1. Primer pairs used for detection and characterization of group A rotaviruses

Rotavirus A gene segments	Primer	Sequence (5' to 3')	Size (bp)	Annealing Temperature (°C)	References
VP4 (P)	Con3F Con2R	TGGCTTCGCTCATTTATAGACA ATTTCGGACCATTTATAACC	877	53	41
	Wang F20 Wang 1185R	TGGCTTCGCTCATTTATATAFAC GACTGGCCATGCACCTACAGGT	1185	53	42
VP7 (G)	9con1F 9con2R	TAGCTCCTTTTAATGTATGG GTATAAAATACTTGCCACCA	1030	52	43
	Vp7-F59 Vp7-R998	GCTCCTTTTAATGTATGGTAT ARTGAYCKTGATCKTTTGGACAT	960	54.5	44
	TNG C1-F TNG C2-R	GGCTTTAAAAGAGAGAATTTCCGTC TGG GGTCACATCATACAATTCTAATCTAAG	1065	52.5	45
VP6 (I)	Gen VP6 F Gen VP6 R	GGCTTTWAAACGAAGTCTTC GGTCACATCCTCTCACT	1340	48	3
	Ghosh VP6 F Ghosh VP6 R	GGCTTTAAAACGAAGTCTTC GGTCACATCCTCTCACT	1340	52	46
NSP4 (E)	Gen Nsp4 F Nsp4 722R	TAAAAGTTCTGTTCCGAGAGAG TTAAGACCGTTCCTTCCATT	722	52	13

3. Results

3.1. Incidence of RVA

RVA is identified by its characteristic 11-banded pattern (4:2:3:2) on RNA-PAGE, whereas amplification of the *VP6* gene is confirmed by the production of 1340 bp amplicons using RT-PCR. Out of the 100 stool samples from children, 32 tested positive for RVA, whereas the remaining 68 did not yield any RV identification profiles through either method. Four RNA-PAGE-positive samples failed to be amplified using RT-PCR. Among the 32 RVA-positive samples, 21 (65.63%) could be detected by RNA-PAGE, whereas 28 (87.5%) could be identified by RT-PCR. In addition, seven samples that were detected positive by RT-PCR were not detected by RNA-PAGE. However, all but four of the RVA-positive samples identified through PAGE were also confirmed to have the *VP6* gene by RT-PCR. On the other hand, field samples demonstrated both short and long electropherotypes of RNA segregation in the RNA-PAGE analysis (Figure 1).

One private hospital in the densely populated city of Bareilly recorded the highest incidence of cases (47.5%) among the five hospitals studied (Table 2). Furthermore, a greater number of hospitalized children with RVA infections (59%) were noted in December and January. The incidence of RVA infections showed an increase from November to January, followed by a decrease from February to March. Thus, the majority of RVA cases were reported during the peak winter months in Bareilly.

Among the 200 stool samples from piglets, a total of 80 samples tested positive for RVA using RNA-PAGE and

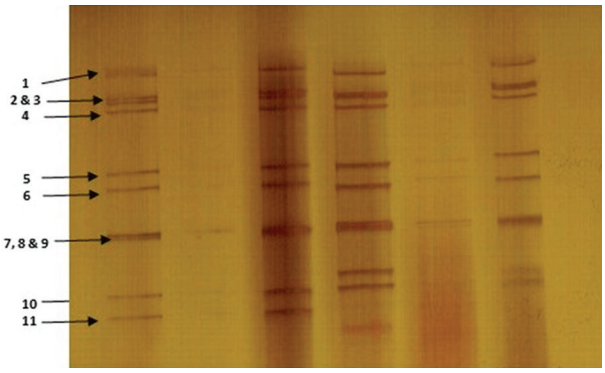

Figure 1. Positive field samples of *Rotavirus* show different migration patterns in ribonucleic acid-polyacrylamide gel electrophoresis.

RT-PCR, whereas the remaining 120 were not detected with any RV through either method used in the study. Out of the 80 samples detected positive by RT-PCR, 51 (37.5%) were also confirmed by RNA-PAGE, all of which were derived from diarrheal stool samples. These results also indicate the greater sensitivity of RT-PCR in RVA detection compared to RNA-PAGE. In contrast, RVA was not detected in any of the 64 non-diarrheal stool samples. All positive samples were sourced from a single pig farm in Rupapur during December and January, whereas the other two farms in Izatnagar and Ismilepur showed no signs of RVA.

3.2. RVA genotyping

The distribution of G, P, I, and E genotypes in RVA from positive human samples are summarized in Table 3, and the amplified products of *VP7*, *VP4*, *VP6*, and *NSP4* genes

Table 2. Incidence of RVA in diarrheal stool samples of humans

Months	(A) (HBI)		(B) (HBM)		(C) (HBS)		(D) (HBG)		(E) (HBD)		Total	
	PAGE (%)	RT-PCR (%)	PAGE (%)	RT-PCR (%)	PAGE (%)	RT-PCR (%)	PAGE (%)	RT-PCR (%)	PAGE (%)	RT-PCR (%)	PAGE (%)	RT-PCR (%)
November-17	1/4	2/4	0/6	0/6	0/8	0/8	0/1	0/1	0/2	0/2	1/21	2/21
December-17	3/6	4/6	1/5	2/5	1/4	1/4	1/6	0/6	2/7	3/7	8/28	10/28
January-18	5/10	6/10	1/1	1/1	0/1	0/1	0/2	0/2	1/3	2/3	7/17	9/17
February-18	3/12	4/12	0/3	0/3	0/2	0/2	0/3	1/3	1/5	2/5	4/25	6/25
March-18	1/8	1/8	0	0	0	0	0	0	0/1	0/1	1/9	1/9
Total	13/40	17/40	2/15	3/15	1/15	1/15	1/12	1/12	4/18	6/18	21/100	28/100
	(32.5)	(42.5)	(32.5)	(13.3)	(6.66)	(6.66)	(8.33)	(8.33)	(22.2)	(33.3)	(21.0)	(28.0)
RVA+Samples	19/40		3/15		1/15		2/12		7/18		32/100	
	(47.5)		(20.0)		(6.66)		(16.6)		(38.8)		(32.0)	

Notes: HBI: Hospital 1; HBM: Hospital 2; HBS: Hospital 3; HBG: Hospital 4; HBD: Hospital 5.
Abbreviation: RVA: *Rotavirus* A; RT-PCR: Reverse transcription polymerase chain reaction; PAGE: Polyacrylamide gel electrophoresis.

are presented in Figure 2. Among the 32 human RVA-positive samples, 28 (87.5%) were detectable using RT-PCR targeting the *VP6* gene. Genotypic determination of these 28 samples targeting the *VP7*, *VP4*, *VP6*, and *NSP4* genes revealed 24 instances of G genotypes, 25 of P genotypes, 28 of I genotypes, and 27 of E genotypes. When further characterizing the *VP7* gene sequences, the G3 (46.42%) genotype was found to be the most prevalent, followed by G1 (28.57%) and G2 (10.71%). In terms of P genotypes, P[8] (60.71%) was the most dominant, compared to P[4] and P[6] (14.28% each). Besides, I1 (78.57%) and E1 (75.0%) were more prevalent compared to I2 and E2 (21.42% each), respectively. Three G genotypes (G1, G2, and G3) were frequently found in combination with three P genotypes (P[4], P[6], and P[8]). This resulted in four G-P combinations, with the predominant circulating strain being G3P[8] (46.42%), followed by G1P[8] (14.28%), G2P[4] (10.71%), and G1P[6] (14.28%), as shown in Table 3.

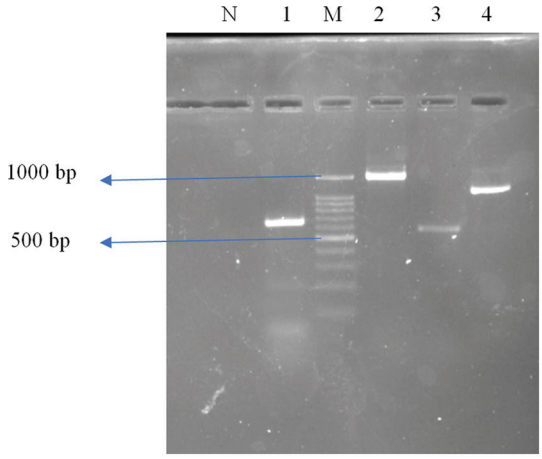

Figure 2. Result of gel electrophoresis shows amplified genes of *Rotavirus* A
Notes: Lane N: Negative control; Lane1: *VP4* (850 bp); Lane M: DNA ladder (100 bp); Lane 2: *VP6* (1060 bp); Lane 3: *NSP4* (700 bp); Lane 4: *VP7* (900 bp).

Furthermore, the RVA strains with G3 genotype were found to consistently coexist with P[8], I1, and E1 genotypes, whereas G2 genotypes showed a consistent combination with P[4], I2, and E2 genotypes. In addition, the coexistence of G1, I1, and E1 genotypes was observed to be accompanied by either P[8] or P[6]. It also appears that I2 and E2 genotypes were found only in strains with P[4] genotype, not in those with P[6] or P[8] (Table 3). In total, out of the 28 human RVA strains in this study, 13 were characterized as G3P[8]I1E1, 4 as G1P[6]I1E1, and 3 each as G1P[8]I1E1 and G2P[4]I2E2 genotypes.

As for the RVA-positive piglet samples, gel electrophoresis revealed a similar pattern for all samples, implying the likelihood of a single and highly virulent RVA strain circulating on the Rupapur farm. Five positive samples from this farm were randomly selected to undergo *VP7*, *VP4*, *VP6*, and *NSP4* gene sequence analysis by BLAST and RotaC program, and they all revealed identical sequences representing the same genotypic constellation, which is G9P[13]-I5-E1. The fact that all stool samples harboring RVA with this genotypic constellation were sourced from a single pig farm in Rupapur indicates an outbreak. In addition, further investigation unveiled that the diarrheal episodes began at the end of November 2017, peaked in December 2017 and January 2018, and subsided by early February 2018, inferring that the outbreak could be related to winter. Approximately 75% of the piglet population on this farm was affected during this episode,

Table 3. Genotypic profiles of *Rotavirus* A from the positive human stool samples

S. No.	Samples ID	*VP7* (G)	*VP4* (P)	*VP6* (I)	*NSP4* (E)	Accession number
1.	HBI-02	G3	P[8]	I1	E1	
2.	HBI-03	G3	P[8]	I1	E1	
3.	HBI-06	--	P[4]	I2	E2	
4.	HBI-09	G2	P[4]	I2	E2	
5.	HBI-10	G3	P[8]	I1	E1	
6.	HBI-11	G1	P[6]	I1	E1	
7.	HBI-12	G3	P[8]	I1	E1	
8.	HBI-14	G3	P[8]	I1	E1	
9.	HBI-16	G1	P[8]	I1	E1	
10.	HBI-18	G1	P[8]	I1	E1	
11.	HBI-19	G2	P[4]	I2	E2	
12.	HBI-20	G3	P[8]	I1	E1	
13.	HBI-24	G3	P[8]	I1	E1	
14.	HBI-25	G3	P[8]	I1	E1	
15.	HBI-37	G3	P[8]	I1	E1	
16.	HBI-38	G1	P[6]	I1	E1	
17.	HBI-39	G3	P[8]	I1	E1	
18.	HBI-40	--	--	I2	E2	
19.	HBG-01	--	--	I1	E1	
20.	HBG-02	--	--	I2	E2	
21.	HBD-03	G1	P[6]	I1*	E1	MH560407
22.	HBD-05	G1	P[8]	I1	--	
23.	HBD-06	G2	P[4]	I2*	E2*	MH560409 MH204127
24.	HBD-07	G3	P[8]	I1*	E1*	MH560410 MH204128
25.	HBD-08	G1	P[8]	I1	E1	
26.	HBM-1	G1	P[6]	I1	E1*	MH204130
27.	HBM-3	G3	P[8]*	I1	E1*	MH560408 MH204131
28.	HBS 2	G3	P[8]	I1	E1*	MH204132
	Total	24	25	28	27	

Notes: HBI: Hospital 1; HBM: Hospital 2; HBS: Hospital 3; HBG: Hospital 4; HBD: Hospital 5.

highlighting the significant impact of the RV strain in terms of morbidity, which was accompanied by stunted growth and substantial economic losses.

4. Discussion

Various studies conducted across different regions of India have indicated a wide range of RV prevalence rates, from 5% to 71%, among hospitalized children under 5 years of age suffering from acute gastroenteritis.[16-18] However, following the introduction of the RV vaccine for children in March 2016, the prevalence has declined by approximately 33.7%, with a reduction in deaths by 38.3%.[1] Notably, this study observed a high incidence rate of 32.0%, which aligns with other research reporting a prevalence of around 35% in various parts of the country.[19,20] The potential impact of the RV vaccination program was further explored by Dhalaria, who found a higher prevalence of 9.1% among children who had not received any doses of the vaccine.[21] Neighboring countries, such as Bangladesh, Nepal, and Pakistan, have also seen varied RV prevalence rates of 32.0%, 28.0%, and 26.8%, respectively.[22-24] Globally, RV is prevalent, contributing to an estimated 199,000 cases, with a 95% uncertainty interval of 165,000 – 241,000 diarrheal deaths, with India accounting for 21357 of those fatalities.[25] RV stands out as the leading cause of diarrheal deaths in children under 5 years in developing countries; however, no fatalities were reported in our study among cases of RV diarrhea.

RVA infections are known to hinder growth in piglets, especially during the pre- and post-weaning stages, leading to decreased profitability in India's pig farming sector.[26] In our study, porcine RVAs were identified in 58.82% (80/136) of samples collected from three different farms in Uttar Pradesh. This high prevalence is primarily attributed to a single farm affected by a highly virulent strain (G9P[13]-I5-E1), from which most samples were taken. It appears that this could be an outbreak impacting approximately 70 – 75% of the piglet population on that farm. Few outbreaks of piglet diarrhea have been documented globally. A recent significant outbreak of acute diarrhea in piglets occurred in Henan province, China, where RV B (strain HNLY-2022) with the genotype G6-P[6]-I4-R6-C6-M6-A7-N5-T7-E5-H4 was identified as the causative agent. Half of its genotypes (P[6], R6, C6, M6, T7, and E5) were newly reported, indicating a reassortment in the RV strain.[27] Notably, the porcine strain identified in the present study (G9P[13]-I5-E1) was also determined to be a reassortant strain. Previous research documented this genotypic combination (G9P[13]-E1) in pigs, revealing that G9 genotype sequences exhibited over 96 – 98.9% similarity with human isolates, whereas P[13] sequences formed a distinct cluster with porcine sequences from India and other regions, suggesting interspecies transmission.[9]

We observed that long electropherotypes predominated in RNA-PAGE results from both human and animal samples,[28,29] but a higher positivity rate was recorded by RT-PCR compared to RNA-PAGE. In our study, 87.5% of the 32 RV-positive samples from children and 100% of

[1] https://theprint.in/health/rotavirus-vaccine-has- curbed-deaths-in-india-by-a-third-deterred-antibiotic-misuse-finds-us-study/2236105/, accessed on 06.11.2024

the 80 samples from piglets were detected by RT-PCR, as opposed to 65.63% and 63.75% detected by RNA-PAGE, respectively. This finding aligns with Kylla, who also noted a lower detection rate of RVA using RNA-PAGE (4.81%) compared to RT- PCR (7.43%).[30] Moreover, RV was found only in diarrheal samples, corroborating previous studies that reported similar results. For instance, Kylla found that 9.14% of samples from diarrheal pigs tested positive for RVA, compared to just 2.54% from non-diarrheal piglets.[28] All diarrhea cases of RVA in piglets were reported during the winter season, which corresponds to the name "winter diarrhea."

