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# Double-edged swords: important factors connecting metabolic disorders and cancer development - from basic research to translational applications, volume II

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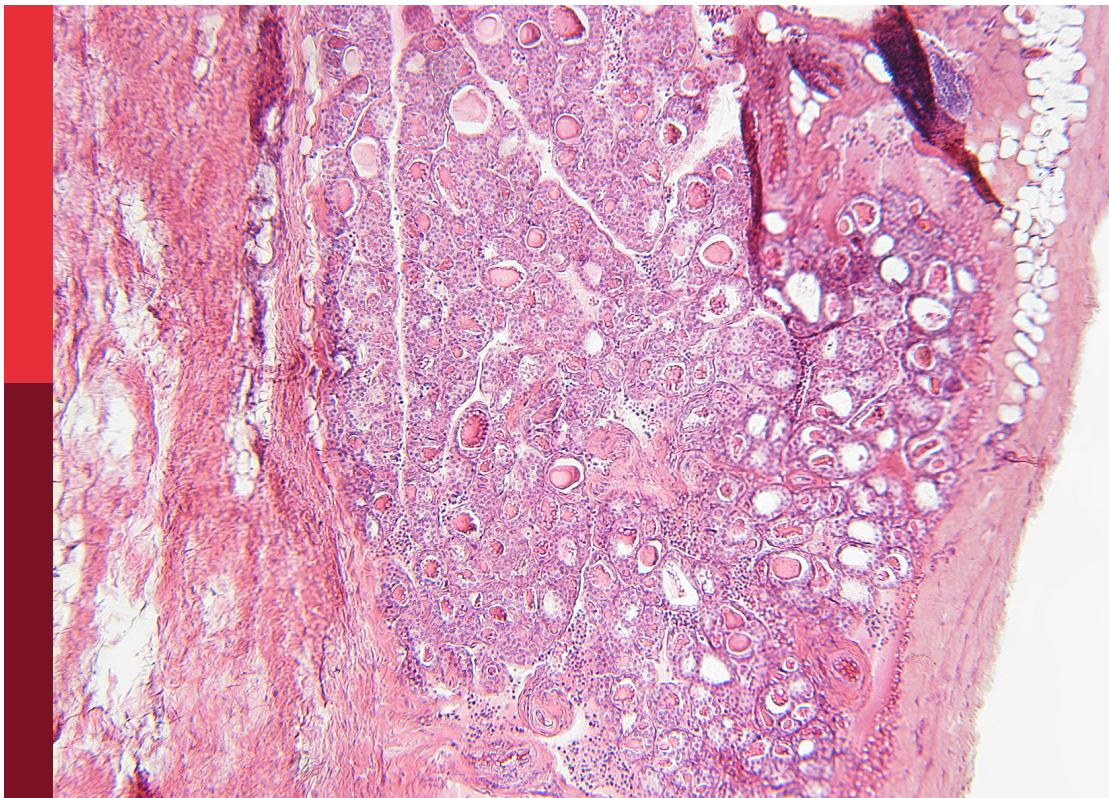

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# Double-edged swords: important factors connecting metabolic disorders and cancer development - from basic research to translational applications, volume II

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# Editorial: Double-edged swords: important factors connecting metabolic disorders and cancer development – from basic research to translational applications, volume II

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**metabolic disorder, cancer, translational research, basic research, metabolism**

Editorial on the Research Topic

Double-edged swords: important factors connecting metabolic disorders and cancer development - from basic research to translational applications, volume II

Building upon the previous two series of articles (Double-edged Swords: Genetic Factors That Influence the Pathogenesis of Both Metabolic Disease and Cancer; Double-Edged Swords: Important Factors Connecting Metabolic Disorders and Cancer Development - From Basic Research to Translational Applications) discussing the intersections between metabolic dysfunction and cancer development, this research topic highlights new challenges and opportunities with our expanded knowledge.

Drug resistance is a significant issue in cancer therapy (1). Tirendi et al. reviewed the recent literature between 1988 and 2022 regarding therapeutic strategies and challenges of colorectal cancer (CRC) in "Colorectal cancer and therapy response: a focus on the main mechanisms involved". The authors discussed mechanisms contributing to CRC resistance, including metabolic reprogramming in cancer stem cells. To overcome CRC resistance, metabolic adaptors such as metformin and nanoparticle-based systems have been developed to improve treatment efficacy and delivery, respectively.

In "Co-administration of MDR1 and BCRP or EGFR/PI3K inhibitors overcomes lenvatinib resistance in hepatocellular carcinoma", Sun et al. described novel strategies to overcome resistance of hepatocellular carcinoma (HCC) to lenvatinib, a tyrosine kinase inhibitor used in patients with unresectable HCC. Following the development of lenvatinib resistance (LR), multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) transporters were upregulated, and the epidermal growth factor receptor

(EGFR) and PI3K/AKT pathways were activated. As the result, combining lenvatinib with MDR1/BCRP dual inhibitor elacridar or EGFR inhibitor gefitinib proved to be effective strategies to overcome LR.

To develop novel treatments against HCC, new opportunities have been presented by our expanded understanding of the interaction between immunity and metabolism. Tu et al. described in "Hepatic macrophage mediated immune response in liver steatosis driven carcinogenesis" how liver macrophages produce inflammatory mediators to cause lipid dysfunction, steatosis and ultimately liver cancer. Treatments targeting this pathway, such as AMPK activators or dietary interventions, may be beneficial when integrated in HCC therapy.

Connections between metabolic syndrome and thyroid cancer (TC) have been described (2). In "Do metabolic factors increase the risk of thyroid cancer? a Mendelian randomization study", Liang and Sun provided additional contexts by using Mendelian Randomization (MR) to analyze genome-wide association studies (GWAS) dataset. Their analysis revealed a protection role for high-density lipoprotein (HDL) on TC, suggesting that strategies targeting HDL regulations could have therapeutic values. Other metabolites can also be linked to TC. In "Alterations in the amino acid profile in patients with papillary thyroid carcinoma with and without Hashimoto's thyroiditis", Hellmann et al. used high-performance liquid chromatography-triple stage quadrupole-mass spectrometry (HPLC-TSQ-MS) to profile amino acids (AA) in the serum of patients with papillary thyroid carcinoma (PTC) with or without Hashimoto's thyroiditis (HT). Despite sharing similar AA profiles compared with healthy controls, serum of PTC patients with HT (PTC1) can be distinguished from those without HT (PTC0) by lysine and alanine profiles, suggesting diagnostic values of AA in TC.

Some metabolites, such as 2-Hydroxyglutarate (2HG), also possess pro-tumorigenic functions (3). In "Renal oncometabolite L-2-hydroxyglutarate imposes a block in kidney tubulogenesis: Evidence for an epigenetic basis for the L-2HG-induced impairment of differentiation", Taub et al. showed that knockdown of L-2HG dehydrogenase (L2HGDH) in Renal Proximal Tubule (RPT) cells resulted in increased 2HG level and reduced tubulogenesis by RPT cells. This result was accompanied with reduced expression of cell differentiation factors and altered methylation status of chromatin. It suggests that 2HG functions as an oncometabolite by suppressing normal differentiation.

Our understanding about the role of glucose metabolism in human diseases have spanned from diabetes to cancer (4). In "The "sweet" path to cancer: focus on cellular glucose metabolism", Iacobini et al. reviewed the current literature contextualizing the role of aerobic glycolysis, or Warburg effect, in cancer, inflammation, and diabetes. They highlighted two important factors, the hypoxia-inducible factor-1α (HIF-1α) and M2 isoform of pyruvate kinase (PKM2), in promoting glucose metabolic rewiring to shape the immune and endocrine environments during disease progression.

Both HIF-1α and PKM2 are metabolic enzymes critical for functions in normal and cancerous cells. Mahé et al. did a deep dive, in "Genetics of enzymatic dysfunctions in metabolic disorders and cancer", into our current knowledge about how genetic alterations in metabolic enzymes contribute to human diseases. They explored a variety of functional pathways, including the urea cycle, glycogen storage, lysosome storage, fatty acid oxidation, and mitochondrial respiration among others, that can be hijacked by dysregulation of metabolic enzymes to promote the development of metabolic disorders and cancers.