Studies conducted from 1983 to 1997 on human rotaviruses showed that G1 and G2 strains were more prevalent than G3 strains, a trend that shifted from 1996 to 1998 when G2 became predominant, followed by G1 and G9.[15] A similar pattern continued from 1998 to 2000, with G1 strains predominating over G2, G3, and G4 strains.[31] Overall, G1P[8] strains (31.4 – 46.1%) and G2P[4] strains (20.2%) were consistently reported as dominant throughout the years studied, except in 2009 when G9P[8] strains peaked at 15.3%, particularly from Pune in Western India.[32] Consequently, G1P[8] was identified as the dominant strain in Pune during 1992 – 1993 and again from 2006 to 2008. Similar trends were seen in other parts of India, including northern regions, where G1 was predominant, followed by G2, G-untypeable, and G9 strains.[33-36] Interestingly, between 2016 and 2019, the dominance shifted to G3P[8] (44.09%), followed by G1P[8] (32.65%), G2[P4] (5.10%), G1[P6] (3.06%), and G9[P4] (1.02%).[37] In our research, we similarly found that the G3P[8] RVA strain was predominant at 46.42%, followed by G1[P8] (14.28%), G2P[4] (10.71%), and G1P[6] (14.28%) among children. In addition, I1 (78.57%) and E1 (75.0%) genotypes were more common than I2 and E2 (21.42% each) in this investigation. Notably, I2 and E2 genotypes were consistently associated with the G2P[4] strain, but not with G1P[6] or G3P[8], suggesting specific linkages among these strains. Similar relationships among these genes have shown both concordance (G2-P[4]-I2-E2) and discordance (G9P[4]-I2-E6).[38]

The single circulating piglet rotaviral genotype G9P[13]-I5-E1 identified in this study was highly virulent and associated with severe diarrheal outbreaks. Research into the pathogenesis and genome analysis of the G9P[13] RV strain from Ohio revealed a human-like G9 VP7 genotype that shared a higher overall nucleotide identity with historical porcine RV (PRV) strains. This strain led to longer rectal virus shedding and RV RNAemia in pigs compared to HRV Wa G1P[8] and provided complete short-term cross-protection against HRV or PRV challenges, whereas HRV Wa G1P[8] offered only partial protection.[39] Another study indicated that the genomic segments of G9P[13] RVAs were closely linked to porcine or porcine-like human RVAs, demonstrating the existence of porcine-human reassortant strains.[40]

5. Conclusion

This study identified the predominant RVA strain G3P[8], followed by G1P[8], G1P[6], and G2P[4], circulating among children in Bareilly and surrounding regions. This highlights a shifting landscape in the epidemiology and genotypic distribution of RV related to childhood diarrhea. In addition, the emerging G9P[13]-I5-E1 RVA strain in pigs may pose a risk for potential outbreaks among piglets on farms near Rupapur, as well as for children living in close proximity to these farms. Thus, it is crucial to regularly assess the effectiveness of current RV vaccines and to maintain ongoing surveillance of RV genotypes in diarrheal cases affecting both children and animals.

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Conflict of interest

The authors declare that they have no competing or conflict of interests.

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Ethics approval and consent to participate

IBSC approval was obtained from ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India, for the research project on "Genotypic diversity of human, bovine and porcine group A rotaviruses" under which the present study was carried out (proceedings of the IBSC meeting held on July 6, 2018). IVRI Institute Animal Ethical Committee (IAEC) is giving Ethical approval to the research where the laboratory animal experiments are carried out. However, IVRI IBSC approval is required for handling any pathogens and samples in the laboratory of the Institute. As the laboratory animal experiments are not done in the present research work, took permission from IBSC. Informed consent was obtained from each child's guardian and from the administrators of five childcare hospitals in the Bareilly district of Uttar Pradesh.

Consent for publication

Inform consent in the form of verbal permission was obtained from parents.

Availability of data

Data and material will be made available upon reasonable request to the corresponding authors.

References

1. World Health Organization. *World Health Statistics 2017: Monitoring Health for the SDGs, Sustainable Development Goals*. Geneva: World Health Organization; 2017.
2. Tate RL, Perdices M, Rosenkoetter U, *et al.* The single-case reporting guideline in behavioural interventions (SCRIBE) 2016 statement. *Phys Ther*. 2016;96(7):e1-e10.

   doi: 10.2522/ptj.2016.96.7.e1
3. Matthijnssens J, Ciarlet M, Rahman M, *et al.* Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch Virol*. 2008;153(8):1621-1629.

   doi: 10.1007/s00705-008-0155-1
4. Matthijnssens J, Ciarlet M, McDonald SM, *et al.* Uniformity of *Rotavirus* strain nomenclature proposed by the *Rotavirus* Classification Working Group (RCWG). *Arch Virol*. 2011;156(8):1397-1413.

   doi: 10.1007/s00705-011-1006-z
5. Matthijnssens J, Ciarlet M, Heiman E, *et al.* Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine *Rotavirus* strains and human DS-1-like and bovine *Rotavirus* strains. *J Virol*. 2008;82(7):3204-3219.

   doi: 10.1128/JVI.02257-07
6. Okitsu S, Khamrin P, Thongprachum A, *et al.* Whole-genomic analysis of G3P[23], G9P[23] and G3P[13] *Rotavirus* strains isolated from piglets with diarrhea in Thailand, 2006-2008. *Infect Genet Evol*. 2013;18:74-86.

   doi: 10.1016/j.meegid.2013.05.005
7. Khakha SA, Varghese T, Giri S, *et al.* Whole-genome characterization of common *Rotavirus* strains circulating in Vellore, India from 2002 to 2017: Emergence of non-classical genomic constellations. *Gut Pathog*. 2023;15(1):44.

   doi: 10.1186/s13099-023-00569-6
8. Gleason A, Kumar CK, Klein E, Laxminarayan R, Nandi A. Effect of *Rotavirus* vaccination on the burden of *Rotavirus* disease and associated antibiotic use in India: A dynamic agent-based simulation analysis. *Vaccine*. 2024;42(22):126211.

   doi: 10.1016/j.vaccine.2024.126211
9. Abass G, Dubal ZB, Rajak KK, *et al.* Molecular characterization of porcine *Rotavirus* A from India revealing zooanthroponotic transmission. *Anim Biotechnol*. 2020;33:1073-1085.

   doi: 10.1080/10495398.2020.1868486
10. Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD. Rapid diagnosis of *Rotavirus* infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J Clin Microbiol*. 1982;16(3):473-477.

    doi: 10.1128/jcm.16.3.473-477.1982
11. Svensson L, Uhnoo I, Grandien M, Wadell G. Molecular epidemiology of *Rotavirus* infections in Uppsala, Sweden, 1981: Disappearance of a predominant electropherotype. *J Med Virol*. 1986;18(2):101-111.

    doi: 10.1002/jmv.1890180202
12. Djikeng A, Halpin R, Kuzmickas R, *et al.* Viral genome sequencing by random priming methods. *BMC Genomics*. 2008;9:5.

    doi: 10.1186/1471-2164-9-5
13. Maes P, Matthijnssens J, Rahman M, Van Ranst M. RotaC: A web-based tool for the complete genome classification of group A rotaviruses. *BMC Microbiol*. 2009;9:238.

    doi: 10.1186/1471-2180-9-238
14. Chitambar SD, Arora R, Chhabra P. Molecular characterization of a rare G1P[19] *Rotavirus* strain from India: Evidence of reassortment between human and porcine *Rotavirus* strains. *J Med Microbiol*. 2009;58(Pt 12):1611-1615.

    doi: 10.1099/jmm.0.012856-0
15. Ghosh S, Kobayashi N. Whole-genomic analysis of *Rotavirus* strains: Current status and future prospects. *Future Microbiol*. 2011;6(9):1049-1065.

doi: 10.2217/fmb.11.90

16. Jain V, Das BK, Bhan MK, Glass RI, Gentsch JR, Indian Strain Surveillance Collaborating Laboratories. Great diversity of group A *Rotavirus* strains and high prevalence of mixed *Rotavirus* infections in India. *J Clin Microbiol*. 2001;39(10):3524-3529.

doi: 10.1128/JCM.39.10.3524-3529.2001

17. Broor S, Husain M, Chatterjee B, Chakraborty A, Seth P. Temporal variation in the distribution of *Rotavirus* electropherotypes in Delhi, India. *J Diarrhoeal Dis Res*. 1993;11(1):14-18.

18. Kelkar SD, Ayachit VL. Circulation of group A *Rotavirus* subgroups and serotypes in Pune, India, 1990-1997. *J Health Popul Nutr*. 2000;18(3):163-170.

19. Pol SS, Dedwal AK, Ranshing SS, Chitambar SD, Pednekar SN, Bharadwaj RS. Prevalence and characterization of rotaviruses in children hospitalized for diarrheal disease in a tertiary care hospital, Pune. *Indian J Med Microbiol*. 2017;35(1):33-36.

doi: 10.4103/ijmm.IJMM_16_94

20. Giri S, Nair NP, Mathew A, *et al. Rotavirus* gastroenteritis in Indian children < 5 years hospitalized for diarrhoea, 2012 to 2016. *BMC Public Health*. 2019;19(1):69.

doi: 10.1186/s12889-019-6406-0

21. Dhalaria P, Kapur S, Singh AK, Verma A, Priyadarshini P, Taneja G. Potential impact of *Rotavirus* vaccination on reduction of childhood diarrheal disease in India: An analysis of National Family Health Survey-5. *Vaccine X*. 2023;14:100319.

doi: 10.1016/j.jvacx.2023.100319

22. Haque W, Haque J, Barai D, *et al.* Distribution of *Rotavirus* genotypes in Dhaka, Bangladesh, 2012-2016: Re-emergence of G3P[8] after over a decade of interval. *Vaccine*. 2018;36(43):6393-6400.

doi: 10.1016/j.vaccine.2018.08.081

23. Shrestha S, Thakali O, Raya S, Shrestha L, Parajuli K, Sherchand JB. Acute gastroenteritis associated with *Rotavirus* A among children less than 5 years of age in Nepal. *BMC Infect Dis*. 2019;19(1):456.

doi: 10.1186/s12879-019-4092-2

24. Sadiq A, Bostan N, Bokhari H, *et al.* Molecular characterization of human group A *Rotavirus* genotypes circulating in Rawalpindi, Islamabad, Pakistan during 2015-2016. *PLoS One*. 2019;14(7):e0220387.

doi: 10.1371/journal.pone.0220387

25. Troeger, CE Khalil IA, Blacker BF, *et al.* Quantifying risks and interventions that have affected the burden of diarrhoea among children younger than 5 years: An analysis of the Global Burden of Disease Study 2017. *Lancet Infect Dis*. 2019;20(1):37-59.

doi: 10.1016/S1473-3099(19)30401-3

26. VinodhKumar OR, Sircar S, Pruthvishree BS, *et al.* Cross-sectional study on *Rotavirus* A (RVA) infection and assessment of risk factors in pre- and post-weaning piglets in India. *Trop Anim Health Prod*. 2020;52(1):445-452.

doi: 10.1007/s11250-019-01999-8

27. Li Q, Wang Z, Jiang J, *et al.* Outbreak of piglet diarrhea associated with a new reassortant porcine *Rotavirus* B. *Vet Microbiol*. 2024;288:109947.

doi: 10.1016/j.vetmic.2023.109947

28. Kusumakar AL, Savita, Malik YS, Minakshi, Prasad G. Genomic diversity among group A rotaviruses from diarrheic children, piglets, buffalo and cow calves of Madhya Pradesh. *Indian J Microbiol*. 2010;50(1):83-88.

doi: 10.1007/s12088-010-0016-y

29. Dubal ZB, Bhilegaonkar KN, Barbuddhe SB, *et al.* Prevalence and genotypic (G and P) determination of porcine group A rotaviruses from different regions of India. *Trop Anim Health Prod*. 2013;45(2):609-615.

doi: 10.1007/s11250-012-0267-1

30. Kylla H, Dutta TK, Roychoudhury P, Mandakini R, Subudhi PK. Detection and characterization of Genogroup 5 *Rotavirus* associated with piglet diarrhoea in the North East Region of India. *Vet Arhiv*. 2018;88(4):453-466.

doi: 10.24099/vet.arhiv.170613a

31. Das S, Varghese V, Chaudhuri S, *et al.* Genetic variability of human *Rotavirus* strains isolated from Eastern and Northern India. *J Med Virol*. 2004;72(1):156-161.

doi: 10.1002/jmv.10542

32. Pradhan GN, Chitambar SD. Genetic analysis of *Rotavirus* G2P[4] strains in Pune, Western India: Circulation of a novel reassortant bearing E6 *NSP4* genotype. *Arch Virol*. 2018;163(5):1391-1394.

doi: 10.1007/s00705-018-3710-4

33. Kang CI, Kim SH, Park WB, *et al.* Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: Risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. *Antimicrob Agents Chemother*. 2005;49(2):760-766.

doi: 10.1128/AAC.49.2.760-766.2005

34. George J, Lim JS, Jang SJ, *et al.* Comprehensive genomic profiles of small cell lung cancer. *Nature*. 2015;524(7563):47-53.

doi: 10.1038/nature14664

35. Kumar A, Basu S, Vashishtha V, Choudhury P. Burden of *Rotavirus* diarrhea in under five Indian children. *Indian Pediatr*. 2016;53(7):607-617.

doi: 10.1007/s13312-016-0896-y

36. Saluja S, Silverstein A, Mukhopadhyay S, *et al.* Using the consolidated framework for implementation research to implement and evaluate national surgical planning. *BMJ Glob Health.* 2017;2:e000269.

doi: 10.1136/bmjgh-2016-000269

37. Shrivastava AK, Reddy NS, Giri S, *et al.* Burden and molecular epidemiology of *Rotavirus* causing diarrhea among under-five children: A hospital-based study from Eastern India. *J Glob Infect Dis*. 2019;11(4):147-152.

doi: 10.4103/jgid.jgid_16_19

38. Tate R, Kennedy M, Ponsford J, *et al.* INCOG recommendations for management of cognition following traumatic brain injury, part III: Executive function and self-awareness. *J Head Trauma Rehabil*. 2014;29(4):338-352.

doi: 10.1097/HTR.0000000000000068

39. Shao L, Takeda K, Kato S, Mori S, Kodama T. Communication between lymphatic and venous systems in mice. *J Immunol Methods*. 2015;424:100-105.

doi: 10.1016/j.jim.2015.05.007

40. Li Y, Wang F, Kan R, *et al.* Genetic and immunological characterization of G9 group A porcine rotaviruses in China. *Zoonoses Public Health*. 2022;69(6):694-703.

doi: 10.1111/zph.12958

41. Gentsch JR, Glass RI, Woods P, *et al.* Identification of group A *Rotavirus* gene 4 types by polymerase chain reaction. *J Clin Microbiol*. 1992;30(6):1365-1373.

doi: 10.1128/jcm.30.6.1365-1373.1992

42. Wang YH, Zhou X, Ghosh S, *et al.* Prevalence of human *Rotavirus* genotypes in Wuhan, China, during 2008-2011: Changing trend of predominant genotypes and emergence of strains with the P[8]b subtype of the *VP4* gene. *Arch Virol*. 2011;156(12):2221-2231.

doi: 10.1007/s00705-011-1122-9

43. Das BK, Gentsch JR, Cicirello HG, *et al.* Characterization of *Rotavirus* strains from newborns in New Delhi, India. *J Clin Microbiol*. 1994;32(7):1820-1822.

doi: 10.1128/jcm.32.7.1820-1822.1994

44. Abe M, Ito N, Morikawa S, *et al.* Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine *Rotavirus* bearing new P and G genotypes. *Virus Res*. 2009;144(1-2):250-257.

doi: 10.1016/j.virusres.2009.05.005

45. Taniguchi K, Wakasugi F, Pongsuwanna Y, *et al.* Identification of human and bovine *Rotavirus* serotypes by polymerase chain reaction. *Epidemiol Infect*. 1992;109(2):303-312.

doi: 10.1017/s0950268800050263

46. Ghosh S, Alam MM, Ahmed MU, Talukdar RI, Paul SK, Kobayashi N. Complete genome constellation of a caprine group A *Rotavirus* strain reveals common evolution with ruminant and human *Rotavirus* strains. *J Gen Virol*. 2010;91(Pt 9):2367-2373.

doi: 10.1099/vir.0.022244-0

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

FXR1 modulates the expression of oncogenes and tumor suppressor genes associated with poor cancer prognosis

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Abstract

Post-transcriptional regulation by RNA-binding proteins (RBPs) is critical for mRNA stability, localization, and translation, primarily through interaction with the 3′-untranslated region. Dysregulation of RBPs has been associated with various cancers, with fragile X-related protein 1 (FXR1) emerging as a critical RBP involved in tumorigenesis through its interactions with target mRNAs. Despite its significance, the specific role of FXRI in cancer progression remains underexplored. In this study, we investigated FXR1's function using SH-SY5Y cells. RNA immunoprecipitation (RNA-IP) assay was employed to isolate RNA complexes associated with FXR1. We generated stable cell lines with either FXR1 overexpression or silencing to assess the impact of FXRI binding to mRNA complexes. Subsequent analyses, including quantitative reverse transcription polymerase chain reaction, correlation analysis, gene expression profiling, and survival analysis, were performed to validate the interactions of FXR1 with target mRNAs. Our RNA-IP analysis identified several mRNAs significantly enriched in FXR1-bound RNA complexes, including *SHISAL1*, *SLC43A3*, *NBAT1*, *PDZK1IP1*, *ACKR3*, *KCNN3*, *NECAB2*, *ANO5*, *ATOH8*, *IGFBP7*, *LEMD1*, *GPR35*, *WNT7A*, and *F2RL3*. Notably, we observed changes in the expression levels of these genes following FXR1 overexpression or depletion, indicating FXR1-mediated functional regulation. Co-expression analysis further supported FXR1's association with these target mRNAs. These findings highlight the significant role of FXR1 in regulating the stability and expression of key mRNAs implicated in malignancies. The dysregulation of FXR1 and its interaction with these target transcripts suggest that FXR1 plays a critical role in tumor biology, potentially offering new avenues for therapeutic interventions. This study provides a deeper understanding of FXR1's involvement in cancer and underscores the importance of RBPs in the post-transcriptional regulatory landscape of cancer progression.