In "Is MG53 a potential therapeutic target for cancer?", Du et al. discussed the roles MG53 plays as a target in cancer therapy. As a member of the tripartite-motif (TRIM) protein family with glucose-regulating functions, MG53 has been shown to play beneficial roles in cancer treatment. Restoring or elevating MG53 levels could enhance efficacy of chemo- and immuno-therapy while limiting associated tissue injuries. MG53's role in metabolic regulation, however, has also been implicated in insulin resistance and cancer cachexia, leading to detrimental effects during cancer treatment.

Drug repurposing represents a promising strategy to discover novel therapies for cancer (5). In "The magic bullet: Niclosamide", Jiang et al. reviewed the potential of niclosamide, an FDA-approved drug for tapeworm treatment, in cancer therapy considering its recently discovered ability to modify the global epigenetic landscape through metabolic reprogramming (6). With its distinctive effects on epigenetic regulation, metabolic programming, and other oncogenic and tumor suppressive mechanisms, such as Wnt/β-catenin, NF-κB, p53, and AMPK pathways, niclosamide is a promising candidate for combination therapies.

Racial disparity plays a significant role in disease progression and therapy (7). In "Population-enriched innate immune variants may identify candidate gene targets at the intersection of cancer and cardio-metabolic disease", Yeyeodu et al. discussed the roles innate immunity and inflammation play in differential susceptibilities to metabolic disorder and cancer among racial populations. Genetic inheritances and adaptions, in response to geographically defined environmental stresses, shape the innate immune profiles in different ethnic groups. It offers important insights in development of precision therapies.

With the variety of topics covered, our discussion and learning about links between metabolic functions and cancer continue, from basic science to translational applications.

## Author contributions

C-PK: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing. TB: Project administration, Writing – original draft, Writing – review & editing. C-HY: Project administration, Writing – original draft, Writing – review & editing. IB: Project administration, Writing – original draft, Writing – review & editing. MM: Project administration, Supervision, Writing – original draft, Writing – review & editing.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Renal oncometabolite L-2-hydroxyglutarate imposes a block in kidney tubulogenesis: Evidence for an epigenetic basis for the L-2HG-induced impairment of differentiation

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2-Hydroxyglutarate (2HG) overproducing tumors arise in a number of tissues, including the kidney. The tumorigenesis resulting from overproduced 2HG has been attributed to the ability of 2HG alter gene expression by inhibiting α-ketoglutarate (αKG)-dependent dioxygenases, including Ten-eleven-Translocation (TET) enzymes. Genes that regulate cellular differentiation are reportedly repressed, blocking differentiation of mesenchymal cells into myocytes, and adipocytes. In this report, the expression of the enzyme responsible for L2HG degradation, L-2HG dehydrogenase (L2HGDH), is knocked down, using lentiviral shRNA, as well as siRNA, in primary cultures of normal Renal Proximal Tubule (RPT) cells. The knockdown (KD) results in increased L-2HG levels, decreased demethylation of 5mC in genomic DNA, and increased methylation of H3 Histones. Consequences include reduced tubulogenesis by RPT cells in matrigel, and reduced expression of molecular markers of differentiation, including membrane transporters as well as HNF1α and HNF1β, which regulate their transcription. These results are consistent with the hypothesis that oncometabolite 2HG blocks RPT differentiation by altering the methylation status of chromatin in a manner that impedes the transcriptional events required for normal differentiation. Presumably, similar alterations are responsible for promoting the expansion of renal cancer stem-cells, increasing their propensity for malignant transformation.

KEYWORDS

**L-2-Hydroxyglutarate, proximal tubule, matrigel (MA), differentiation, renal cell carcinoma**

# 1 Introduction

Specific cancer cells including gliomas, secondary glioblastomas, and acute myeloid leukemia (AML) overproduce D-2-Hydroxyglutarate (D-2HG) due to point mutations in cytosolic Isocitrate Dehydrogenase 1 (IDH1) (1). Renal carcinomas similarly have been observed to overproduce 2HG; however, the L isoform is overproduced in these tumors, presumably due to reduced expression of L-2HG dehydrogenase, which normally oxidizes L-2HG to αKetoglutarate (αKG) (2). While a number of mechanisms have been proposed, the most validated mechanisms by which 2HG accelerates oncogenesis are epigenetic. 2HG potently inhibits αKG-dependent dioxygenases by preventing αKG binding (3). Included amongst the αKG-dependent dioxygenases which are affected are the Ten-Eleven Translocation (TET) enzymes, which demethylate 5-methylcytosine (5mC) residues in genomic DNA (1), as well as the Jumonji C (JmjC) domain-containing histone demethylases (JMDHs). The consequence of TET inhibition is hypermethylation of DNA, while the consequence of JMDH inhibition is hypermethylation of histones. Ultimately, the increased DNA and histone methylation changes gene expression. Of particular interest in these regards is that these changes in gene expression have been observed to block cellular differentiation into adipocytes and muscle cells (4, 5). These observations have been made with cells that overproduce D-2HG due to IDH1mutations (1). However, the question has not been addressed as to whether L2HG-producing cells have a similar block in differentiation. This question is of particular importance with regard to clear cell renal carcinomas (CCRCCs), given that the studies of Shelar et al. (2) indicate that L2HG contributes to the development of these tumors.

Here we examine the hypothesis that elevated 2HG affects the differentiation of normal renal cells, altering their ability to undergo tubulogenesis. Of particular interest in these regards are renal proximal tubule (RPT) cells, the cells of origin of ccRCCs (6). In our previous studies, we observed that normal RPT cells, which have just been removed from the animal, form monolayer cultures that exhibit differentiated functions when cultured on plastic in defined medium (7). However, when RPT cells are cultured in a reconstituted basement membrane, matrigel, tubulogenesis occurs in response to either EGF or TGFα (8). Furthermore, the newly formed tubules possess the capacity for transepithelial transport (9), similar to developing nephrons (8, 10). Included amongst the initial events which occur during kidney development is the induction of the metanephric mesenchyme by WNT signals, followed by the induction of transporters specific to the RPT (11, 12). Notably, these developmental events are dependent upon DNA and histone methylation (13–16). For this reason, we have examined whether a) renal proximal tubulogenesis, and the expression of developmentally regulated transporter genes is altered by 2HG, and b) whether 2HG-mediated alterations can be attributed to changes in DNA and histone methylation.

# 2 Materials and methods

## 2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 Medium (F12), growth factor depleted matrigel, soybean trypsin inhibitor, 0.05% EDTA/0.53 mM trypsin in phosphate-buffered saline (PBS), EGF, lipofectamine, siRNA, RNA-4PCR kits, TURBO DNase I, Superscript Vilo kits, DNA oligos, and tissue culture plasticware were from Thermo Fisher (Waltham, MA). TransIT-LT1 transfection reagent was from Mirus Biotechnology Co. (Madison, WI). Tissue culture plate inserts, 24 wells, with PET membranes, 8.0 μm, were from VWR (Radnor, PA). Monarch Genomic DNA Purification Kits were obtained from New England BioLabs (Ipswich, MA). Hybond-N+ membranes were from GE Healthcare Biosciences (Chicago, IL). Nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). The Sirius Western Bright detection kit was from Advansta, San Jose, CA. The 5-hydroxy methyl cytosine (5-hmC) rabbit polyclonal antibody was from Active Motif (Carlsbad, CA), and the rabbit polyclonal antibodies against di- and tri-methylated histones (included in Histone Sampler Kits 9783 and 9847) were from Cellular Signaling Technologies, Danver, MA. The rabbit anti-NPT2a antibody was from alpha Diagnostics International (San Antonio, TX), while the rabbit anti-L2HGDH antibody (Cat # 15707-1-AP) and the rabbit anti-SGLT2 antibody (Cat. # 24654-1-AP) were from Proteintech (Rosemont, IL). The mouse monoclonal anti-beta actin antibody was from Santa Cruz Biotech (Dallas, TX). The secondary antibodies (Horseradish Peroxidase (HRP)-coupled Goat anti-Rabbit and HRP-coupled Goat anti-Mouse), as well as the SsoAdvanced Universal Syber Green Supermix, were obtained from Bio-Rad Laboratories (Hercules, CA). Collagenase Class IV was from Worthington (Freehold, NJ). Western Blocking Reagent, produced by Roche, as well as bovine insulin, human transferrin, hydrocortisone, and other chemicals was from Sigma Aldrich Chemical Corp. (St. Louis, MO). Selenium was from Difco laboratories (Detroit, MI). New Zealand White rabbits, 4-5 lb, male, were from Charles River (Wilmington, MA). Prism 9 software was from GraphPad, Inc. (San Diego, CA).

## 2.2 Plasmids

The pLK0.1 TRC cloning vector (17), the pLKO.1-TRC vector (17), and the scramble shRNA vector in pLKO.1 (18) were obtained from Addgene (Watertown, Mass). The pMD2.G vector [expressing VSV-G envelop; Addgene plasmid # 12259; http://n2t.net/addgene:12259;RRID:Addgene_12259), and psPAX2 (a lentiviral packaging vector; Addgene plasmid #

12260; http://n2t.net/addgene:12260;RRID:Addgene_12260)] were gifts from Didier Trono.

## 2.3 Cell culture

The basal medium, which consists of a 50:50 mixture of Dulbecco`s Modified Eagle`s Medium and Ham's F12 Medium containing 15 mM HEPES and 20 mM sodium bicarbonate (DMEM/F12) (pH 7.4), is supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, $5 \times 10^{-8}$ M hydrocortisone, 92 U/ml penicillin, and 0.01% kanamycin (i.e., Medium RK-1). Water used for medium and growth factor preparations was purified using a Milli-Q deionization system. Cultures were maintained in a humidified 5% $CO_2$/95% air mixture at 37°C.

Primary rabbit RPT cell cultures were initiated from rabbit kidneys, as previously described (7). Animal use was reviewed and approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. After their removal from the animal, rabbit kidneys were perfused *via* the renal artery, with DMEM/F12 containing 0.5% iron oxide (w/v), until the kidney turned gray black in color. Renal cortical slices were removed, disrupted with a sterile glass homogenizer, and the material was separated sequentially through 253 μm and 83 μm nylon sieves. Tubules and glomeruli on the 83 μm sieve were transferred into DMEM/F12, glomeruli (containing iron oxide) removed with a stir bar, and remaining proximal tubules incubated in DMEM/F12 containing 0.05mg/ml collagenase IV/0.5 mg/ml soybean trypsin inhibitor (2′; 23° C). Dissociated tubules were centrifuged, resuspended in DMEM/F12, and plated into culture dishes (or 12-well plates) containing Medium RK-1. The medium was changed the day after plating, and every 2 days thereafter.

## 2.4 Treatment of primary cultures with either L2HGDH siRNA or L2HGDH shRNA

The sequence of rabbit L2HGDH stealth siRNA (UUACAGUACUCAUACAUGAGGGCUG, positive strand) and scrambled (scr) control stealth siRNA (UUAGGCAU GAACUCACAUGAGUCUG, positive strand) was determined using Stealth siRNA software (Thermo Fisher), whereas the sequence of rabbit L2HGDH Silencer Select siRNA (GAUGCUUACUGUUUUGGAAtt) was determined using Silencer Select siRNA software (Thermo Fisher). Silencer Select Negative Control siRNA #1 was used in parallel with L2HGDH silencer select siRNA. Primary RPT cells were transfected with either Rabbit L2HGDH siRNA or a Control siRNA (scrambled stealth siRNA) using lipofectamine, while transfections with L2HGDH Silencer Select siRNA or a Control siRNA (Silencer Select Negative Control siRNA #1, ThermoFisher) were conducted using lipofectamine RNAiMAX. Two days later, the cultures were either used experimentally or transfected a second time with the siRNAs.

A rabbit L2HGDH shRNA oligo, generated by RNAi Consortium Software, the Broad Institute, (CCGGAA GATGGGATGAAATATCCAATTCTCGAGTTGGATATTT CATCCCATCTTTTTTTG), was inserted into a pLK0.1 TRC cloning vector. The sequence was verified by the Roswell Park Cancer Institute Sequencing Facility (Buffalo, NY). The pLKO.1-TRC vector and the scramble shRNA vector in pLKO.1 were used controls. To prepare lentivirus, 292 T cells were cotransfected, using a TransIT-LT1 transfection agent, with a pLK0.1 TRC vector, pMD2.G, and psPAX2 followed by medium change (after 24 h). Medium containing virus was collected 48 and 72 h after transfection. and the virus was titered using HT1080. Primary RPT cell cultures were transduced with lentiviral particles, and transformants selected for 7 days using puromycin.

## 2.5 Matrigel cultures

Growth factor depleted matrigel, prepared as described by Taub et al. (8), was stored at -20°C. Prior to its use, matrigel was thawed and maintained at 4°C. Prior to the addition of the cultures, 12-well plates were coated with matrigel. Subsequently, monolayer cultures of primary RPT cell cultures were detached from their dishes using EDTA/trypsin. Trypsin action was inhibited using 0.1% soybean trypsin inhibitor in PBS. The cells were suspended in DMEM/F12 and pelleted at 500×*g* for 5 min. After resuspension in DMEM/F12, the cell number was determined using a Coulter counter, and the cells were added to matrigel at 4°C. The cells in matrigel were plated into individual wells of matrigel-coated 12 well plates at $2 \times 10^4$ cells/well. The matrigel cultures were maintained in a humidified 5% $CO_2$ incubator in a humidified 5% $CO_2$/95% air environment at 37° C. DMEM/F12 medium containing 5 μg/ml bovine insulin, 5 μg/ml human transferrin (DMEM/F12-IT), and other pertinent factors (including 5 ng/ml EGF) was added the day after plating. The matrigel cultures were incubated with EGF, and/or other appropriate supplements. One week later, the number of tubules was determined in each of 25 microscope fields/well, in three wells per condition, and compared to control values in the absence of added growth factor (unless otherwise stated).

## 2.6 Realtime PCR

RNA was purified from the cultures using an RNA-4PCR kit. Subsequently, genomic DNA was removed using TURBO DNase I, and cDNA was synthesized using a Superscript Vilo kit. Transcripts were amplified using a BioRad CFX96 RealTime System using SsoAdvanced Universal Syber Green Supermix containing 5 μM forward and reverse primers complementary to cDNA templates. Ct values (obtained using BioRad software) were determined in

quadruplicate. Relative mRNA levels were calculated using the Ct values, as described by Pfaffl (19) using beta actin mRNA as an internal control. Primers were designed by using Primer-BLAST (NCBI website) and synthesized by ThermoFisher.

## 2.7 Western analysis

Cell lysates were prepared in RIPA buffer containing protease inhibitors, as previously described (20), and protein levels were determined using the micro BCA protein assay (21). The samples, equalized with respect to protein, were separated by electrophoresis through 7.5% SDS/polyacrylamide gels, and transferred to nitrocellulose, as previously described (22). The blots were incubated first with a primary antibody and, subsequently, with an HRP-conjugated secondary antibody, also as previously described (22). Following an incubation with WesternBright Sirius Chemiluminescent HRP substrate, bands were visualized in a BioRad Chemidoc MP. Band intensities were compared using ImageLab Software.

## 2.8 5-Hydroxymethylcytosine slot blots

Slot blots were employed to probe for 5-hydroxymethylcytosine (5hmC) in genomic DNA, using serial dilutions of known quantities of genomic DNA, a semi-quantitative method described by Liu et al. (23) and Jia et al. (24). To summarize, genomic DNA was purified using a Monarch Genomic DNA Purification Kit and quantitated using a Nanodrop. In order to expose the bases, the DNA was denatured at 99°C for 5 min, and quick cooled. Dilutions of the samples were applied onto an Hybond-N+ membrane, using a HybriSlot Manifold, and baked at 80°C for 1 h. The membrane was blocked 1 h in 1% Blocking Solution (1% Western Blocking Reagent in Tris Buffered Saline (TBS)), followed by 1 h incubation with 5-hydroxy methyl cytosine (5-hmC) rabbit polyclonal antibody in 0.5% Blocking Solution. The membrane was washed twice in TBS + 0.1% Tween 20 (TBST) and incubated for 1 h in 0.5% blocking solution containing a Horseradish Peroxidase (HRP)-coupled Goat anti-Rabbit secondary antibody. After four washes with TBST, bands were developed using a Sirius Western Bright detection kit and visualized using a BioRad Chemidoc MP.