Keywords: FXR1; RNA immunoprecipitation; Carcinogenesis; Overexpression; Knockdown; Correlation analysis; Survival analysis

1. Introduction

Post-transcriptionally, RNA-binding proteins (RBPs) regulate the transcript levels of various RNAs by interacting with specific regions, particularly within the 3'-untranslated region (UTR). Accumulating evidence suggests that dysregulation of RBPs significantly contributes to carcinogenesis. In this context, fragile X-related protein 1 (FXR1) is increasingly recognized as an important RBP in various cancers.[1-5] Due to its diverse RNA-binding domains and functional flexibility, FXR1 is implicated in controlling multiple post-transcriptional processes, including mRNA transport, translation, and degradation, by binding to adenylate/uridylate-rich elements and G-quartet regions of RNA.[6-8] In addition to its RNA-binding capabilities, FXR1 is known to interact with unbound ribosomes and polysomes.[9] Like many other RBPs, FXR1 is primarily localized in the cytoplasm,[10] but it can undergo nuclear reorientation under certain conditions.[11]

Several types of cancers, including head and neck squamous cell carcinoma (HNSCC) and lung squamous cell carcinoma (LSCC), exhibit elevated FXR1 expression.[12,13] FXR1 deficiency has been shown to induce apoptosis in LSCC,[13] while in HNSCC, it promotes cellular senescence.[11] Previous research has demonstrated that FXR1 targets the 3'-UTR of the tumor suppressor gene p21 for degradation, thereby facilitating the development and proliferation of HNSCC by evading senescence.[12] In addition, FXR1 has been shown to prevent the degradation of miR301a-3p by exoribonucleases. In FXR1-deficient cells, the exonuclease polyribonucleotidyltransferase 1 degrades miR301a-3p, resulting in increased p21 transcript levels in squamous cell carcinoma.[14] These findings suggest that inhibiting FXR1, combined with anti-microRNA (miRNA) oligonucleotide therapies and chemotherapy, could represent a more effective strategy for treating HNSCC.[15-17]

FXR1 interacts with a diverse array of mRNA transcripts, regulating their stability and translation, which is essential for various physiological functions. In muscle development, FXR1 regulates the expression of key myogenic factors, such as MyoD and myogenin, thereby facilitating myogenesis.[18] In addition, FXR1 plays a pivotal role in cellular stress responses, particularly through the regulation of heat shock protein mRNAs, which are crucial for cell survival under oxidative stress conditions.[19,20] Furthermore, FXR1 contributes significantly to neuronal function by modulating mRNAs encoding glutamate receptors, influencing synaptic plasticity and cognitive processes, including learning and memory.[21-23] Moreover, FXR1 has been implicated in the immune response through its interaction with mRNAs encoding inflammatory cytokines, suggesting a role in mediating host defense mechanisms against pathogenic challenges.[24,25] In this study, we demonstrate that FXR1 overexpression enhances the oncogenic activity of several cancer-related genes while decreasing the levels of tumor-suppressing genes. Conversely, FXR1 knockdown results in reduced oncogene activity and increased expression of tumor suppressors. Our results reveal that FXR1 directly regulates the expression of oncogenes, such as *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5*, while also targeting tumor suppressor genes, such as *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8*, and *IGFBP7*. These findings provide new insights into the role of FXR1 in cancer progression and highlight its potential as a therapeutic target.

2. Materials and methods

2.1. Cell culture

The SH-SY5Y cell line (Fenghbio Biological Ltd., China) was cultured in a 1:1 mixture of Minimum Essential Medium (MEM) and F12 media. The growth medium was supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin to ensure adequate nutrition for cell growth and protection against contamination. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. Cell passages were performed when the cultures reached approximately 80 – 90% confluence, and the media was refreshed every 2 – 3 days to sustain optimal growth conditions. Regular morphological assessments were performed to monitor cellular health and morphology throughout the culture period. All experiments utilized cells between passages 5 and 15 to ensure reproducibility and minimize genetic drift.

2.2. Construction of plasmid and lentiviral packaging

Lentiviral packaging was conducted using Lipofectamine 3000 transfection reagent (Thermo Fisher, United States). HEK293T cells were seeded in 10 cm dishes to achieve 40 – 50% confluency. A total of 12 μg plasmid DNA, comprising the target plasmid (pLVX-shRNA2-puro or pCDH-GFP+puro), helper plasmid psPAX2, and helper plasmid pMD2.G in ratios of 5:4:1 or 4:3:2, was transfected in a serum- and antibiotic-free medium. After 6 h, the medium was replaced with a fresh culture medium. The supernatant was collected 72 h post-transfection, centrifuged at 1,000 rpm for 4 min at room temperature, and filtered through a 0.45 μm membrane to obtain the lentiviral solution. The lentiviral solution was aliquoted and stored at −80°C. The sequences used for FXR1 knockdown were shFXR1#1 (5'-GCTAGAGGTTTCTTGGAATTT-3'), shFXR1#2 (5'-CGCCAGGTTCCATTTAATGAA-3'),

and the negative control shRNA (shNC, 5'-GTTCT CCGAACGTGTCACGTT-3') obtained from the Public Protein/Plasmid Library. The FXR1 overexpression construct was generated by cloning the human FXR1 coding sequence (NM_001013439.3) into a vector, which was subsequently packaged into a lentivirus.

2.3. Stable transfection protocol

For stable gene knockdown, proliferating SH-SY5Y cells were seeded in a 6-well plate and transduced with lentiviral particles containing shRNAs targeting the gene of interest. The lentiviral supernatant was added directly to the cells at a final concentration of approximately 30 μL of virus-containing medium in serum-free MEM. To enhance transduction efficiency, polybrene was added to the culture medium at a final concentration of 5 – 8 μg/mL. The cells were incubated with the lentivirus for 6 h to facilitate efficient viral uptake and gene silencing. Following this incubation period, the medium was replaced with MEM/F12 medium supplemented with 10% FBS, antibiotics (100 μg/mL streptomycin, 100 U/mL penicillin), and additional nutrients to support cell recovery. The cells were cultured for 48 h to allow stable integration of the shRNA-expressing constructs. To select successfully transduced cells, puromycin (2 μg/mL) was added to the culture medium, with daily media changes until non-transduced cells were eliminated and only puromycin-resistant clones remained. Selection typically lasted 5 – 7 days and was considered complete when no further cell death was observed. Stable cell lines were maintained in the puromycin-containing medium for downstream experiments.

For overexpression experiments, SH-SY5Y cells were transduced with lentiviral vectors carrying the gene of interest. The lentiviral supernatant was added directly to the cells at an appropriate concentration in serum-free MEM. Polybrene was also included at a final concentration of 5 – 8 μg/mL to improve transduction efficiency. The cells were incubated with the lentivirus for 6 h to ensure efficient transduction and gene expression. After this incubation, the medium was replaced with MEM/F12 medium supplemented with 10% FBS and antibiotics to promote cell recovery. Cells were cultured for an additional 48 h to ensure optimal gene expression before proceeding with further experimental assays. Both protocols were carried out using cells in the logarithmic growth phase to maximize transduction efficiency and minimize variability.

2.4. FXR1 RNA immunoprecipitation assay

FXR1 RNA immunoprecipitation (RNA-IP) was performed with slight modifications as previously described.[26] Briefly, SH-SY5Y cells were lysed in RNase inhibitor-supplemented RIP lysis buffer (20 mM Tris-HCl, pH 8.2, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% triton X-100, and a protease inhibitor cocktail). The lysates were pre-cleared using centrifugation at 12,000× g for 10 min at 4°C. For RNA-IP, a polyclonal FXR1 antibody (rabbit, Proteintech, China) was utilized, with an isotype-matched rabbit IgG antibody (Proteintech, China) serving as the negative control to account for non-specific binding.

The pre-cleared cell lysates were incubated with the antibody for 4 h at 4°C to allow binding of FXR1 and associated mRNAs. All steps were performed under RNase-free conditions to preserve RNA integrity. The samples were gently rotated during the incubation to facilitate interaction between the FXR1-mRNA complexes. The protein A/G+ agarose beads (Thermo Fisher, United States) were added to the cell lysate and incubated at 4°C for 40 min on an orbital shaker. After incubation, the beads were washed extensively with RNA-IP buffer to remove unbound material. To prevent reabsorption of mRNA and ensure maximum efficiency of the RNA-IP, the RNA-bound complexes were resuspended in 1 mL of proteinase K buffer (containing 10 mM Tris-HCl, pH 8, 50 mM EDTA, 0.5% SDS, 50 mM NaCl), supplemented with proteinase K enzyme at a ratio of 4:100 (v/v) and RNase inhibitor (1:100 v/v). The mixture was then incubated for 30 min at 50°C to degrade proteins bound to the beads and release the RNA for subsequent analysis. The resulting RNA was extracted using the TRIzol extraction method and purified for downstream analysis, such as polymerase chain reaction (PCR), quantitative reverse transcription (qRT)-PCR, or RNA sequencing, to identify mRNA targets bound by FXR1.

2.5. RNA extraction, quantitative reverse transcription polymerase chain reaction, and western blotting

Total RNA was isolated using TRI reagent (Takara, China), following the manufacturer's guidelines. Briefly, cells were lysed in TRI reagent, and the homogenized lysate was phase-separated using chloroform. After centrifugation at 12,000 × g for 15 min at 4°C, the aqueous phase containing RNA was carefully collected and precipitated with isopropanol. The RNA pellet was washed with 75% ethanol, air-dried, and resuspended in RNase-free water. The concentration and purity of the isolated RNA were determined using a NanoDrop spectrophotometer by measuring absorbance at 260/280 nm and 260/230 nm ratios. RNA integrity was assessed through agarose gel electrophoresis to ensure high-quality RNA for downstream applications.

For complementary DNA (cDNA) synthesis, 1 μg of total RNA was reverse transcribed into cDNA using the 1st Strand cDNA Synthesis Kit (Vazyme, China) according to the manufacturer's instructions. The reverse

transcription reaction utilized oligo (dT) primers to selectively synthesize cDNA from polyadenylated mRNA transcripts. The reaction mixture was incubated at 37°C for 45 min, followed by enzyme inactivation at 85°C for 5 s. The resulting cDNA was diluted 1:5 in RNase-free water and stored at -20°C for subsequent PCR or qRT-PCR analysis.

To evaluate the expression levels of target mRNAs, qRT-PCR was conducted using Applied Biosystems SYBR Green Master Mix (Vazyme, China). Each qRT-PCR reaction was performed in a 10 μL volume containing 1 μL of diluted cDNA, 5 μL of SYBR Green Master Mix, 0.5 μL of each forward and reverse primer (10 μM), and 3 μL of RNase-free water. The reactions were run in a 96-well plate on an Applied Biosystems Real-Time PCR System under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Melting curve analysis was performed at the end of each run to confirm the specificity of the PCR products, ensuring no primer-dimer formation or non-specific amplification. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* or β-actin as the internal control for normalization. Primer sequences for the target mRNA transcripts are provided in Table 1. Each sample was run in triplicate, and all experiments were repeated at least three times to ensure the reproducibility and reliability of the results.

For western blot analysis, cells were lysed in RIPA lysis buffer supplemented with a complete protease inhibitor cocktail (ab271306, Abcam, United Kingdom). Protein concentration was determined using a bicinchoninic acid reagent (Cat no. 23225, Thermo Scientific, United States). Equal amounts of each protein sample were separated using electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to a polyvinylidene fluoride (PVDF) membrane on ice. The PVDF membranes were blocked with 5% fat-free milk powder in tris-buffered saline containing 0.1% Tween-20 (Sigma-Aldrich, Germany) for an hour. Subsequently, the protein blots were incubated overnight at 4°C with the respective primary antibodies. Following incubation, the blots were incubated with secondary antibodies. After incubation with secondary antibodies, the protein bands were visualized using the enhanced chemiluminescence technique (Millipore, USA). β-actin served as the internal control, and untreated cells were used as the blank.

2.6. Bioinformatics analysis of FXR1 correlation and target gene expression

We performed a comprehensive bioinformatics analysis to evaluate the correlation between FXR1 and its target genes, as well as their independent expression across various cancer types and their impact on overall survival (OS). The RBPsuite tool (http://www.csbio.sjtu.edu.cn/bioinf/RBPsuite/) was used to identify potential FXR1 binding sites on the 3'UTR of target genes. Correlation analysis between FXR1 and its target genes was conducted using the Gene Multiple Association Network Integration Algorithm (GeneMANIA) database. Gene expression data and survival analysis were obtained from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) using the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/). Pairwise correlation analyses were performed to assess the association between FXR1 and the selected target genes, while their expression levels in tumor versus normal tissues were visualized using boxplots. The Kaplan-Meier survival curves were generated to evaluate the impact of high and low gene expression on OS, and statistical significance was determined using log-rank tests. Correlation coefficients were computed to quantify the strength of the association between FXR1 and the expression levels of target genes.

3. Results

3.1. Investigating FXR1 binding to mRNAs of selected genes through RNA immunoprecipitation assay

To explore whether FXR1 directly binds to the mRNAs of key genes, we selected several of the significantly upregulated and downregulated genes for further analysis. These genes were chosen based on prior computational predictions and their relevance to the biological pathways under investigation. we performed RNA-IP using an FXR1-specific antibody on SH-SY5Y cells (Figure 1A). This cell line was selected due to its well-characterized nature and high expression levels of FXR1, making it ideal for studying RNA-protein interactions and post-transcriptional regulatory roles.

The RNA-IP results revealed that the mRNAs of several selected genes, including *SHISAL1*, *SLC43A3*, *NBAT1*, *PDZK1IP1*, *ACKR3*, *KCCN3*, *NECAB2*, *ANO5*, *ATOH8*, *IGFBP7*, *LEMD1*, *GPR35*, *WNT7A*, and *F2RL3*, showed significant binding to FXR1 compared to the IgG control beads. This suggests that these mRNAs are likely targets of FXR1 and may be involved in post-transcriptional regulation mediated by FXR1. However, a subset of mRNAs analyzed in the same experiment, *MIR3142HG*, *MUSK*, *SLPI*, *CA1*, *CALB2*, and *PAEP*, did not show binding to the FXR1 protein under identical conditions. This discrepancy could be attributed to variations in FXR1 binding affinity, the presence of specific sequence motifs, or structural properties of these mRNAs that hinder efficient recognition by FXR1. The results of the RNA-IP experiment

Table 1. Primer sequences of the genes used in the present study

Gene	Gene full name	Accession no.	Primer sequence (5' – 3')
FXR1	Fragile X-related protein 1	NM_001013439.3	F: GAGAGAAGATTTAATGGGCCTGG
			R: GCTCAATGGCGGTAACTCCA
SHISAL1	Shisa like 1	NM_001099294.2	F: TTTTTCGTGTTGATGCTGCT
			R: CGGGTGCTTCAGATCTCATC
SLC43A3	Solute carrier family 43 member 3	NM_014096.4	F: CCTCACCTTCATCCTGCAAGT
			R: GGAAGATGAACAAAACCATCC
NBAT1	Neuroblastoma-associated transcript 1	NR_034143.1	F: ACCATCAGCATGCCTTATAC
			R: TTTTTGCATCTCCATTAAAAAT
PDZK1IP1	PDZK1 interacting protein 1	NM_005764.4	F: CAAGGCCTGGGGAACCTT
			R: GCCCACTATTGCAGATTCCA
ACKR3	Atypical chemokine receptor 3	NM_020311.3	F: CGTGGTGGTCTTCCTTGTCT
			R: CCCATCACCCTGTTCAAAAA
KCNN3	Potassium calcium-activated channel subfamily N member 3	NM_001204087.2	F: GACCTGGAGAAGCAGATTGG
			R: AGCGACCAGGAGAGAGTTGA
NECAB2	N-terminal EF-hand calcium-binding protein 2	NM_019065.3	F: TCAGATGAAGATGGCACCAA
			R: CGGGATTTCCTTCACAAGAA
MIR3142HG	MIR146A host gene	NR_132748.1	F: AGCATGTTGCAAAATAACTTGG
			R: ATCTCAAATCCCACCACGTC
MUSK	Muscle-associated receptor tyrosine kinase	NM_001166280.2	F: TAACCGCTACACTACAGAGTC
			R: ATTCCCCTGGCTCACAGAGT
ANO5	Anoctamin 5	NM_001142649.2	F: ATGCAATTCTGGCATGTCCT
			R: TGGCCCTAAAACCAGAGGAT
SLPI	Secretory leukocyte peptidase inhibitor	NM_003064.4	F: AATCTGCCCAGTGCCTTAGA
			R: AAAGGACCTGGACCACACAG
ATOH8	Atonal bHLH transcription factor 8	NM_032827.7	F: ATCAAAGCCCTGCAGCAGAC
			R: GACTCAGCGAGCTCACCTTG
IGFBP7	Insulin-like growth factor binding protein 7	NM_001553.3	F: CATCTGGAACAAGGTAAAAAGG
			R: GGCAAGAACAGGTAATGTAGTT
CA1	Carbonic anhydrase 1	NM_001128830.4	F: AACGAGCCCCATTCACAAAT
			R: ATTGATTTGAAGGCATGCTG
LEMD1	LEM domain containing 1	NM_001199050.2	F: GAGGCTTCCACCACTAAACG
			R: TTTTGGTTCTTTCCTGAAGCA
GPR35	G protein-coupled receptor 35	NM_001195382.2	F: GTGTTCGTGGTCTGCTTCCT
			R: TGCTACTGGTTCCAGCTTCC
WNT7A	Wnt family member 7A	NM_004625.4	F: AAGAAGCCACTGTCGTACCG
			R: GTCCTCCCAGCAATCTGACT
F2RL3	F2R like thrombin or trypsin receptor 3	NM_003950.4	F: GGCAACCTCTATGGTGCCTA
			R: ATTCTGAGGTCCCAGGAAGG
CALB2	Calbindin 2	NM_001740.5	F: CCTGCCTGTCCAGGAAAACT
			R: GTCTGGGTAGACGCATCAGG
PAEP	Progestagen-associated endometrial protein	NM_001018049.3	F: ACTATACGGTGGCGAACGAG
			R: GGTGGGAGTCTGGTCTTCCT

were validated through conventional PCR (Figure 1B) and qRT-PCR (Figure 1C), providing compelling evidence that FXR1 binds to a diverse range of mRNAs and suggesting its significant role in gene expression regulation. p21 was used as a positive control based on a previous study,[12] and β-actin served as a negative control. Further investigations are necessary to explore the functional consequences of these interactions and to identify additional FXR1 targets that may have been overlooked in this study.