Following 5hmC blotting, the blots were stained with methylene blue, in order to visualize total DNA on the blots, within the limits of its sensitivity.

## 2.9 Analysis of histones

Primary RPT cell cultures in 60 mm dishes were treated with either a) L2HGDH stealth siRNA in parallel with scrambled (scr) control siRNA or b) L2HGDH Silencer Select siRNA in parallel with Silencer Select Negative Control #1 siRNA. Two days later, histones were extracted from the cultures using a Histone Extraction Kit (ab113476; Abcam, Cambridge, MA), and protein was determined as described by Scopes (25), employing a Nanodrop. Purified histones, equalized with respect to protein, were separated on 12.5% SDS-polyacrylamide gels, transferred to Nitrocellulose, and subjected to Western analysis (using rabbit polyclonal antibodies against di- and tri-methylated histones), as previously described (22).

## 2.10 Determination of L- and D-2HG, and glutamine

Primary RPT cell cultures in 100 mm dishes were treated with either a) L2HGDH Stealth siRNA or scrambled control siRNA, or b) L2GDH Silencer Select siRNA or Negative Control 1 siRNA, as described above. Two days later, the cultures were harvested as follows. The medium was changed 2 h prior to harvesting. The cultures were treated with EDTA/trypsin. Dislodged cells were transferred into a 2 ml screwtop tube (removing a sample to determine cell number). The cells were centrifuged (3,000×*g*), washed with PBS, and flash-frozen in dry ice/ethanol. Samples were then utilized for the determination of L- and D- 2HG levels. Subsequently, samples were homogenized in a bead mill homogenizer, L- and D-2HG were derivatized using acidified R-2-butanol, and derivatized L- and D-2HG were separated by GC-MS, as described by Li and Tennessen (26). The identity of the peaks for L- and D-2HG was verified using *L,D*-[2,3,3-$^{2}H_{3}$]-2-hydroxyglutarate) as an internal standard, and peak areas for L- and D-2HG were determined, also as described by Li and Tennessen (26), The final concentration of L- and D-2HG (determined in nanomoles) was standardized with respect to cell number, as determined using a Coulter Counter. Final values are averages of triplicate determinations +/- the SEM.

In order to examine the effect of glutaminase inhibitor CB-839 on glutamine levels, intracellular glutamine and glutamate were determined in triplicate cultures using the Promega Glutamine/Glutamate-Glo Assay. Cultures were grown in 96-well plates in Medium RK-1. The medium was changed to either Medium RK-1 with diluent (DMSO), or Medium RK-1 supplemented with 1 μM CB-839. After a 3-day incubation, the cultures were lysed in 0.1N HCl. A portion of the lysate was treated with glutaminase, while the other portion was untreated. Subsequently, the glutamate level was determined both in glutaminase-treated and untreated lysates using the Glutamate-Glo Assay. Emitted light was quantitated using a Biotek Plate Reader. The glutamine level was determined by subtracting the glutamate level measured in an untreated lysate from the glutamate level determined in the portion of the lysate treated with glutaminase. Values are averages +/- SEM of triplicate determinations.

## 2.11 Transwell migration assay

Primary RPT cells were trypsinized and plated ($5 \times 10^4$ cells/0.5 ml) into matrigel coated tissue culture plate inserts with PET membranes in 12-well plates. DMEM/F12 containing chemoattractant was added to the bottom chamber. The next day, the transwell was removed and washed with PBS, and the cells were removed from the membrane side facing the upper chamber using a cotton swab. After fixing the cells with formalin, the transwell was washed twice with PBS, the cells were stained with Hoechst (10 μg/ml), and the transwells were washed twice with PBS. Images were captured (in at least 25 microscope fields) using a Zeiss Axio Observer Inverted Microscope. The cells in each of the images were automatically counted using NIH ImageJ software. The average number of cells/10 microscope fields was determined in each of the three transwells/condition.

## 2.12 Statistical analysis

Statistical analyses were conducted using Prism software. Statistical results are expressed as means +/- SEM. Statistical differences between groups were determined using a two-tailed t-test. Differences between means were considered statistically significant when $p < 0.05$.

# 3 Results

## 3.1 Effect of L2HGDH KD on tubulogenesis

The effect of an L2HGDH Knockdown (KD) on renal proximal tubulogenesis *in vitro* was examined. Towards these ends, primary RPT cell cultures were transduced with lentiviral particles containing vectors expressing either L2HGDH shRNA, or the Control TRC shRNA vector, and selected with puromycin. Subsequently, transduced cells were introduced into matrigel and cultured in the presence of EGF. Figure 1A shows tubules observed in Control TRC transduced cultures, as compared with the structures in cultures transduced with L2HGDH shRNA. Quantitative studies of parallel cultures indicated that both the number of tubules and the L2HGDH mRNA levels were reduced by 80% (Figures 1B, C, respectively). Similar results were obtained when primary RPT cell cultures were transfected with Silencer Select L2HGDH siRNA, in comparison with Negative Control siRNA (as shown in Figures 1D–F).

Previously, Shelar et al. (2) conducted studies which indicated that L2HG is primarily generated from Glutamine (Gln) in RCCs. The proposed pathway is illustrated in Figure 2A. Consistent with this hypothesis, the glutaminase inhibitor CB-839 was observed to reduce L-2HG levels in ccRCC cells (2). Thus, the effect of the glutaminase inhibitor 1 μM CB-839 on tubulogenesis by primary RPTs was examined. Figure 2B shows that incubation of primary RPTs transduced with lentiviral L2HGDH shRNA with 1 μM CB-839 prevented the decrease in the number of tubules caused by L2HGDH shRNA. In contrast, 1 μM CB-839 did not significantly affect the number of tubules in matrigel cultures transduced with the Control TRC vector. Similarly, as shown in Figure 2C, CB-839 significantly alleviated the decrease in tubulogenesis caused by Silencer Select L2HGDH siRNA. Figure 2D shows that 1 μM CB-839 caused a significant increase in intracellular glutamine levels, indicating that glutaminase was significantly inhibited under these conditions. Thus, these results are consistent with the hypothesis that the decrease in tubule formation normally caused by the L2HGDH shRNA can be attributed to an increase in the L2HG level, and that this increase no longer occurs in the presence of CB-839.

In order to evaluate this hypothesis further, the effect of extracellular L2HG on tubule formation was examined. The intracellular level of L2HG has been reported to vary dramatically in normal cells derived from different tissues, including 0.4 μM L-2HG in macrophages (27) and 43.79 μM in HEK293FT cells, derived from human kidney (28), and 2HG levels high as 700 μM in white blood cells (29). While the level of D- and L-2HG in the renal microenvironment has not been precisely determined, 1.37 μM 2HG has been measured in serum (30).

Thus, initially the effect of the cell permeable octyl L-2HG on tubulogenesis was examined. Figure 3A shows the typical impairment in tubule formation in matrigel cultures treated with 1 μM octyl L-2HG. As shown in Figure 3B as the octyl L-2HG concentration was gradually increased to 100 μM, the number of tubules decreased to 0. In contrast, as the L-2HG concentration was increased to 100 μM, tubule formation only decreased by 50%. Figure 3C shows that the 75% decrease in tubule formation observed at 10 μM octyl L-2HG was associated with a forty-fold increase in intracellular L-2HG. Presumably then, the inhibitory effect of octyl L-2HG can be attributed to the increased intracellular L-2HG, which inhibits αKG-dependent dioxygenases. Consistent with this hypothesis, the inhibitory effect of octyl L-2HG on tubule formation was alleviated by 5-octyl-αKetoglutarate (αKG) (as shown in Figure 3D). This latter observation can be explained if 5-ocytl-αKG successfully competes with the elevated L-2HG for binding to αKG-dependent dioxygenases, thereby preventing the inhibitory effect of the elevated L-2HG on αKG-dependent dioxygenases.

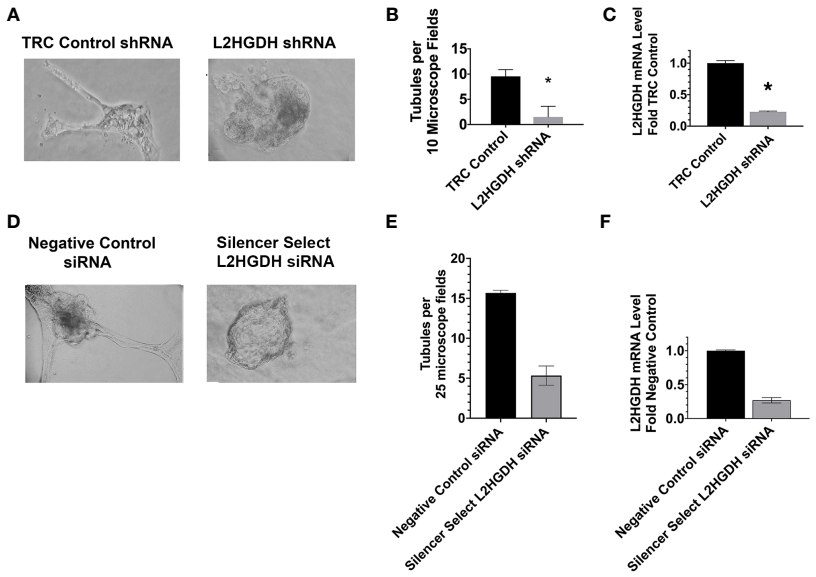


FIGURE 1
Inhibition of tubulogenesis by L2HGDH shRNA AND L2HGDH siRNA. Primary RPT cell cultures were either **(A)** transduced with lentivirus containing either L2HGDH shRNA or Control TRC shRNA vectors, or **(D)** transfected with either Silencer Select L2HGDH siRNA, or Negative Control siRNA. Prior to culturing in matrigel, the shRNA transduced cells were selected 1 week with 1.6 µg/ml puromycin, while the siRNA transfected cells were cultured 1 day to allow for gene expression. Subsequently, the primary cultures were trypsinized and passaged into matrigel in DMEM/F12-IT further supplemented with 5 ng/ml EGF, as described in Materials and Methods. Representative microscope fields of matrigel cultures are illustrated including (A1) matrigel cultures transduced with either Control TRC shRNA or L2HGDH shRNA, and (B1) matrigel cultures transfected with either Silencer Select L2HGDH siRNA or Negative Control siRNA. **(B)** the effect of the L2HGDH shRNA on number of tubules was quantitated, relative to Control shRNA, and **(C)** the relative levels of L2HGDH mRNA determined in the two conditions. Similarly, in **(E)** the effect of Silencer Select L2HGDH siRNA on the number of tubules was quantitated, relative to Negative Control siRNA, and **(F)** the relative levels of L2HGDH mRNA determined in the same 2 conditions. Values are averages +/- SEM of triplicate determinations. (*) $p < 0.05$ relative to either the TRC control shRNA **(B, C)** or the Negative Control siRNA **(E, F)**, respectively. Scale Bars, 50 µm.

## 3.2 Effect of L2HGDH KD on L- and D-2HG levels

In order to determine a) whether L2HGHD siRNA causes a significant increase in L-2HG, and b) whether the L-enantiomer, rather than the D- enantiomer is affected, the level of both L- and D-2HG was determined in primary cultures treated with either L2HGDH Stealth siRNA or Scrambled control siRNA by means of a GC-MS analysis. Figure 4A shows that in control primary cultures treated with Scr siRNA D- was the major enantiomer of 2HG (0.34 +/- 0.07 nmol D-2HG/$10^6$ cells vs. 0.16 +/- 0.01 nmol L-2HG/$10^6$ cells). In contrast, in primary cultures treated with L2HGDH stealth siRNA, L- was the major enantiomer. This can be attributed to a 4.4-fold increase in the L-enantiomer of 2HG in primary RPT cells treated with L2HGDH stealth siRNA (to 0.70 +/- 0.06 nmol/$10^6$ cells), unlike the D-enantiomer, which did not change significantly (0.40 +/- 0.07 nmol/$10^6$ cells). Figure 4B shows that similarly, L-2HG became the predominant enantiomer in primary RPT cultures treated with L2HGDH silencer select siRNA (vs. Negative Control siRNA). This can be explained by a significant increase in the level of the L- rather than the D- enantiomer of 2HG in primary RPTs treated with L2HGDH silencer select siRNA. These results are consistent with the hypothesis that L2HGDH stealth siRNA caused a specific increase in L2HG due to a reduction in the level of the L2HGDH enzyme without affecting D2HGDH enzyme levels.

## 3.3 Effect of L2HGDH KD on DNA and histone methylation

Previously, a knockdown of L2HGDH was observed not only to cause an increase in L2HG levels, but in addition to cause alterations in the level of DNA hydroxymethylation and histone methylation in renal carcinomas and other cultured renal cells (31). In this respect, an increase in L2HG has similar consequences to those caused by increases in D2HG (and a decrease in D2HGDH) observed in other types of cancers (32). Such changes in DNA and histone methylation have been attributed to the ability of 2HG to inhibit αKG-dependent dioxygenases that control gene expression (32). Included amongst these dioxygenases are TET methylcytosine dioxygenases, as well as Jumonji domain containing histone

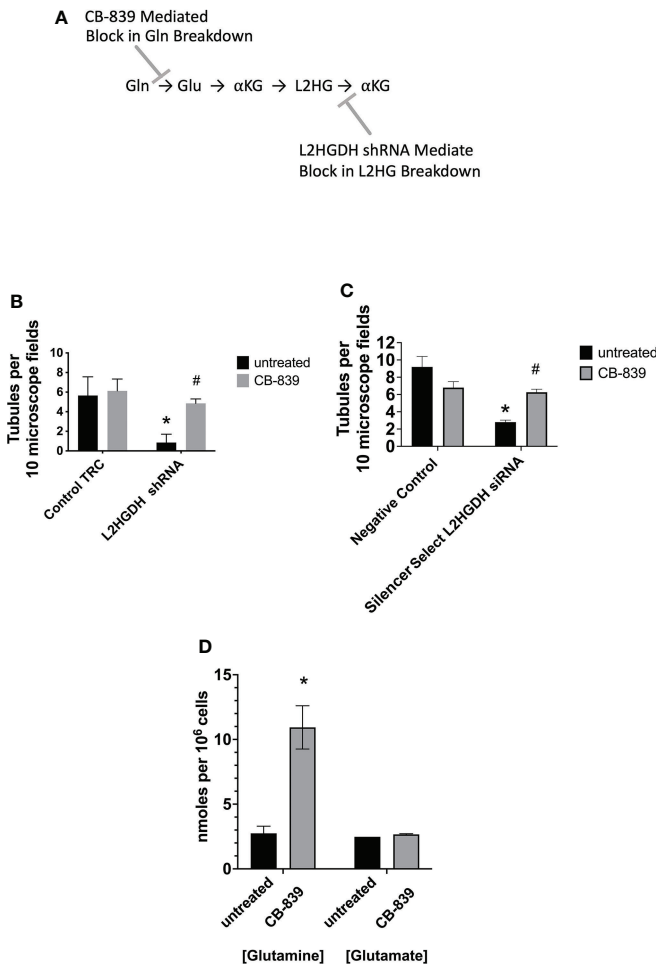


FIGURE 2
Effect of Glutaminase Inhibitor CB-839 on tubulogenesis. **(A)** Model for the Effect of CB-839 on Gln metabolism. Shelar et al. (2) previously presented evidence indicating that L2HG in RCCs primarily originates from Gln, initially occurring *via* the metabolism of Gln to Glu by Glutaminase. L2HG is subsequently metabolized to αKG by L2HGDH. **(B)** Primary RPT cultures were transduced with lentivirus containing either L2HGDH shRNA or Control TRC shRNA, selected with puromycin, and passaged into matrigel, as described in the Figure 1 legend. **(C)** Primary RPT cell cultures were transfected with Silencer Select L2HGDH siRNA or Negative Control siRNA, and passaged into matrigel 1 day later, as described in the Figure 1 legend. In parts **(B, C)** Matrigel cultures were incubated with DMEM/F12- IT further supplemented with 5 ng/ml EGF and either 1 μM CB-839 or no further supplement, the day after cultures were initiated in matrigel. In parts **(B, C)**, tubules were counted as described in the Figure 1 legend. Values are averages +/- SEM of triplicate determinations. (*) $p < 0.05$ relative to untreated Control TRC; (#) $p < 0.05$ relative to the untreated L2HGDH condition. **(D)** The level of glutamine and glutamate was determined in primary RPT cell cultures treated with either 1 μM CB-839 or untreated, as described in Materials and Methods. Values are averages +/- SEM of triplicate determinations. (*) $p < 0.05$ relative to untreated control in the glutamine condition.

demethylases (JMDHs), a family of histone demethylases. Thus, the hypothesis was examined that the inhibition of these two families of αKG-dependent dioxygenases resulting from an L2HGDH knockdown could be responsible for the inhibition of tubulogenesis caused by an L2HGDH knockdown in normal renal cells, as well as other related alterations.