3.2. FXR1 modulates the expression of the key selected genes

Next, we overexpressed FXR1 and conducted stable knockdown experiments to explore the potential regulatory relationship between FXR1 and the expression of target genes (Figure 2). The orginal (uncropped) WB images are provided in supplementary Figures R1 and R2. Total RNA was isolated, and qRT-PCR assays were performed to quantify the expression levels of the selected genes under conditions of FXR1 depletion and overexpression. The data indicated significant alterations in the expression of these selected genes when FXR1 expression was either knocked down or enhanced (Figures 3 and 4). Specifically, silencing FXR1 resulted in a marked reduction in the expression of several genes, including *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5* (Figure 3). Conversely, overexpression of FXR1 led to decreased mRNA levels of other genes, such as *SHISAL1*, *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8*, and *IGFBP7* (Figure 4). This dual modulation highlights the critical role of FXR1 in both upregulating and downregulating key genes involved in cellular processes. These findings suggest that FXR1 acts as a central regulator of gene expression, influencing the balance between oncogenic and tumor-suppressive pathways.

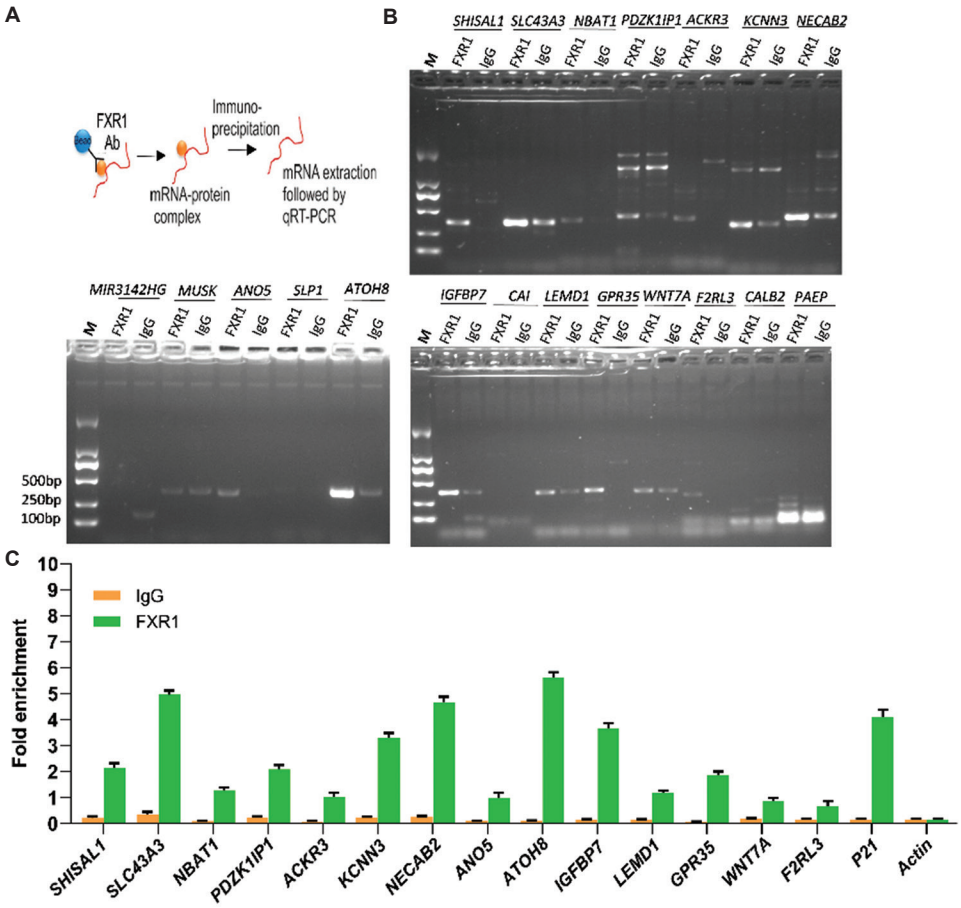

Figure 1. RNA immunoprecipitation (RIP) analysis. (A) A schematic representation illustrates the workflow of the RNA-IP assay, highlighting the use of an FXR1 antibody to isolate RNA-protein complexes. (B) Total RNA was extracted from RNA-IP samples, followed by PCR to assess the enrichment of specific mRNAs within the immunoprecipitated complex. (C) Total RNA was extracted from RNA-IP samples, followed by qRT-PCR to assess the enrichment of specific mRNAs within the immunoprecipitated complex.
Abbreviations: Ab: Antibody; IgG: Immunoglobin G; M: Marker; PCR: Polymerase chain reaction; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

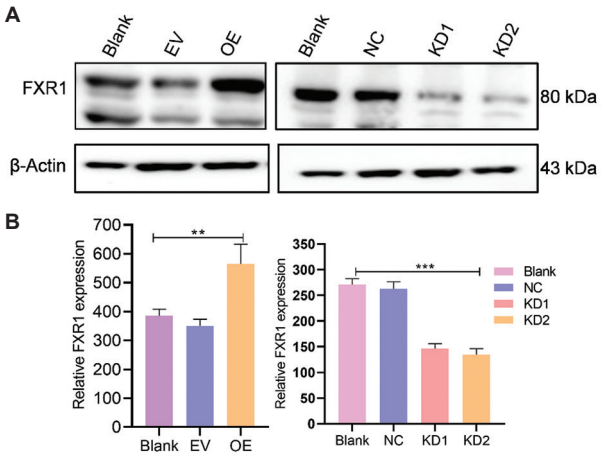

Figure 2. Validation of FXR1 mRNA and protein expression using qRT-PCR and western blotting. (A) Protein lysates were prepared from cells using RIPA lysis buffer, and FXR1 protein expression was analyzed through western blot using FXR1 antibody, with β-actin as the loading control. (B) Total RNA was isolated using TRIzol reagent, and cDNA was synthesized using reverse transcription. FXR1 mRNA levels were quantified using qRT-PCR, normalized to *GAPDH*, and analyzed for relative expression using the $2^{-\Delta\Delta Ct}$ method. Note: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

Abbreviations: EV: Empty vector, KD: Knockdown; NC: Negative control; OE: Overexpression; PCR: Polymerase chain reaction; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

3.3. Computational mapping of FXR1 binding sites on different mRNAs

To identify potential FXR1 binding sites on various mRNAs, we utilized the full-length mRNA sequences for RBP binding site prediction using RBPsuite. This tool facilitates accurate prediction of binding sites based on RNA sequence data. The process begins by segmenting the full-length mRNA sequences into smaller fragments, which are then analyzed by RBPsuite to predict possible FXR1 binding sites. A cut-off score of 0.5 was used to ensure a reliable threshold for identifying true binding interactions. Based on this computational approach, several mRNAs were found to contain one or more FXR1 binding sites. Specifically, *SHISAL1*, *SLC43A3*, *NBAT1*, *PDZK1IP1*, *ACKR3*, *KCNN3*, *ATOH8*, *IGFBP7*, *GPR35*, *WNT7A*, and *F2RL3* were predicted to harbor one or two FXR1 binding sites (Table 2). These predictions were supported by existing crosslinking immunoprecipitation sequencing (CLIP-seq) data, which independently validated the presence of FXR1 binding regions within the CLIP-seq peaks on these mRNAs, further substantiating the accuracy of the predictions. In contrast, no FXR1 binding sites were identified on the mRNAs of *NECAB2*, *LEMD1*, and *ANO5*, suggesting that these particular transcripts may not be direct targets of FXR1 under the conditions tested (Table 2). The absence of binding sites on these mRNAs may indicate specificity in FXR1's interaction with certain mRNA subsets and highlight the importance of specific structural or sequence conditions in identifying binding motifs. This computational mapping provides a foundational understanding of FXR1's mRNA interactions and underscores the need for further experimental validation to gain insights into the role of FXR1 in post-transcriptional regulation across various mRNA targets.

3.4. Correlation analysis of FXR1 with selected genes

Using the GEPIA database, we conducted a comprehensive pairwise gene correlation analysis utilizing expression data from both the TCGA and GTEx datasets. This analysis aimed to explore the relationship between FXR1 and a curated list of selected upregulated and downregulated genes across various cancer types and normal tissues. Our results identified both positive and negative correlations between the expression levels of FXR1 and the selected genes. These correlations were statistically significant in multiple cancer types, providing evidence that FXR1 may play a key regulatory role in the expression of these genes. Specifically, the positive correlations suggest that FXR1 could be involved in promoting the expression of oncogenes, such as *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5*, potentially driving cancer progression. In contrast, the negative correlations may imply that FXR1 suppresses the expression of tumor suppressor genes or genes involved in pathways antagonistic to tumor development, such as *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8*, and *IGFBP7*. These results provide crucial insights into the molecular mechanisms by which FXR1 may influence cancer biology, offering a basis for further experimental validation and functional studies. These insights could be particularly valuable for understanding the dual roles of FXR1 in different cancer types and its potential as a therapeutic target (Figure 5).

Next, we performed co-expression analysis using GeneMANIA, identifying oncogenes, such as *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5*, along with tumor suppressors, such as *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8*, and *IGFBP7*, that are potentially co-expressed with FXR1 (Figure 6). This complex network involves multiple interaction types, including co-expression and genetic interactions (Table 3). Functional enrichment analysis indicated that these co-expressed genes are primarily associated with mRNA splicing, cellular stress response, physiological function, and tumorigenesis. The results suggest that FXR1 may regulate RNA stability and translation, potentially enhancing cancer progression by stabilizing mRNAs

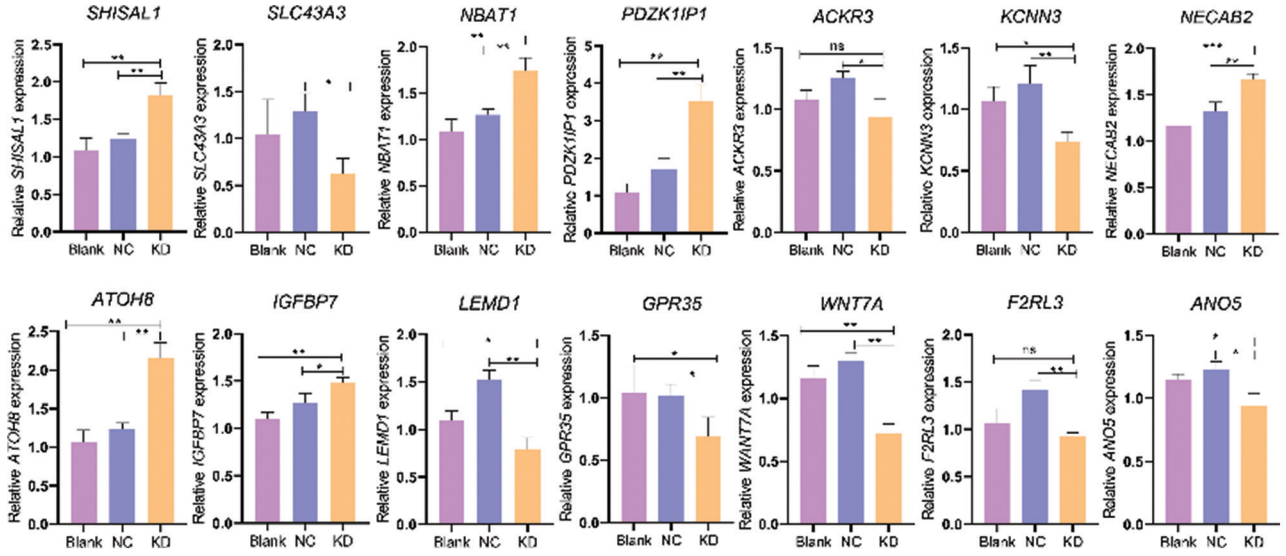

Figure 3. Validation of differential gene expression in FXR1 knockdown cells using qRT-PCR. The expression levels of the selected genes were analyzed in FXR1 knockdown and control cells using qRT-PCR. Total RNA was extracted from the cells using TRIzol reagent, followed by cDNA synthesis. The qRT-PCR reactions were performed with gene-specific primers and normalized to *GAPDH* as the internal control. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, ensuring accurate quantification of the fold change in gene expression. Note: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

Abbreviations: KD: Knockdown; NC: Negative control; ns: Not significant; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

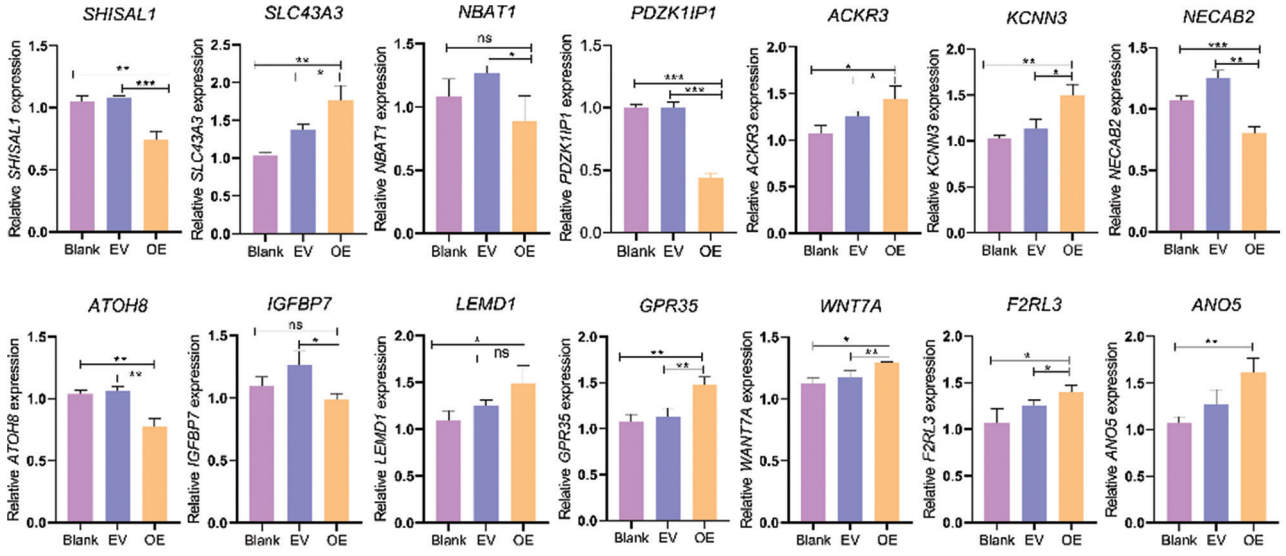

Figure 4. Validation of differential gene expression in FXR1 over-expressing cells using qRT-PCR. Gene expression analysis was conducted in FXR1-overexpressing cells using qRT-PCR to evaluate changes in the expression of target genes. Total RNA was extracted using TRIzol reagent, followed by cDNA synthesis. Gene-specific primers were designed, and *GAPDH* was used as the internal control for normalization. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Significant changes in the expression of multiple genes were observed in FXR1-overexpressing cells compared to controls. Note: $^{*}P < 0.05$, $^{***}P < 0.01$, $^{**}P < 0.01$ compared with the control group.

Abbreviations: EV: Empty vector; ns: Not significant; OE: overexpression; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

critical for cell survival and proliferation. These findings are consistent with existing literature on FXR1's role in RNA metabolism, highlighting the need for future studies to explore FXR1's regulatory pathways in specific cancers, such as esophageal cancer.