The TET methylcytosine dioxygenases (including TET1, TET2, and TET2) are involved in the demethylation of 5-methylcytosine (5mC) in genomic DNA (1). The reaction initially involves the formation of 5-hydroxymethyl cytosine (5hmC) from 5mC, to determine whether this enzymatic activity is altered in primary RPT cultures were treated with either L2HGDH shRNA or Scrambled shRNA. Subsequently, genomic DNA was purified from these primary cultures, and the level of 5hmC in the genomic DNA was examined by means of slot blots. Figure 5A shows that the level of 5hmC is indeed reduced in genomic DNA derived from cultures treated with L2HGDH shRNA, as compared with Scrambled Controls. Similar results were obtained when the primary cultures were treated with Silencer Select L2HGDH siRNA, as

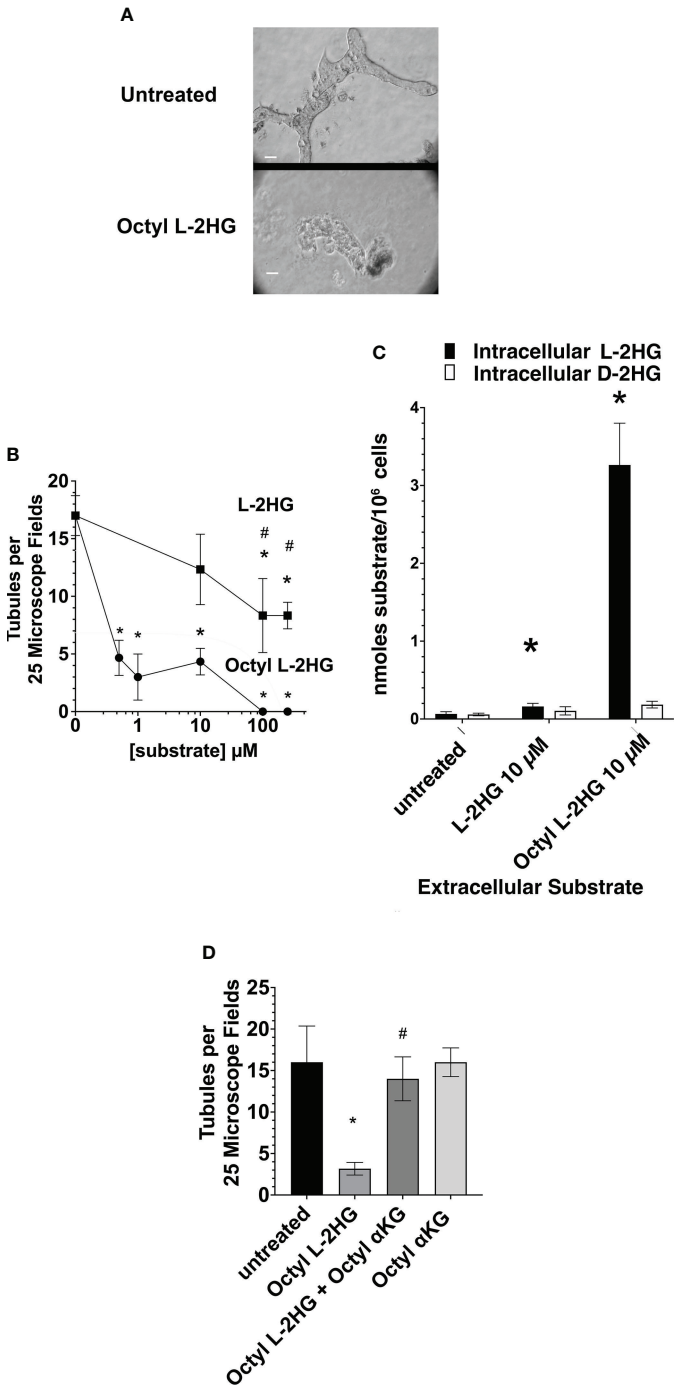


FIGURE 3
Inhibition of tubulogenesis by Octyl L-2HG. **(A)** Representative microscope fields of either control matrigel cultures in DMEM/F12-IT supplemented with 5 ng/ml EGF alone, or further supplemented with 100 μM Octyl L-2HG. **(B)** The frequency of tubule formation as a function of the concentration of either Octyl L-2HG or L-2HG. **(C)** Effect of 10 μM L-2HG or Octyl L-2HG on the intracellular L- and D-2HG concentration. **(D)** Effect of 5-octyl-αKG (250 μM) on the Octyl L-2HG (100 μM)-induced inhibition of tubulogenesis. Values are averages +/- SEM of triplicate determinations. (*) $p < 0.05$ relative to untreated cultures; (#) $p < 0.05$ relative to Octyl L2HG. Scale Bar, 50 μm.

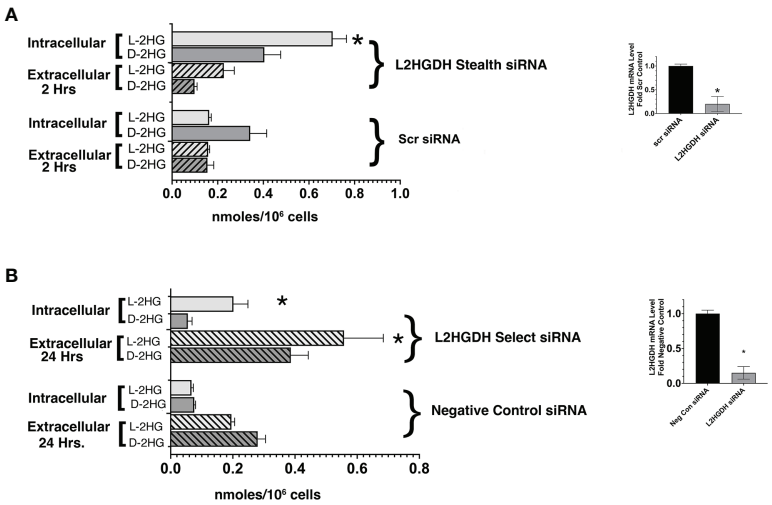


FIGURE 4
Effect of L2HGDH shRNA an L2HGDH siRNA on the intra- and extracellular L- and D-2Hydroxyglutarate Level. **(A)** Primary RPT cells were transfected with either L2HGDH Stealth siRNA or the corresponding Scrambled siRNA. The medium was changed on Days 1, 2 and 3 post-transcription. **(A)** Two hours after the medium change on Day 3 post-transfection, frozen cell pellets were prepared, and medium was collected. **(B)** Primary cultures were transfected either with L2HGDH Silencer Select siRNA or Negative Control siRNA. As in part A, the medium was changed on Days 1, 2 and 3 post-transfection. The medium that was changed on Day 3, however, was collected and frozen, while frozen pellets were prepared 2 h after the final medium change. The L- and D-2HG in each of the samples (i.e. cell pellets and medium) was derivatized, separated by GC/MS, and quantitated, as described in Materials and Methods. Values are averages (+/- SEM) of triplicate determinations. In part A, *p < 0.05 relative to intracellular L-2HG with Scr siRNA, while in part B, *p < 0.05 relative to Negative Control siRNA in the same condition (i.e. either intracellular L-2HG, or extracellular L-2HG). In the insets, L2HGDH mRNA levels were determined as described in Materials and Methods. (*) p < 0.05 relative to Control condition.

compared with Negative Control-treated cultures (as show in Figure 5B). These results are consistent with reduced TET enzymatic activity in primary RPT cell cultures treated with L2HGDH shRNA.

Inhibition JMDHs would also be expected to occur following an increase in L-2HG levels, which would prevent the demethylation of some classes of histones. Thus, the effect of L2HGDH siRNA on methylated histones was examined. Figure 6A shows that there was a generalized increase in the level of methylated histones in primary cultures treated with L2HGH Stealth siRNA, as compared with parallel cultures with Scrambled Control siRNA. Not only did the level of K4 dimethyl histone H3 increase, but in addition, there was an increase in the level of both K27 dimethyl and trimethyl histones in L2HGDH Stealth siRNA treated cultures. The level of K36 dimethyl histone increased in an analogous manner in the L2HGDH Stealth siRNA treated cultures. In addition, K79 dimethyl and trimethyl Histone H3 levels increased in primary RPT cells treated with L2HGDH Stealth siRNA. As shown in Figure 6B, similar increases in histone methylation were observed in primary cultures treated with L2HGDH Silencer Select siRNA. Thus, our results are consistent with the hypothesis that both DNA and histone methylation increase in primary RPT cell cultures with an L2HGDH KD.

## 3.4 Effect of an L2HGDH KD on the expression of differentiated transporters

The expression of RPT transporters is induced during the tubulogenesis which occurs during kidney development (12, 33). Included amongst these transporters are the $Na^+$/phosphate cotransporter (NPT2a), the p-Aminohippurate transporter (OAT1), Aquaporin 1 (AQP1), and the $Na^+$/glucose cotransporter (SGLT2). Initially, the effect of an L2HGDH KD was examined in monolayer cultures. Figure 7A. shows the reduced NPT2a, OAT1 and SGLT2 mRNA levels in monolayer cultures of primary RPT cells transduced with lentiviral L2HGDH shRNA. Transporter mRNA levels were similarly reduced in primary RPT monolayers transduced with L2HGDH Stealth siRNA (vs. Scrambled Controls) (Figure 7B), as well as L2HGDH Silencer Select siRNA (Figure 7C).

The effect of an L2HGDH KD at the protein level was also examined. Figures 8A, B show that in primary RPTs treated with L2HGDH shRNA, the level of the NPT2a and SGLT2 proteins was reduced by 67 +/- 11% and 58 +/- 11%, respectively, as compared with Con TRC shRNA-treated controls. Similar reductions in the level of NPT2a and SGLT2 proteins were observed in primary cultures treated with either L2HGDH Stealth siRNA or L2HGDH Silencer Select siRNA, as compared with their respective controls. Figures 8A, B also shows a substantial reduction in the L2HGDH protein level in

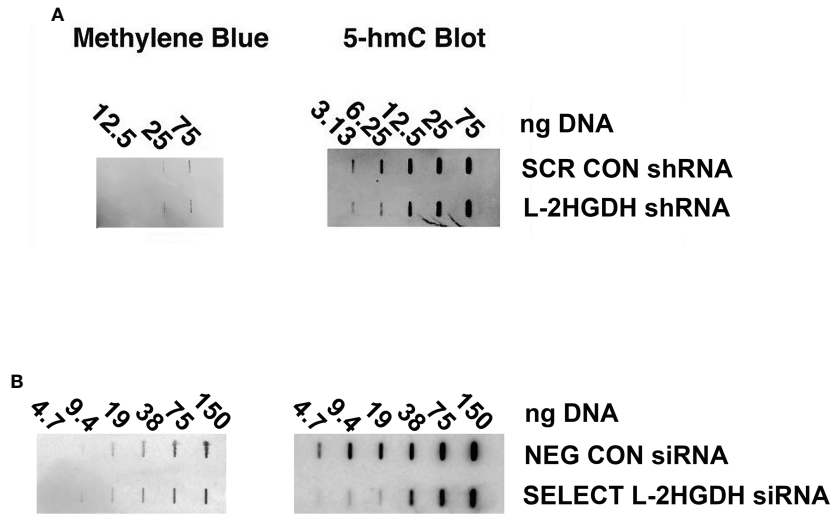


FIGURE 5
Effect of L2HGHD shRNA and siRNA on DNA methylation. The effect of L 2HGDH shRNA and siRNA on 5hmC levels. Genomic DNA was purified from primary cultures **(A)** transduced with lentivirus containing either Scrambled shRNA or L2HGDH shRNA vectors, or **(B)** transfected with either Negative Control siRNA, or Silencer Select L2HGDH siRNA. Serial dilutions of the genomic DNA were applied to slot blots. Subsequently, blots were probed with a 5hmC antibody, and total DNA visualized with methylene blue.

primary cultures treated with L2HGDH shRNA, L2HGDH Stealth siRNA, or L2HGDH Silencer Select siRNA, as compared with their respective controls (Con TRC shRNA, Scrambled Stealth siRNA, and Negative Control siRNA, respectively).

Relative to their respective controls, the L2HGDH protein levels were 16 +/- 4% (L2HGDH shRNA), 29 +/- 7% (L2HGDH Stealth siRNA), and 33 +/- 1% (L2HGDH Silencer Select siRNA).

## 3.5 Effect of basement membrane on gene expression, as well as on alterations caused by L2HGDH knockdowns

### 3.5.1 Effect of L2HGDH KD on Transporter gene expression: Influence of matrigel

Previous studies indicate that the basement membrane components of matrigel promote the differentiation of cells originating from a diverse number of tissues (34). Thus, it is reasonable to determine whether the expression of differentiated renal transporters is altered by basement membrane matrigel, as well as by the process of tubulogenesis itself (35). For this reason, the effect of basement membrane matrigel on the expression of transporter mRNAs and the cellular response to L2HGDH shRNA was examined, including studies both in Control RPT cell cultures (treated with lentiviral Con TRC shRNA), as well as in RPT cell cultures treated with lentiviral L2HGDH shRNA.

As shown in Figure 9, the expression of SGLT2 mRNA and NPT2a mRNA increased when Control primary RPT cell cultures were maintained in matrigel (where they form tubules) as compared to plastic (as shown in Figures 9A, B, respectively). The expression of SGLT2 and NPT2a mRNA was reduced in matrigel as well as in monolayer cultures transduced with L2HGDH shRNA (Figures 9A, B). In contrast, Figure 9C shows that the AQP1 mRNA level increased in matrigel cultures transduced with L2HGDH shRNA, unlike the Control TRC cultures, which exhibited a decreased level of AQP1 mRNA in matrigel. This unexpected observation with AQP1 mRNA (which distinguishes AQP1 from the two other transporters studied) may possibly be attributed to the role of AQP1 in cell adhesion and migration, in addition to transport (36).

### 3.5.2 Effect of L2HGDH KD on HNF transcription factor expression: Influence of matrigel

Hepatocyte nuclear factors (HNFs) not only regulate the expression of a number of renal transporters (13) but also play a role in kidney development (37). Thus, the decreased expression of renal transporter mRNAs observed in cultures treated with L2HGDH shRNA may possibly be explained by reduced expression of HNFs in RPT cell cultures treated with lentiviral L2HGDH shRNA. To examine this hypothesis, the expression of the mRNAs encoding for 2 HNFs, HNF1α, and HNF1β was examined both in monolayer and matrigel cultures treated with either lentiviral L2HGDH shRNA or Control TRC shRNA. The level of HNF1α and HNF1β mRNA was significantly reduced in matrigel cultures transduced with L2HGDH shRNA (vs. Control TRC cultures), as shown in Figures 10A, B, respectively. In