3.5. FXR1 overexpression and its impact on oncogenes and tumor suppressors in cancer

SLC43A3, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5* were found to be significantly overexpressed in tumor tissues compared to normal tissues,

Table 2. The score of FXR1 binding site on different genes

Sequence name	Accession no.	Sequence	Score
SHISAL1	NM_001099294.2	AGUGGCUGAAUUGGAAAUGAUAAAAUAAAAUGAAAUUUUAGGAGCUCGCUGGCUGGGACAGGCCUGGACU	0.93541616
		UCAUUACAGUCUACAGAAUGAAAGAGUAACCUGUCCCAGAGGAGAGGCAGGUGAAAGACUCCACAGCAUG	0.51442057
SLC43A3	NM_0012782062	CUCUUUGGCUGGCCUUCACUAGUGUUUGUCUUCAAGAAUGAAGAUUACUUUAAGGAUCUGUGUGGACCAG	0.6119528
		AGCUUCUUUAUGAAAAAGGCAUCAGCCUCAGGGCCUCCUUCAUCUUCAUCUCUGUCUGCAGUACCUGGCA	0.52791125
NBAT1	lncRNA gene	AACAAACGAUUGAGGCAGGAUUUGGAUAUUGCUCUACAUGACGGGAAAGCCUGUGCUCUUGGAAUUACAG	0.55741394
PDZK1IP1	NM_005764.4	GCCAUGUCGGCCCUCAGCCUCCUCAUUCUGGGCCUGCUCACGGCAGUGCCACCUGCCAGCUGUCAGCAA GGCCUGGGGAACCUUCAGCCCUGGAUGCAGGG	0.530119
		CCGUCGGAAACAAGGCAGAUGGAGUCCUGGUGGGAACAGAUGGAAGGUACUCUUCGAUGGCGGCCAGUUU CAGGUCCAGUGAGCAUGAGAAUGCCUAUGAG	0.6568217
ACKR3	NM_0203113	AGAGGGGAGCACGUGCCCCCUGCAUCCAUUCUCUCUUUCUCUUGAUGACGCAGCUGUCAUUUGGCUGUGC	0.5021565
KCNN3	NM_0012040872	UAGAGAUUUAGUCUUAAUUUCAACCAAAUGACAUGCAAGAUUCCACUGCCACUCUUCCUGGCCAAAAGUG	0.9143031
		ACUCAUCUACAGGAAUUAAAUGGAAAAAACAGGCUAGGAUUUCUCAGCACACUUUAGGAGUGAAUGAAAA	0.52305186
NECAB2	NM_001329749.2	NA	
ANO5	NM_2135993	GAAACAACGACAAGCCAGACUGGAAUAUGAAUGGGACCUGGUGGACUUUGAAGAGGAACAGCAGCAGCUU	0.5148203
ATOH8	NM_0032827.7	UCCUGAGGAACUUGAUAGACAAACAAUGACAGUGUUUUCCAGAACUGUGGGUACGUGUCUAAUCUCAGAU GGUACUAUGAAUUCCUGGAGAUCAAAGUUUG	0.9388821
		UCAAGGAGACCCUCAGCAAAAUAUAAAUGACGAGGAGCUGCCCUCAUGGGGCCCUGUGAAAGCACUUUGCA GUCCAGCCUUGGGUUUGUGGUCACAGAGU	0.936801
		GACCAGCUGCCUGUCAGACACUAAGAUAGGCAGUGGGAAUGAAGAGAUGGAUCUUGUGUCAUGCAUGGCAU CACGGAGCUCUGGGUUCUGUACGGAGGGUG	0.5166229
IGFBP7	NM_0015533	AAGGGCACCUGCGAGCAAGGUCCUUCCAUAGUGACGCCCCCCAAGGACAUCUGGAAUGUCACUGGUGCCC	0.51078486
		AGAAUUGUAUUGAAGUGACCCCACUUCUCUACCCCAGAAAAAGACAAAGAUCUGGUACUGUAGAUAAUGA	0.5004961
ANO5	NM_213599.3	NA	
LEMD1	NM_0010015525	NA	
GPR35	NM_0011953823	UCUUAUGAAGAAGGUUCCUUGCUUCCCCUUCACCUUCUGCCAUGAUUGUAAGUUUCCUGAGGCCUCCCUA	0.54091585
		CUGCUGGUUUCUGCCUCUCCUGUACUCCUGCAUGGAGGGCAUCUCGAAACCCAAGCUGGAAGGACAGGGC	0.540495
WNT7A	NM_0046254	UUCUGCAAUCAAAGUGGACUGCUUGCUUUCCUAGCAGGAUGAUUUUGUUGCUAGGACAAGGAGCCGUGUA	0.5815808
		UCACAGGGCUGUUGUGAGGAUCCUCCCAGUAAUGACUGUAUGGCACUUUGAGUUGCAAAGCAAUGGAUGA	0.51607025
F2RL3	NM_0039504	UUAAUUAAUUAAUUUAAUUCAAUUUUAAAAAGACGAAAAGUGACGGCCAGGUGCAGUGGCUCACGCCUAU	0.9999931

Note: The sequence highlighted in red indicates the FXR1 potential binding site in the target mRNAs.
Abbreviations: lncRNA: Long non-coding RNA; NA: Not available.

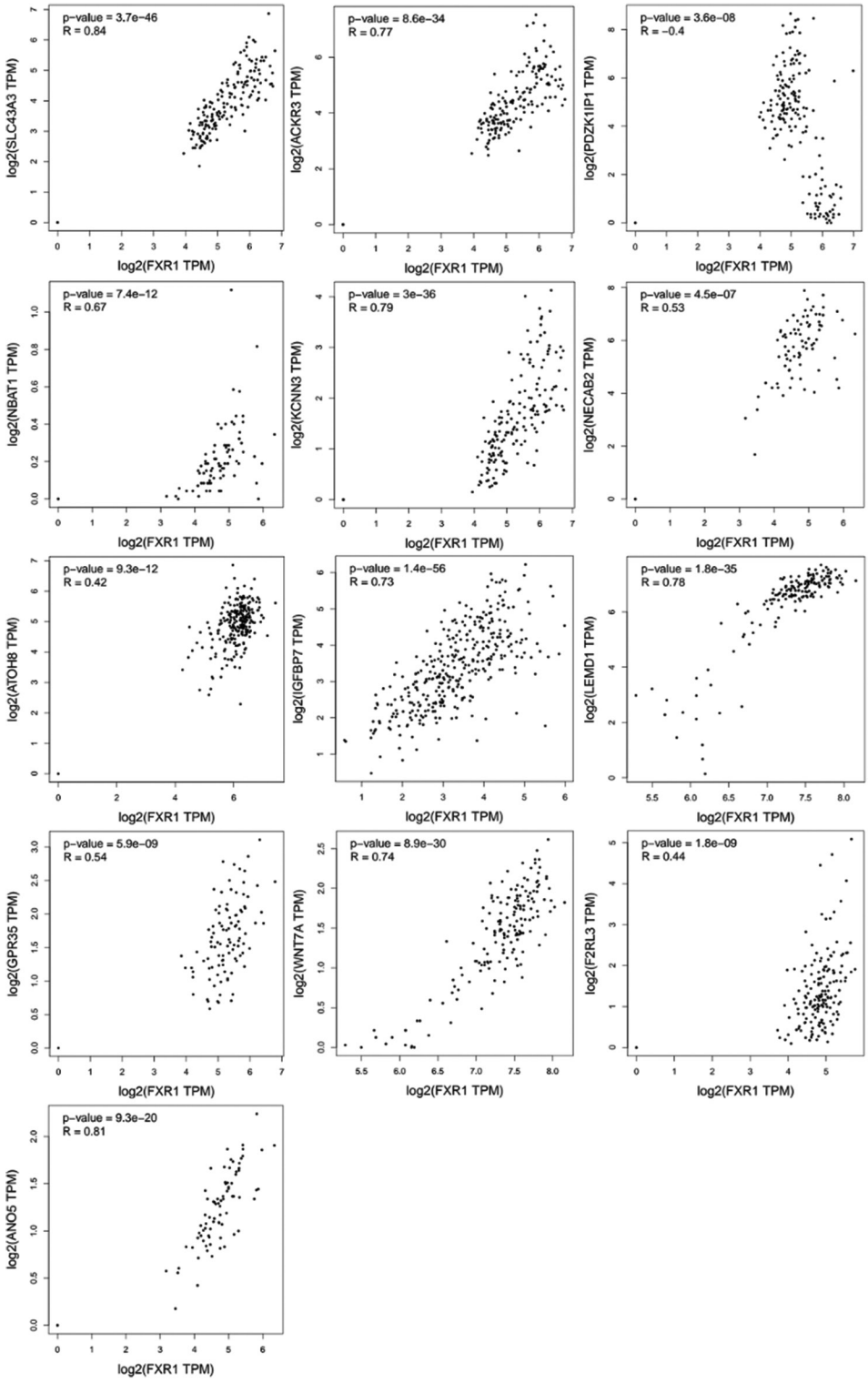

Figure 5. Correlation analysis of FXR1 with selected genes. Scatter plots illustrate the correlation between FXR1 expression and the selected genes. The x-axis represents FXR1 expression levels, while the y-axis displays the expression levels of each selected gene, both presented as log2 (transcript per million [TPM]). Pearson correlation coefficients (r) quantify the strength and direction of the relationships, with *P*-values indicating statistical significance. These plots provide visual and statistical evidence of potential associations between FXR1 and target gene expressions.

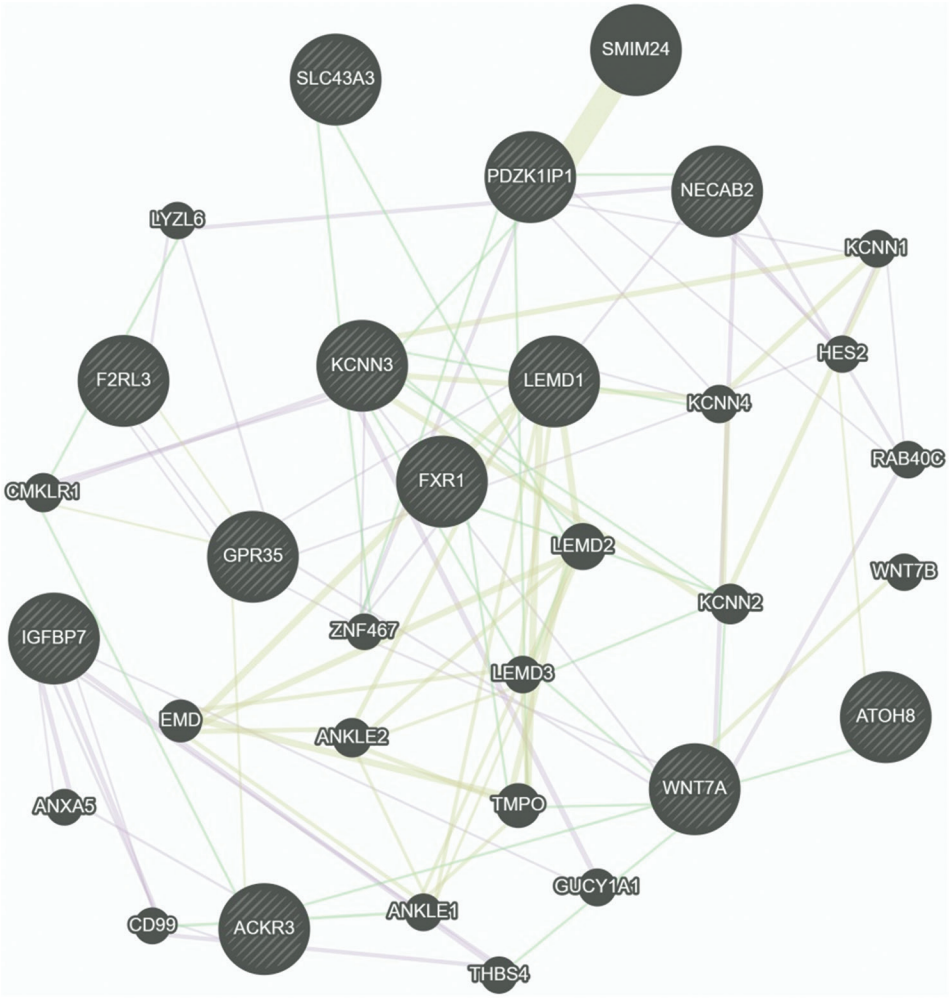

Figure 6. Co-expression network analysis of FXR1 using GeneMANIA. The network, generated using GeneMANIA, illustrates FXR1's functional interactions, including co-expression, physical interactions, shared protein domains, and predicted relationships with other genes. The red lines indicate physical interactions, the purple lines represent co-expression, the blue lines represent co-localization, the green lines indicate genetic interactions, the orange lines represent predicted interactions, and the gray lines denote other biological associations.

as shown in the boxplots (Figure 7A). This overexpression suggests a potential oncogenic role for these genes. Transcriptome sequencing data from the TCGA and GTEx databases identified *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5* as independent risk factors for OS. The Kaplan-Meier survival curve demonstrated that patients with high expression of these genes had significantly shorter OS than those with low expression levels (Figure 7A). The high-expression group shows a rapid decline in survival probability over time, suggesting that overexpression of these genes is associated with poor prognosis in cancer patients. Furthermore, as previous analyses indicate, FXR1 plays a regulatory role in the expression of these genes. FXR1 likely promotes the stabilization or upregulation of these mRNAs, contributing to their oncogenic potential. These findings suggest that the high expression of these genes, driven by FXR1, correlates with poorer prognosis, making them potential biomarkers for cancer aggressiveness and candidates for targeted therapy.

In contrast, we found that *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8,* and *IGFBP7* were downregulated in tumor tissues compared to normal tissues, as shown by the boxplots (Figure 8). The Kaplan-Meier survival curve demonstrated that patients with low expression of these genes had shorter OS compared to those with higher expression levels (Figure 8). Notably, FXR1 is also implicated in regulating the expression of these genes; it likely destabilizes or promotes the degradation of these mRNAs, leading to their reduced expression in tumor tissues. These findings indicate that low expression of these genes, influenced by

Table 3. Summary of co-expressed genes associated with FXR1

Gene 1	Gene 2	Network group		
		Co-expression	Genetic interaction	Shared protein domains
FXR1	*SLC43A3*		0.0019075415	
	PDZK1IP1	0.024462251	0.0005016976	
	ACKR3	0.020030404	0.0055209743	
	KCNN3	0.030047113	0.0013293843	
	NECAB2	0.019942889	0.002363983	0.008422975
	ATOH8	0.00041934196		
	IGFBP7	0.022652313	0.003947871	
	LEMD1	0.00039458455	0.0019075415	
	ANO5		0.041739117	
	GPR35	0.019942889		0.006434719
	WNT7A		0.0008893441	0.006434719
	F2RL3	0.002897449	0.0024557738	

FXR1 activity, correlates with poorer prognosis, identifying them as potential biomarkers for cancer progression and candidates for therapeutic intervention.

Figure 7B and 7C illustrates that FXR1 is upregulated in several cancers, reinforcing its potential involvement in oncogenic pathways, particularly in regulating RNA stability and translating key mRNAs. These findings align with previous studies that have identified FXR1 as a critical RBP associated with cancer development, highlighting its importance as a potential therapeutic target in various malignancies. Further investigation into FXR1's functional roles across different cancer types is warranted to understand its contribution to tumorigenesis better.

4. Discussion

Post-transcriptional regulation mechanisms play a pivotal role in gene expression, with RBPs frequently interacting with target mRNA, particularly through their 3'UTR regions. RBPs are central to regulating various events in RNA metabolism, including splicing, translation, stability, transport, and degradation of mRNAs, as well as non-coding RNAs, such as circular RNAs, long non-coding RNAs (lncRNAs), and miRNAs. These proteins form ribonucleoprotein complexes, thereby influencing gene expression through their interactions with specific RNA targets.[5,27-30] Among RBPs, FXR1 has emerged as a key player, modulating the stability and expression of numerous mRNAs involved in both physiological and pathological processes. Our study has revealed that FXR1, a known regulator of mRNA stability, exerts a significant influence on a range of oncogenes, tumor suppressor genes, and genes involved in cell cycle regulation.

FXR1 has been identified as an overexpressed cancer promoter gene in various malignancies. Previous research by Majumder *et al.*[12] demonstrated FXR1's regulation of p21 and *TERC* RNA in oral squamous cell carcinoma, where it contributes to the evasion of p53-induced cellular senescence.[12] Similarly, our findings highlight that FXR1 modulates the expression of key genes and that the overexpression or silencing of FXR1 significantly alters their transcriptional activity. In particular, the upregulation of FXR1 correlates with the overexpression of several oncogenes in cancer tissues, while its knockdown leads to the repression of oncogenic targets. This direct modulation of gene expression by FXR1 suggests that it plays an integral role in the dysregulation of key cancer-related pathways.

Our data support that FXR1 acts not only as a promoter of oncogene expression but also regulates the degradation of tumor suppressor mRNAs. This is consistent with previous studies identifying RBPs, such as CPEB4 and HuR, as drivers of cancer progression through stabilization of oncogenic transcripts in glioma and breast cancer, respectively.[31,32] Furthermore, osteosarcoma cells exhibited elevated levels of the RBP Lin28A; its knockdown resulted in lower cell migration/invasion and proliferation while enhancing cell death by stabilizing the lncRNA *MALAT1*.[33] Fan *et al.*[34] demonstrated that FXR1 controlled transcription and was crucial for the progression of TP53/FXR2 homozygous deletion-associated cancers in humans.[34] Similarly, our study showed that FXR1 significantly alters the expression of both oncogenes and tumor suppressor genes, further supporting the hypothesis that FXR1 functions as a master regulator of RNA metabolism in cancer. Specifically, we have recently discovered that FXR1 overexpression destabilizes *PDZK1IP1* and *ATOH8* mRNAs, thereby promoting the progression of esophageal cancer. Mechanistically, FXR1 directly interacts with the 3'UTRs of *PDZK1IP1* and *ATOH8* transcripts, facilitating their degradation and negatively regulating their expression.[35] These findings highlight the oncogenic effects of FXR1 through the PDZK1IP1/ATOH8 pathway, underscoring its potential as a diagnostic or therapeutic target in cancer.

In this study, the discovery that FXR1 regulates a diverse set of genes, including oncogenes, such as *SLC43A3*, *ACKR3*, *KCNN3*, *LEMD1*, *GPR35*, *WNT7A*, *F2RL3*, and *ANO5,* as well as tumor suppressors, such as *NBAT1*, *PDZK1IP1*, *NECAB2*, *ATOH8,* and *IGFBP7*, highlights its significant role in cancer progression and patient prognosis. The observed correlations in this analysis underscore the functional role of FXR1 as a potential regulator of gene

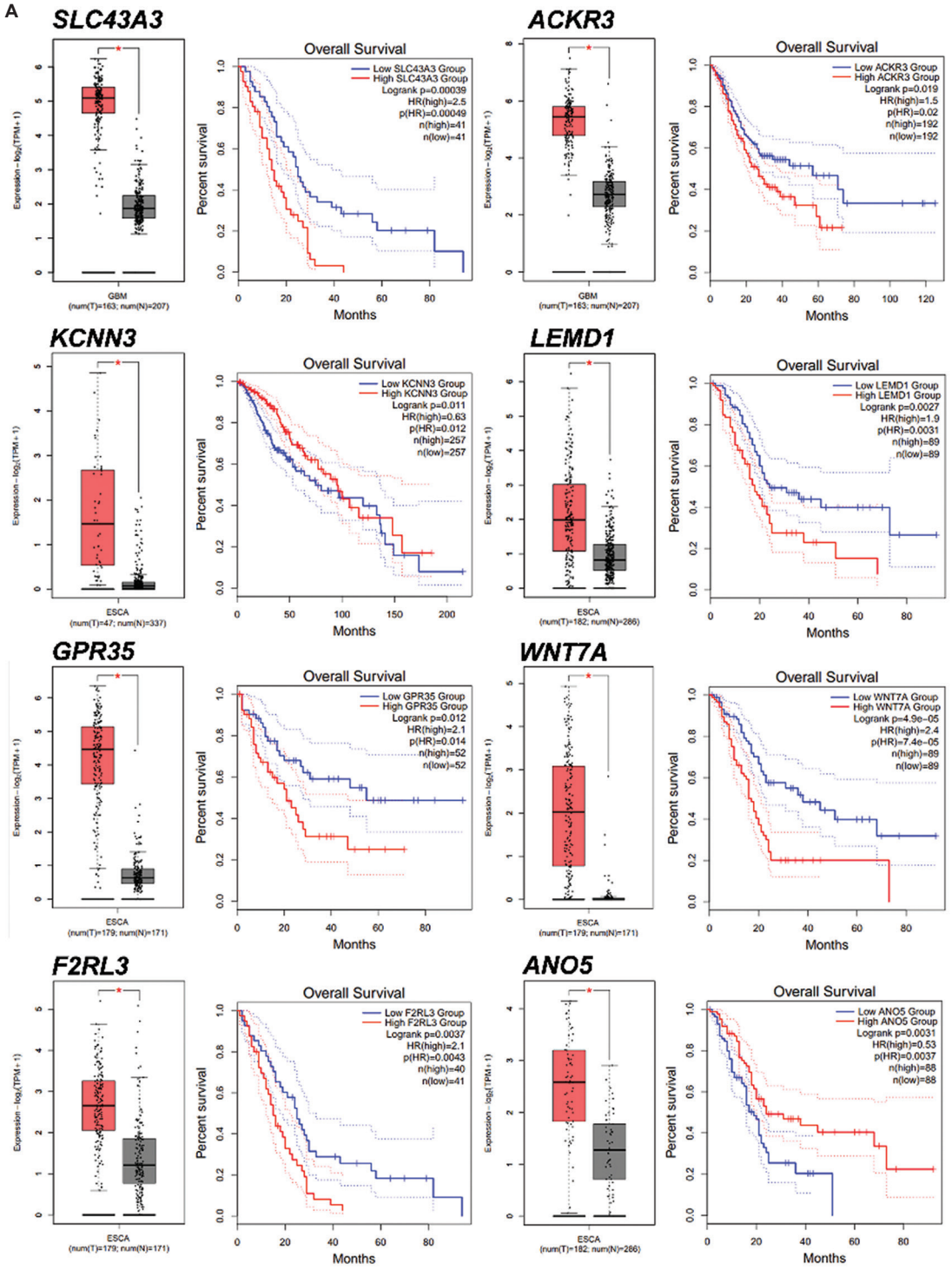

Figure 7. Expression analysis of selected upregulated genes and their impact on overall survival (OS) in cancer. (A) The expression levels of the selected upregulated genes and their association with OS were analyzed using The Cancer Genome Atlas datasets. Gene expression is depicted as box plots comparing tumor and normal tissues (left panel), highlighting significant differences in expression. Kaplan-Meier survival curves (right panel) illustrate the correlation between gene expression levels and overall patient survival across various cancer types, with statistical significance indicated. (B) FXR1 expression across different cancers was examined, with hazard ratios and *p*-values provided to assess the prognostic significance of its upregulation concerning patient outcomes. (C) The most prevalent mutation of FXR1 in cancers is amplification. Note: * $P < 0.05$.

Abbreviation: DLBC: Diffuse large B cell lymphoma; ESCA: Esophageal cancer: GBM: Glioblastoma; LUAD: Lung adenocarcinoma; OV: Ovarian cancer; TPM: Transcript per million.

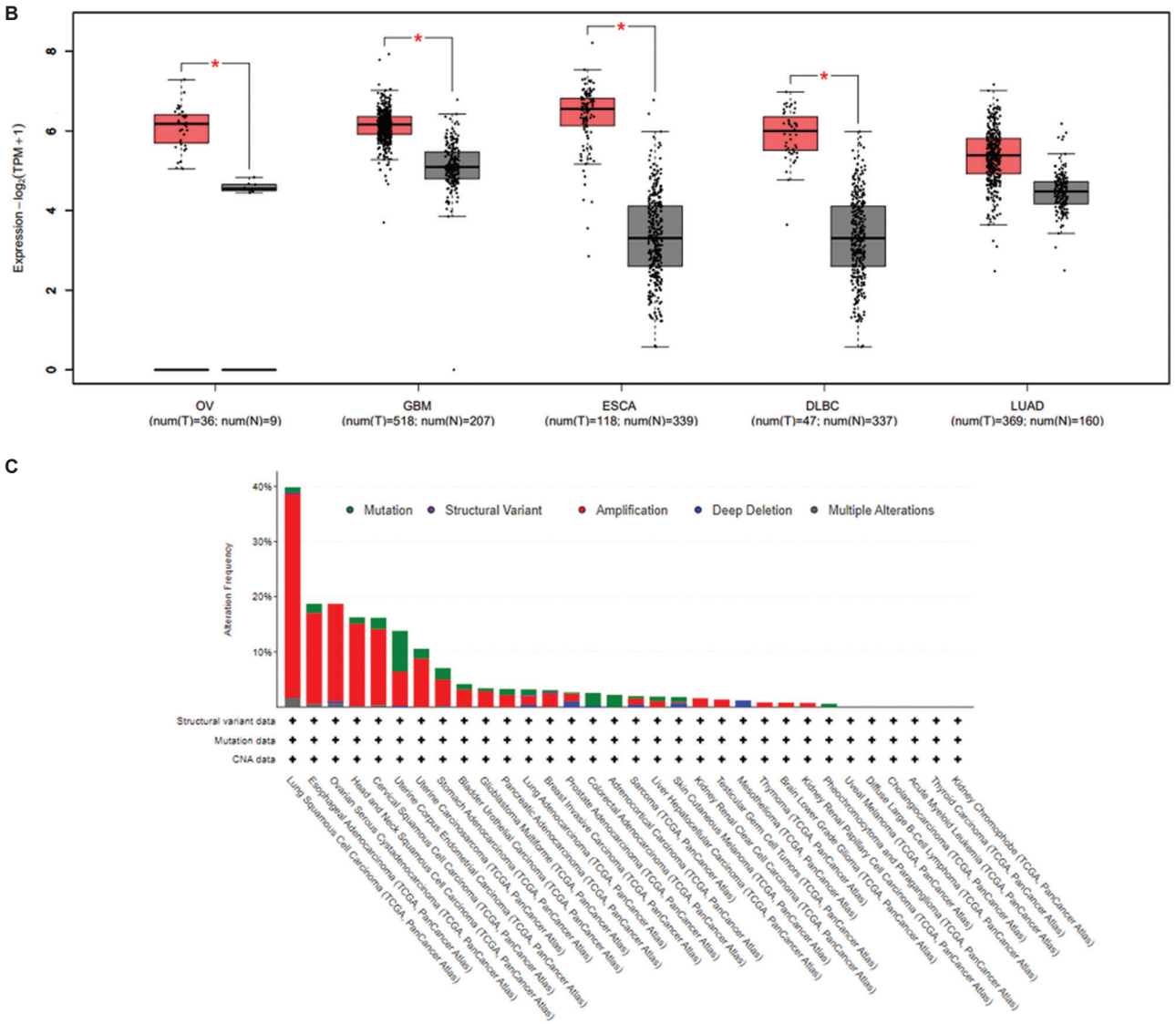

Figure 7. *(Continued)*

expression, either through direct interactions with mRNA transcripts or by modulating upstream signaling pathways. These findings are consistent with previous reports demonstrating FXR1's involvement in post-transcriptional regulation, where it influences the stability, translation, or degradation of its target mRNAs. In line with this, our Kaplan-Meier survival analyses revealed that high expression levels of FXR1 and its associated genes correlate with poor overall survival in cancer patients, underscoring its potential as a biomarker for cancer aggressiveness. Moreover, FXR1's ability to modulate gene expression suggests that it could serve as a potential target for therapeutic intervention for restoring the balance between oncogenic and tumor-suppressive pathways.

FXR1, like other members of the FXR family, is known to localize to stress granules under cellular stress conditions. This sequestration modulates its post-transcriptional regulatory activity by preventing interaction with its target mRNAs.[36-38] While FXR1 overexpression promotes oncogenesis by regulating oncogenes and tumor suppressors, excessive FXR1 levels or stress-induced granule formation may lead to its functional inactivation. This interplay suggests that FXR1's oncogenic potential is tightly regulated by both its expression levels and its localization dynamics within stress granules.[39] The oncogenic effects of FXR1 overexpression appear to be dose-dependent; moderate overexpression enhances FXR1's interaction with target mRNAs, promoting its oncogenic effects, whereas excessive

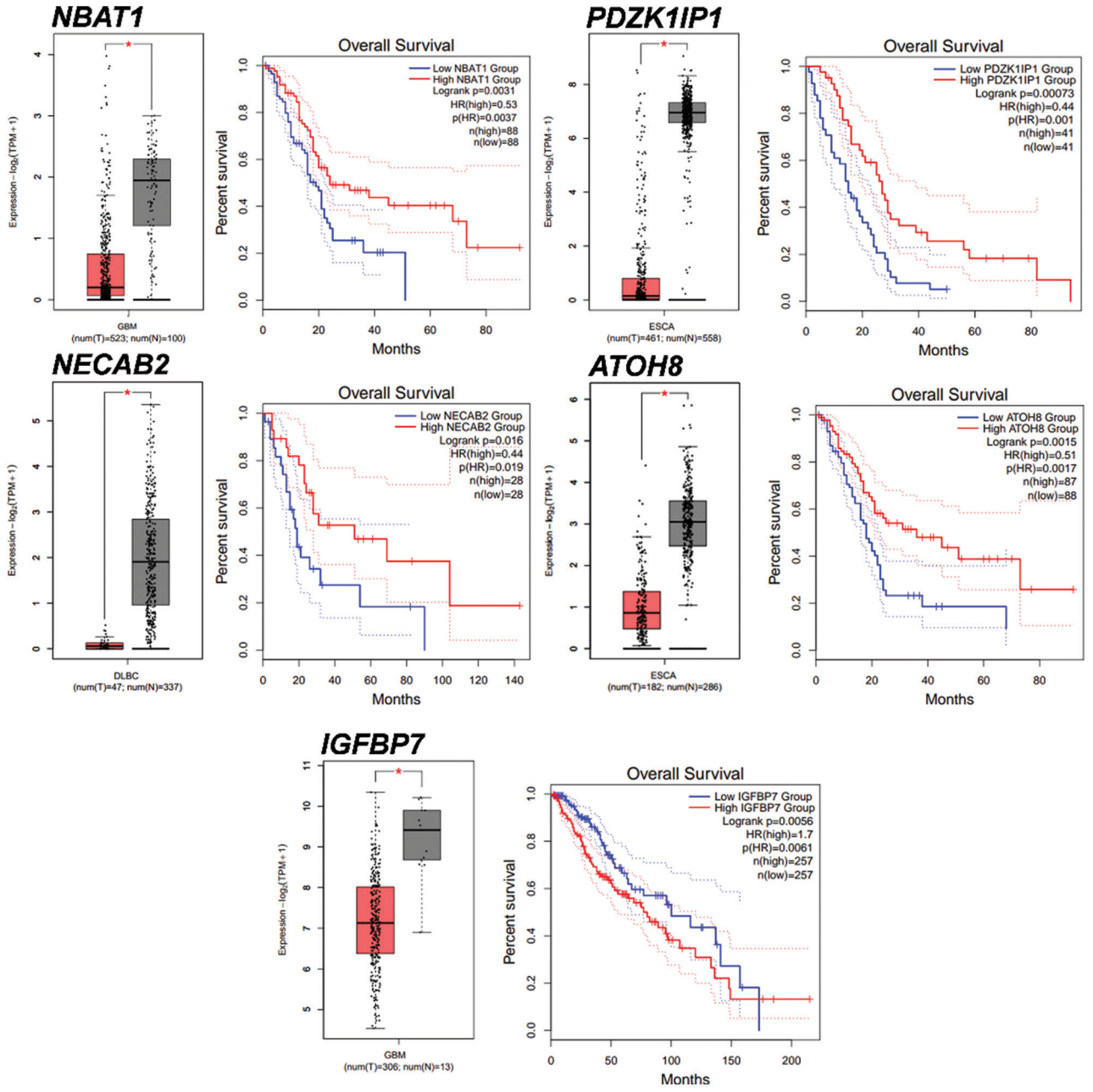

Figure 8. Expression analysis of selected downregulated genes and their impact on overall survival (OS) in cancer. The expression levels of the selected downregulated genes and their association with OS in various cancer types were analyzed using The Cancer Genome Atlas datasets. Gene expression data are represented as box plots, comparing tumor and normal tissue samples (left panel). Kaplan-Meier survival curves were generated to assess the correlation between gene expression and overall survival in specific cancer types, with hazard ratios and *P*-values provided to evaluate the prognostic significance of each gene's expression concerning patient survival outcomes. Note: $^*P < 0.05$.
Abbreviation: DLBC: Diffuse large B cell lymphoma; ESCA: Esophageal cancer; GBM: Glioblastoma; TPM: Transcript per million.

overexpression may lead to a saturation effect, driving FXR1 into stress granules and diminishing its regulatory activity.[40] Interestingly, stress granule formation itself may contribute to cancer progression by facilitating cancer cell adaptation to stress.[41] The sequestration of FXR1 into stress granules could enhance cancer cell survival by protecting stress-sensitive mRNAs or delaying apoptosis through a selective translation of pro-survival factors.[42] Future studies are needed to elucidate the specific conditions under which FXR1 transitions between active and sequestered states and to understand how these dynamics impact cancer progression and cellular stress adaptation.

Such investigations will provide deeper insights into the functional role of FXR1 in cancer biology and its potential as a therapeutic target.

5. Conclusion

The present investigation demonstrates a strong association between alterations in FXR1 activity and the expression of genes involved in tumor progression, emphasizing the clinical importance of this RBP. FXR1's role in regulating the stability and degradation of oncogenes and tumor suppressors highlights its potential as a diagnostic marker and therapeutic target in cancer. While our study provides novel insights into the regulatory functions of FXR1, further research is necessary to fully elucidate the molecular mechanisms by which FXR1 modulates these mRNAs in various cancers. Future investigations will pave the way for the development of FXR1-based diagnostic tools and targeted therapies aimed at mitigating FXR1's oncogenic potential across different cancer types.

Acknowledgments

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Conflict of interest

Xin-Ying Ji is an Editorial Board Member of this journal but was not involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

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Investigation: Faiz Ali Khan
Methodology: Faiz Ali Khan
Supervision: Xin-Ying Ji, Shaoping Ji, Jingcheng Dong
Writing – original draft: Faiz Ali Khan
Writing – review & editing: Faiz Ali Khan

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

All data generated or analyzed during this study are included in this published article and are available upon request from the corresponding author.

References

1. Re A, Joshi T, Kulberkyte E, Morris Q, Workman CT. RNA-protein interactions: An overview. *Methods Mol Biol*. 2014;1097:491-521.

   doi: 10.1007/978-1-62703-709-9_23

2. Gerstberger S, Hafner M, Tuschl T. A census of human RNA-binding proteins. *Nat Rev Genet*. 2014;15(12):829-845.

   doi: 10.1038/nrg3813

3. Pereira B, Billaud M, Almeida R. RNA-binding proteins in cancer: Old players and new actors. *Trends Cancer*. 2017;3(7):506-528.

   doi: 10.1016/j.trecan.2017.05.003

4. Hentze MW, Castello A, Schwarzl T, Preiss T. A brave new world of RNA-binding proteins. *Nat Rev Mol Cell Biol*. 2018;19(5):327-341.

   doi: 10.1038/nrm.2017.130

5. Khan FA, Fang N, Zhang W, Ji S. The multifaceted role of fragile X-related protein 1 (FXR1P) in cellular processes: An updated review on cancer and clinical applications. *Cell Death Dis*. 2024;15(1):72.

   doi: 10.1038/s41419-023-06413-8

6. Bechara E, Davidovic L, Melko M, *et al*. Fragile X related protein 1 isoforms differentially modulate the affinity of fragile X mental retardation protein for G-quartet RNA structure. *Nucleic Acids Res*. 2007;35(1):299-306.

   doi: 10.1093/nar/gkl1021

7. Schaeffer C, Bardoni B, Mandel JL, Ehresmann B, Ehresmann C, Moine H. The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. *EMBO J*. 2001;20(17):4803-4813.

   doi: 10.1093/emboj/20.17.4803

8. Vasudevan S, Steitz JA. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell*. 2007;128(6):1105-1118.

   doi: 10.1016/j.cell.2007.01.038

9. Darnell JC, Fraser CE, Mostovetsky O, Darnell RB. Discrimination of common and unique RNA-binding activities among Fragile X mental retardation protein paralogs. *Hum Mol Genet*. 2009;18(17):3164-3177.

   doi: 10.1093/hmg/ddp255

10. Tamanini F, Willemsen R, van Unen L, *et al*. Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis. *Hum Mol Genet*. 1997;6(8):1315-1322.

doi: 10.1093/hmg/6.8.1315

11. Zarnescu DC, Gregorio CC. Fragile hearts: New insights into translational control in cardiac muscle. *Trends Cardiovasc Med*. 2013;23(8):275-281.

doi: 10.1016/j.tcm.2013.03.003

12. Majumder M, House R, Palanisamy N, *et al*. RNA-binding protein FXR1 regulates p21 and TERC RNA to bypass p53-mediated cellular senescence in OSCC. *PLoS Genet*. 2016;12(9):e1006306.

doi: 10.1371/journal.pgen.1006306

13. Qian J, Hassanein M, Hoeksema MD, *et al*. The RNA binding protein FXR1 is a new driver in the 3q26-29 amplicon and predicts poor prognosis in human cancers. *Proc Natl Acad Sci U S A*. 2015;112(11):3469-3474.

doi: 10.1073/pnas.1421975112

14. Majumder M, Palanisamy V. RNA binding protein FXR1-miR301a-3p axis contributes to p21WAF1 degradation in oral cancer. *PLoS Genet*. 2020;16(1):e1008580.

doi: 10.1371/journal.pgen.1008580

15. Muddashetty RS, Nalavadi VC, Gross C, *et al*. Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. *Mol Cell*. 2011;42(5):673-688.

doi: 10.1016/j.molcel.2011.05.006

16. Li Y, Tang W, Zhang LR, Zhang CY. FMRP regulates miR196a-mediated repression of HOXB8 via interaction with the AGO2 MID domain. *Mol Biosyst*. 2014;10(7):1757-1764.

doi: 10.1039/c4mb00066h

17. Cao S, Zheng J, Liu X, *et al*. FXR1 promotes the malignant biological behavior of glioma cells via stabilizing MIR17HG. *J Exp Clin Cancer Res*. 2019;38(1):37.

doi: 10.1186/s13046-018-0991-0

18. Davidovic L, Durand N, Khalfallah O, *et al*. A novel role for the RNA-binding protein FXR1P in myoblasts cell-cycle progression by modulating p21/Cdkn1a/Cip1/Waf1 mRNA stability. *PLoS Genet*. 2013;9(3):e1003367.

doi: 10.1371/journal.pgen.1003367

19. Qi F, Meng Q, Hayashi I, Kobayashi J. FXR1 is a novel MRE11-binding partner and participates in oxidative stress responses. *J Radiat Res*. 2020;61(3):368-375.

doi: 10.1093/jrr/rraa011

20. Kedersha N, Stoecklin G, Ayodele M, *et al*. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J Cell Biol*. 2005;169(6):871-884.

doi: 10.1083/jcb.200502088

21. Darnell JC, Jensen KB, Jin P, Brown V, Warren ST, Darnell RB. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell*. 2001;107(4):489-499.

doi: 10.1016/s0092-8674(01)00566-9

22. Schenck A, Bardoni B, Langmann C, Harden N, Mandel JL, Giangrande A. CYFIP/Sra-1 controls neuronal connectivity in *Drosophila* and links the Rac1 GTPase pathway to the fragile X protein. *Neuron*. 2003;38(6):887-898.

doi: 10.1016/s0896-6273(03)00354-4

23. Prieto M, Folci A, Martin S. Post-translational modifications of the fragile X mental retardation protein in neuronal function and dysfunction. *Mol Psychiatry*. 2020;25(8):1688-1703.

doi: 10.1038/s41380-019-0629-4

24. Garnon J, Lachance C, Di Marco S, *et al*. Fragile X-related protein FXR1P regulates pro-inflammatory cytokine tumor necrosis factor expression at the post-transcriptional level. *J Biol Chem*. 2005;280(7):5750-5763.

doi: 10.1074/jbc.M401988200

25. Herman AB, Vrakas CN, Ray M, *et al*. FXR1 is an IL-19-responsive RNA-binding protein that destabilizes pro-inflammatory transcripts in vascular smooth muscle cells. *Cell Rep*. 2018;24(5):1176-1189.

doi: 10.1016/j.celrep.2018.07.002

26. Bierhoff H. Analysis of lncRNA-protein interactions by RNA-protein pull-down assays and RNA immunoprecipitation (RIP). *Methods Mol Biol*. 2018;1686:241-250.

doi: 10.1007/978-1-4939-7371-2_17

27. Khan FA, Nsengimana B, Khan NH, *et al*. Chimeric peptides/proteins encoded by circRNA: An update on mechanisms and functions in human cancers. *Front Oncol*. 2022;12:781270.

doi: 10.3389/fonc.2022.781270

28. Khan FA, Nsengimana B, Khan NH, *et al*. Differential expression profiles of circRNAs in cancers: Future clinical and diagnostic perspectives. *Gene Protein Dis*., 2022;1(2):138.

doi: 10.36922/gpd.v1i2.138

29. Nsengimana B, Khan FA, Ngowi EE, *et al*. Processing body (Pbody) and its mediators in cancer. *Mol Cell Biochem*. 2022;477(4):1217-1238.

doi: 10.1007/s11010-022-04359-7

30. Khan FA, Nsengimana B, Awan UA, Ji XY, Ji S, Dong J. Regulatory roles of N6-methyladenosine (m6A) methylation in RNA processing and non-communicable diseases. *Cancer Gene Ther*. 2024;31(10):1439-1453.

doi: 10.1038/s41417-024-00789-1

31. Zhijun L, Dapeng W, Hong J, Guicong W, Bingjian Y, Honglin L. Overexpression of CPEB4 in glioma indicates a poor prognosis by promoting cell migration and invasion. *Tumour Biol*. 2017;39(4):1010428317694538.

doi: 10.1177/1010428317694538

32. Tan S, Ding K, Chong QY, *et al*. Post-transcriptional regulation of ERBB2 by miR26a/b and HuR confers resistance to tamoxifen in estrogen receptor-positive breast cancer cells. *J Biol Chem*. 2017;292(33):13551-13564.

doi: 10.1074/jbc.M117.780973

33. Wang Z, Pang J, Ji B, *et al*. RNA binding protein Lin28A promotes osteocarcinoma cells progression by associating with the long noncoding RNA MALAT1. *Biotechnol Lett*. 2018;40(3):493-500.

doi: 10.1007/s10529-017-2489-9

34. Fan Y, Yue J, Xiao M, *et al*. FXR1 regulates transcription and is required for growth of human cancer cells with TP53/FXR2 homozygous deletion. *Elife*. 2017;6:e26129.

doi: 10.7554/eLife.26129

35. Khan FA, Fouad D, Ataya FS, Fang N, Dong J, Ji S. FXR1 associates with and degrades PDZK1IP1 and ATOH8 mRNAs and promotes esophageal cancer progression. *Biol Direct*. 2024;19(1):104.

doi: 10.1186/s13062-024-00553-3

36. Nsengimana B, Khan FA, Awan UA, *et al*. Pseudogenes and liquid phase separation in epigenetic expression. *Front Oncol*. 2022;12:912282.

doi: 10.3389/fonc.2022.912282

37. Hofmann I, Casella M, Schnölzer M, Schlechter T, Spring H, Franke WW. Identification of the junctional plaque protein plakophilin 3 in cytoplasmic particles containing RNA-binding proteins and the recruitment of plakophilins 1 and 3 to stress granules. *Mol Biol Cell*. 2006;17(3):1388-1398.

doi: 10.1091/mbc.e05-08-0708

38. Linder B, Plöttner O, Kroiss M, *et al*. Tdrd3 is a novel stress granule-associated protein interacting with the Fragile-X syndrome protein FMRP. *Hum Mol Genet*. 2008;17(20):3236-3246.

doi: 10.1093/hmg/ddn219

39. Khan FA, Fouad D, Ataya FS, Saeed U, Ji XY, Dong J. Elevated MRPS23 expression facilitates aggressive phenotypes in breast cancer cells. *Cell Mol Biol (Noisy-le-grand)*. 2025;70(12):65-72.

doi: 10.14715/cmb/2024.70.12.9

40. Taha MS, Haghighi F, Stefanski A, *et al*. Novel FMRP interaction networks linked to cellular stress. *FEBS J*. 2021;288(3):837-860.

doi: 10.1111/febs.15443

41. Glauninger H, Wong Hickernell CJ, Bard JAM, Drummond DA. Stressful steps: Progress and challenges in understanding stress-induced mRNA condensation and accumulation in stress granules. *Mol Cell*. 2022;82(14):2544-2556.

doi: 10.1016/j.molcel.2022.05.014

42. Didiot MC, Subramanian M, Flatter E, Mandel JL, Moine H. Cells lacking the fragile X mental retardation protein (FMRP) have normal RISC activity but exhibit altered stress granule assembly. *Mol Biol Cell*. 2009;20(1):428-437.

doi: 10.1091/mbc.e08-07-0737

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

CASE REPORT

Congenital myopathy-1B caused by a homozygous *RYR1* variant: A case report

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Abstract

Congenital myopathies are a group of clinically and genetically diverse neuromuscular diseases that often present with stable and/or slowly progressive trunk and proximal weakness. Genetic analysis can help diagnose each congenital myopathy more accurately. Although an increasing number of other causative genes have been reported, ryanodine receptor 1 (RYR1)-related myopathy is the most common cause. Herein, we report the clinical presentation of a patient with congenital myopathy-1B (multiminicore disease) that was caused by a c.115 G>A homozygous variant of *RYR1*. The patient had normal cognitive abilities but was developmentally delayed and unable to walk. Electromyography revealed myogenic changes. The c.115G>A variant located in the second exon of *RYR1* was found to be homozygous in the congenital neuromuscular gene panel. The patient's parents and sister both carried the heterozygous variant. Clinical differences between family members with the homozygous and heterozygous variants of *RYR1* highlight the correlation between the genotype and phenotype.

Keywords: Congenital myopathy-1B; Ryanodine receptor 1-related variants; Multiminicore disease

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1. Introduction

Congenital myopathies are a group of clinically and genetically diverse neuromuscular diseases that often present with stable and/or slowly progressive trunk and proximal weakness. Limb deformities, hypotonia, respiratory distress at birth, delayed motor development, scoliosis, periodic muscle stiffness, and paralysis are observed later in life. Although ryanodine receptor 1-related (*RYR1*) myopathy is the most prevalent form of core myopathy, several other causative genes have been reported.[1] Pathogenic variants in *RYR1* are associated with dominantly inherited congenital myopathy 1A, along with susceptibility to malignant hyperthermia (MH) (central core disease [CCD]; MIM 117000),[2] King–Denborough syndrome (MIM 619542),[3] MH susceptibility 1 (MIM 145600),[4] and recessively inherited congenital myopathy-1B (CMYO1B; multiminicore disease; MIM 255320).[5] Multiminicore disease is caused by a homozygous[5] or compound heterozygous mutation in *RYR1*

(OMIM:180901) located on chromosome 19q13.[6] Four types of multiminicore disease with distinguishable characteristic signs and symptoms have been identified so far, namely the antenatal form with arthrogryposis, classic form, progressive form with hand involvement, and ophthalmoplegic form.[1,7,8] Herein, we present the case of a patient with multiminicore disease who exhibited a homozygous variant of *RYR1* and discuss the clinical differences between family members with homozygous and heterozygous variants of *RYR1* to highlight the genotype-phenotype correlation.

2. Case presentation

A mother with a factor V Leiden mutation and a healthy father, who were first-degree cousins, had nine natural pregnancies. The first five pregnancies were aborted. The sixth pregnancy resulted in the birth of a male infant (weight, 2930 g [−0.7 standard deviation (SD)]; length, 47.5 cm (−1.05 SD); and occipitofrontal circumference, 35.5 cm [+0,73 SD]) at 38 weeks through cesarean section due to decreased fetal movements. He was referred to the neonatal intensive care unit (NICU) because of respiratory difficulties. However, he did not require mechanical ventilation and was kept in the NICU for 39 days. The initial examination revealed macrocephaly, micrognathia, poor sucking reflex, hypotonia, and flexion contracture in the third finger of both hands. The child was fed through an orogastric tube throughout his hospital stay, and he was discharged home in the same condition. Magnetic resonance imaging (MRI) of the brain revealed mega cisterna magna and a Dandy–Walker malformation. The child passed away at the age of 1.5 years due to difficulty in swallowing, recurrent pneumonia, and respiratory distress. A karyotype analysis of the child's genes yielded a result of 46, XY. The parents' seventh and eighth pregnancies yielded two healthy daughters. During the ninth pregnancy, increased nuchal lucency was observed on a prenatal ultrasound. However, aneuploidy was not detected during amniocentesis. Due to decreased fetal movements, the child was born at 37 weeks through a planned cesarean section (weight, 3030 g [+0.17 SD]; length, 47 cm [−0.87 SD]; and head circumference, 35 cm [+0.7 SD]). He was transferred to the NICU due to a femur fracture and subdural hemorrhage that resulted from a fall during the delivery. He did not require mechanical ventilation. However, he had a poor sucking reflex and was fed through an orogastric tube. This feeding method was continued for 2 months after discharge. Thereafter, he was fed with a bottle. Physical therapy was initiated because of the flexion contractures in both hands. He achieved head control at 5 months and was able to sit without support at 10 months. However, he never acquired the ability to walk. He began speaking at approximately the same time as his siblings. No mental retardation was detected. He was referred to the Department of Pediatric Genetics at the age of 4 years and was followed up in the Pediatric Neurology Department because of neuromotor developmental delay. The patient's pedigree was drawn at our clinic (Figure 1). The patient's weight was 13.7 kg (−1.51 SD), height was 93 cm (−2.39 SD), and head circumference was 51.5 cm (+0.31 SD). Furthermore, we observed relative macrocephaly; dolichocephaly; posteriorly rotated ears; retrognathia and myopathic face; pectus excavatum; joint contractures in the elbows, knees, and left-hand fingers; simian line on the left hand; partial cutaneous syndactyly on the second and third toes of the left foot; clinodactyly on the second, fourth, and fifth toes of both feet; and cryptorchidism (Figure 2). On neurological examination, the muscle strength was 3 – 4/5 in the upper extremities and 2/5 in the lower extremities. Deep tendon reflexes were diminished in the upper extremities and absent in the lower extremities. No pathological reflexes were observed. There was no restriction of lateral vision or swallowing dysfunction. Only one measurement of the creatine kinase level was 377 U/L; the others ranged between 90 and 100 U/L. The alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase levels were 43, 48, and 189 U/L, respectively. No pathogenic findings were observed on the echocardiogram. MRI of the brain revealed a mega cisterna magna malformation. Ultrasound revealed widespread increased echogenicity of the muscles and decreased volume in the anterior and posterior muscle groups of both thighs as well as in both biceps. Furthermore, electromyography revealed myogenic changes. Thus, the family members were advised to undergo a muscle biopsy. However, they declined it due to its invasive nature. The child's previous karyotype analysis yielded a 46, XY result. The patient's genomic DNA was analyzed using a 40-gene neuromuscular gene panel on the Illumina Next Generation Sequencing platform. The c.115G>A variant, located on the second exon of *RYR1* (NM_000540.3), was homozygous. This missense variant, previously reported as heterozygous and of uncertain clinical significance, results in the substitution of glutamate with lysine at position 39 (Glu39Lys).

In the segregation analysis, the c.115G>A variant of *RYR1* was heterozygous in the mother, father, and sister (Figure 1; II-2, II-3, and III-7, respectively). The father and sister had not undergone any surgery. The mother had previously undergone four cesarean sections and one tonsillectomy without experiencing any complications related to general anesthesia.

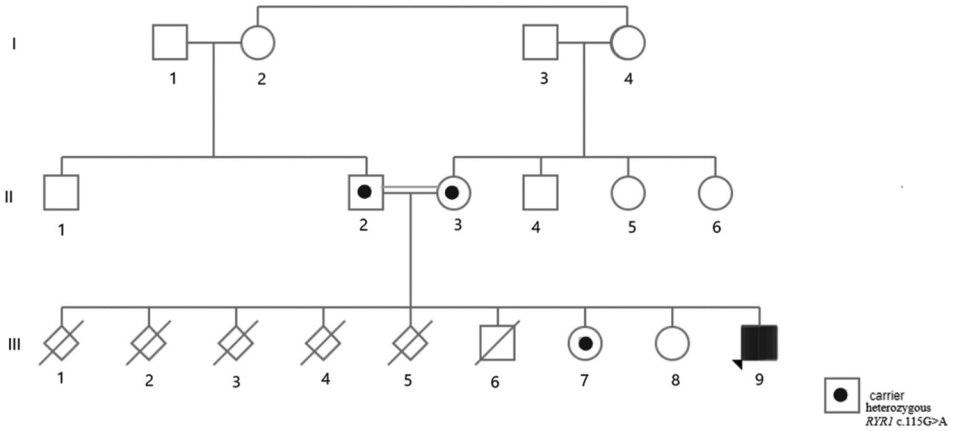

Figure 1. Pedigree of the family. II-2, II-3, and III-7: unaffected carriers with heterozygous ryanodine receptor 1 (*RYR1*) c.115G>A variant. III-9: patient with a homozygous *RYR1* c.115G>A variant.

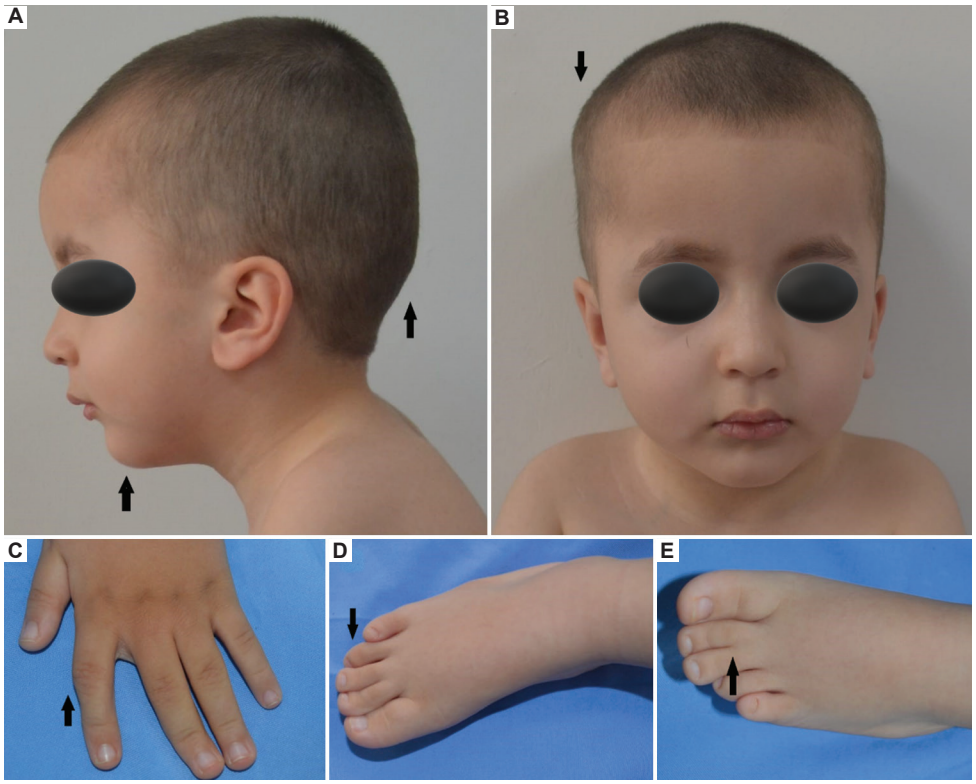

Figure 2. Image of patient's dysmorphic features. (A and B) The patient exhibited dysmorphic facial features such as relative macrocephaly, dolichocephaly, posteriorly rotated ears, retrognathia, and a myopathic face. (C) Joint contractures were observed in the left hand. (D) Partial cutaneous syndactyly was observed in the second and third toes of the left foot. (E) Clinodactyly was observed in the second, fourth, and fifth toes of both feet.

3. Discussion

RYR1 encodes a ryanodine receptor that serves as a calcium release channel in the sarcoplasmic reticulum (SR).[9] When the membrane polarization changes, calcium ions (Ca^{2+}) are released from the SR, triggering muscle contraction at the neuromuscular junction. This involves the opening of the L-type Ca^{2+} channels. The Ca^{2+} release enables the interaction between actin and myosin, leading to an increase in the Ca^{2+} concentration in the cytosol, which initiates the movement of the myosin head.[10] Maintaining appropriate extracellular and intracellular Ca^{2+} levels is crucial for muscle and bone development and function.[11] Individuals with the multiminicore disease group present

in different forms, i.e., classic, progressive, antenatal, and ophthalmoplegic. In the most common classic form, the baby is "floppy" (hypotonic) and may experience feeding problems in the early stages of their lives. The progressive form of multiminicore disease with hand involvement results in joint laxity and muscle weakness in the arms and hands. Antenatal arthrogryposis is characterized by distinctive facial features and rigid joints. The ophthalmoplegic form is characterized by external ophthalmoplegia, causing abnormal eye movements, drooping eyelids (ptosis), and weakness of the proximal muscles.[5,12] In our patient, moderate proximal muscle weakness, delayed motor development, feeding problems, and a homozygous c.115G>A variant in *RYR1* supported the diagnosis of a classic form of multiminicore disease. The deceased brother was believed to have multiminicore disease due to the presence of a homozygous c.115G>A variant in *RYR1*, hypotonicity, dysphagia, and breathing difficulty due to bulbar involvement.

CCD (MIM 117000) mainly presents during infancy or childhood and sometimes in older individuals. It affects the proximal muscles of the lower extremities and causes generalized joint laxity and scoliosis symptoms. The same variants in *RYR1* result in a wide range of phenotypes, from mild to very severe, even within the same family. Patients with N-terminal variants of *RYR1* may exhibit lighter phenotypes.[8,13] Muscle weakness, congenital hip dislocation, generalized joint laxity, and scoliosis are more common and severe. Bulbar, respiratory, and cardiac involvement is uncommon.[1] The heterozygous c.115G>A variant of *RYR1* was found in our patient's mother, father, and sister near the N-terminal region. However, it did not produce any clinical symptoms, indicating that this heterozygous form is not associated with CCD.

MH susceptibility 1 (MIM 145600) is an autosomal dominant skeletal muscle disease. Exposure to certain volatile anesthetic agents, such as halothane, or depolarizing muscle relaxants, such as succinylcholine, can cause an MH crisis, resulting in muscle rigidity, hyperthermia, arrhythmias, respiratory and metabolic acidosis, and rhabdomyolysis.[14] Although the child's mother was exposed to anesthetic agents several times, an MH crisis was not observed. However, we cannot conclude that this RYR1 gene variant will not lead to anesthesia-related deaths, as we cannot predict which anesthetic agent will be used.

Gain-of-function mutations in *RYR1* cause MH susceptibility due to increased Ca^{2+} release from the SR. Furthermore, mutations that cause CCD are typically associated with a loss of function.[15] *RYR1* variants in the central region of the protein increase Ca^{2+}-induced Ca^{2+} release. However, variants in the N-terminal region increase Ca^{2+} release by stabilizing the closed state.[16,17] *RYR1* variants can be associated with either MH or CCD, with some variants being associated with both disorders. However, this has not yet been fully elucidated.

Congenital myopathies are divided into the following five subgroups according to the major morphological features observed in muscle biopsy specimens: core, nemaline, centronuclear, myosin storage, and congenital fiber-type disproportion myopathies. Core myopathies, located at the center or periphery of muscle fibers, are minicore areas of myofibrillar disruption without mitochondria. In CCD, there are typically large and centrally located nuclei in the muscle fibers. In multiminicore myopathy, multiple focal areas devoid of oxidative enzyme activity are observed.[18] Our study's family refused to undergo muscle biopsy because they deemed it an invasive procedure, making it a limitation of this study. However, the 4-year-old patient's clinical picture can be explained by the current molecular findings.

The study results show that pathogenic variants of *RYR1* lead to four distinct types of channel defects. Excitation–contraction coupling in the transverse tubules and SR membrane occurs in a macromolecular complex composed of RYR1, the dihydropyridine receptor (DHPR), and calsequestrin. The first class of these variants makes the channels sensitive to activation due to electrical and pharmacological stimuli, resulting in the clinical presentation of MH. The second class causes depletion of Ca^{2+} from intracellular SR deposits, resulting in CCD. The third class, associated with some types of CCD, results in excitation–contraction uncoupling. Activation of the voltage-sensing DHPR fails to induce Ca^{2+} release from the SR. The fourth class is the reduced expression of mutant RYR1 channels in SR membranes, which is distinct from recessive *RYR1* mutations.[19] Thus, each RYR1 gene variant affects calcium balance differently. However, functional or protein expression studies in these young patients are lacking. Thus, a mutation-specific effect cannot be demonstrated. The identified variants and the resultant clinical findings demonstrated a genotype–phenotype relationship in *RYR1*, highlighting its important neuromuscular features in the clinical presentation of congenital myopathy.

4. Conclusion

Our case report illustrates the clinical presentation of multiminicore disease caused by the c.115 G>A homozygous variant of *RYR1*. The clinical differences between patients with the homozygous and heterozygous variants shed light on the genotype–phenotype correlation.

Furthermore, MH may develop in a patient with a *RYR1* variant. Thus, such patients should be administered non-triggering anesthetic agents to prevent possible adverse outcomes.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Nagehan Bilgeç
Writing–original draft: Nagehan Bilgeç
Writing–review & editing: All authors

Ethics approval and consent to participate

Ethical approval was not required for this study in accordance with the local guidelines. Written informed consent was obtained from the legal guardian of the patient for publication of details of their medical case and any accompanying images.

Consent for publication

The patient's mother provided signed informed consent for the publication of this case report and any accompanying images.

Availability of data

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

References

1. Topaloglu H. Core myopathies-a short review. *Acta Myol*. 2020;39(4):266-273.

   doi: 10.36185/2532-1900-029

2. Shuaib A, Paasuke RT, Brownell KW. Central core disease. Clinical features in 13 patients. *Medicine* (*Baltimore*). 1987;66:389-396.

3. D'Arcy CE, Bjorksten A, Yiu EM, *et al*. King-Denborough syndrome caused by a novel mutation in the ryanodine receptor gene. *Neurology*. 2008;71:776-777.

   doi: 10.1212/01.wnl.0000324929.33780.2f

4. Manning BM, Quane KA, Ording H, *et al*. Identification of novel mutations in the ryanodine-receptor gene (RYR1) in malignant hyperthermia: Genotype-phenotype correlation. *Am J Hum Genet*. 1998;62:599-609.

   doi: 10.1086/301748

5. Ferreiro A, Monnier N, Romero NB, *et al*. A recessive form of central core disease, transiently presenting as multi-minicore disease, is associated with a homozygous mutation in the ryanodine receptor type 1 gene. *Ann Neurol*. 2002;51:750-759.

   doi: 10.1002/ana.10231

6. MacKenzie AE, Korneluk RG, Zorzato F, *et al*. The human ryanodine receptor gene: Its mapping to 19q13.1, placement in a chromosome 19 linkage group, and exclusion as the gene causing myotonic dystrophy. *Am J Hum Genet*. 1990;46(6):1082-1089.

7. Klein A, Lillis S, Munteanu I, *et al*. Clinical and genetic findings in a large cohort of patients with ryanodine receptor 1 gene-associated myopathies. *Hum Mutat*. 2012;33(6):981-988.

   doi: 10.1002/humu.22056

8. Ogasawara M, Nishino I. A review of core myopathy: Central core disease, multiminicore disease, dusty core disease, and core-rod myopathy. *Neuromuscul Disord*. 2021;31(10):968-977.

   doi: 10.1016/j.nmd.2021.08.015

9. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: Elementary events underlying excitation-contraction coupling in heart muscle. *Science*. 1993;262:740-744.

   doi: 10.1126/science.8235594

10. Gehlert S, Bloch W, Suhr F. Ca2+-dependent regulations and signaling in skeletal muscle: From electro-mechanical coupling to adaptation. *Int J Mol Sci*. 2015;16(1):1066-1095.

    doi: 10.3390/ijms16011066

11. Bronner F. Extracellular and intracellular regulation of calcium homeostasis. *ScientificWorldJournal*. 2001;1:919-925.

    doi: 10.1100/tsw.2001.489

12. Wilmshurst JM, Lillis S, Zhou H, *et al*. RYR1 mutations are a common cause of congenital myopathies with central nuclei. *Ann. Neurol*. 2010;68:717-726.

    doi: 10.1002/ana.22119

13. Parker R, Schiemann AH, Langton E, *et al*. Functional characterization of C-terminal ryanodine receptor 1 variants associated with central core disease or malignant hyperthermia. *J Neuromuscul Dis*. 2017;4(2):147-158.

    doi: 10.3233/JND-170210

14. Monnier N, Procaccio V, Stieglitz P, Lunardi J. Malignant-hyperthermia susceptibility is associated with a mutation of

the alpha-1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. *Am J Hum Genet*. 1997;60:1316-1325.

doi: 10.1086/515454

15. Avila G, O'Connell KM, Dirksen RT. The pore region of the skeletal muscle ryanodine receptor is a primary locus for excitation-contraction uncoupling in central core disease. *J Gen Physiol*. 2003;121(4):277-286.

doi: 10.1085/jgp.200308791

16. Murayama T, Kurebayashi N, Ogawa H, *et al*. Genotype-phenotype correlations of malignant hyperthermia and central core disease mutations in the central region of the RYR1 channel. *Hum Mutat*. 2016;37(11):1231-1241.

doi: 10.1002/humu.23072

17. Murayama T, Kurebayashi N, Yamazawa T, *et al*. Divergent activity profiles of type 1 ryanodinereceptor channels carrying malignant hyperthermia and central coredisease mutations in the amino-terminal region. *PLoS One*. 2015;10(6):e0130606.

doi: 10.1371/journal.pone.0130606

18. Harikrishna GV, Padmanabha H, Polavarapu K, *et al*. Phenotype-genotype correlation of a cohort of patients with congenital myopathy: A single centre experience from India. *J Neuromuscul Dis*. 2024;11(5):935-957.

doi: 10.3233/JND-230021

19. Elbaz M, Ruiz A, Bachmann C, *et al*. Quantitative *RYR1* reduction and loss of calcium sensitivity of *RYR1*Q1970fsX16+A4329D cause cores and loss of muscle strength. *Hum Mol Genet*. 2019;28(18):2987-2999.

doi: 10.1093/hmg/ddz092

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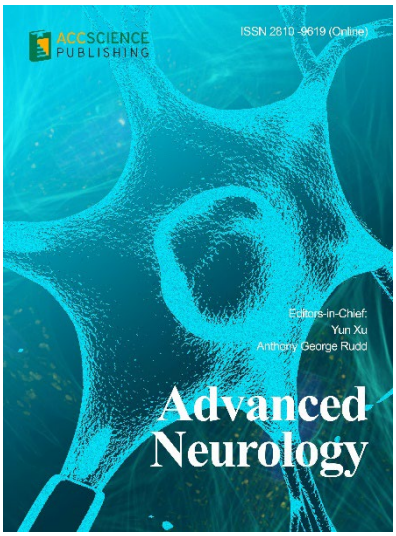

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