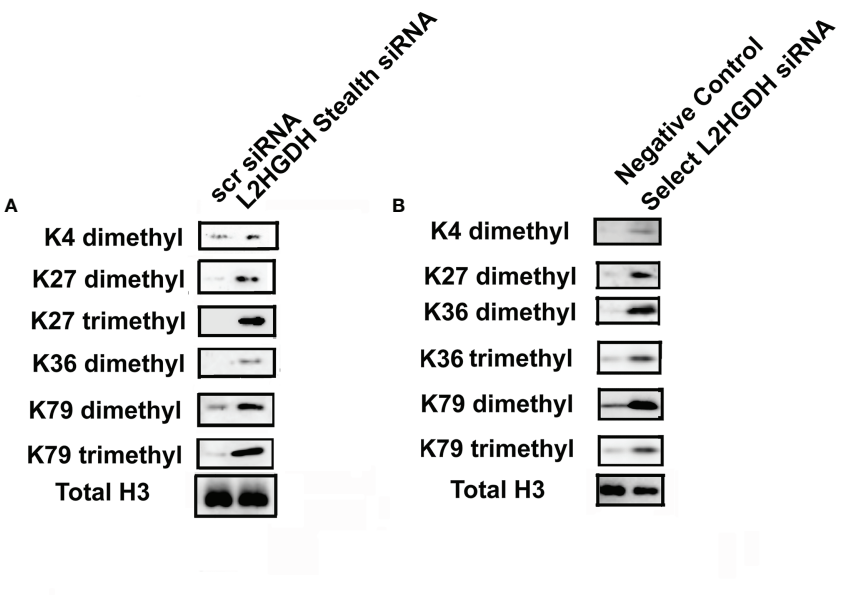


FIGURE 6
Effect of L2HGDH siRNA on histone methylation. Nuclear histones were purified from primary cultures transfected either with **(A)** L2HGDH Stealth siRNA or Scrambled Control Stealth siRNA, or **(B)** Silencer Select L2HGDH siRNA or Negative Control siRNA. Three days later, nuclear histones were purified. Subsequently, nuclear histones were separated by SDS/PAGE, transferred to nitrocellulose, followed by Western analysis, as described in Materials and Methods.

addition, a significant reduction in the HNF1α and HNF1β mRNA was observed in monolayer cultures transduced with L2HGDH shRNA (Figure 10A). Thus, these results are consistent with the hypothesis that a reduction in the level of HNF1α and HNF1β contributes to reduced expression of transporter mRNAs as well as reduced tubulogenesis caused by L2HGDH shRNA.

### 3.5.3 Effect of L2HGDH KD on other genes affecting tubulogenesis: Influence of matrigel

The expression of other genes which affect tubulogenesis was also examined both in matrigel and monolayer cultures. Figure 11A shows that the level of E-Cadherin (CDH1), urokinase type-Plasminogen Activator (PLAU), and Wingless/Integrated 1 (Wnt1) mRNA increased significantly in matrigel, as compared with monolayer cultures, unlike Chibby 1 (CBY1). However, similar increases in the level of CDH1 and PLAU mRNA were not observed in L2HGDH shRNA-derived matrigel cultures (Figures 11B, C). Indeed, in matrigel cultures treated with L2HGDH shRNA, the PLAU mRNA level declined to a level significantly below that observed in Control monolayer cultures. In contrast, Wnt1 mRNA increased substantially in monolayer cultures transduced with lentiviral L2HGDH shRNA, and this increased level of Wnt1 mRNA was maintained in matrigel cultures with an L2HGDH KD (Figure 11D). In contrast, the level of CBY1 mRNA decreased in matrigel cultures with an L2HGDH KD (Figure 11E). CBY1 is a negative regulator of β-catenin-mediated transcriptional activation (38), and thus a reduction in CBY1 gene expression would be expected to stimulate Wnt signaling *via* β-catenin.

Decreases in the expression of E-cadherin and urokinase type-Plasminogen Activator may very well result in reduced cell migration, and, as a consequence, reduced tubulogenesis in EGF treated matrigel cultures. Thus, the effect of EGF on cell migration through transwells was examined. Figure 11F shows that the stimulatory effect of EGF on the migration of RPT cells transduced with lentiviral L2HGDH shRNA was reduced greater than six-fold. This observation is consistent with the hypothesis a reduction in cell migration through matrigel contributes to the reduced tubulogenesis observed in RPT cells transduced with lentiviral L2HGDH shRNA.

## 4 Discussion

Previously, the studies of Shelar et al. (2) indicated that "the L2HG/L2HGDH axis" plays a significant role in the development of RCCs. The expression of a number of genes which possess high-CpG-density promoters was altered because of the elevated L2HG levels in RCCs, including Polycomb proteins, which target developmentally regulated genes (2). The increased L2HG in RCCs may very well contribute to the altered signal transduction pathways observed in these tumors, including signaling pathways involving EGF and Wnt (39–41),

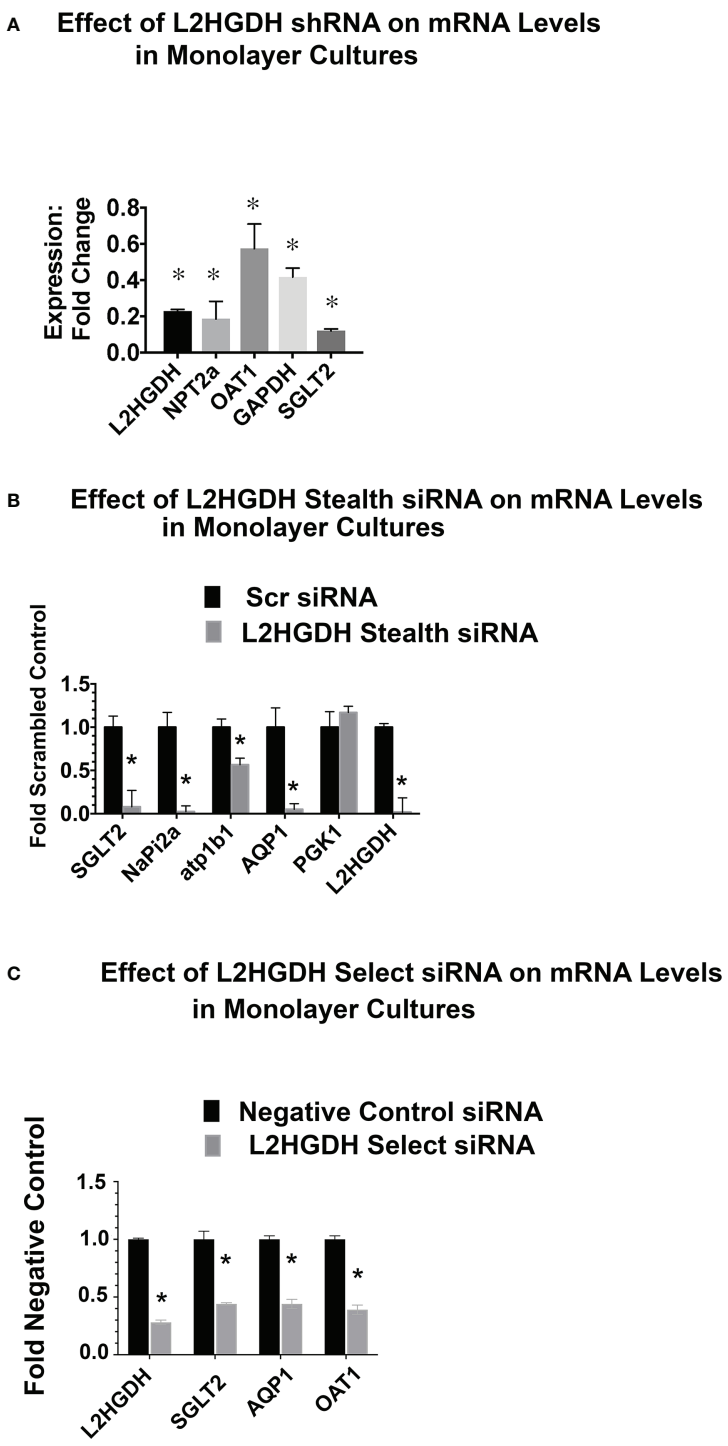


FIGURE 7
Effect of L2HGDH shRNA and L2HGDH siRNA on the expression of transporter mRNAs in monolayer cultures. **(A)** The expression of the mRNAs for L2HGDH, NPT2a, OAT1, GAPDH and SGLT2 was determined in primary cultures transduced with lentivirus containing either an L2HGDH shRNA or Control TRC vector. The relative expression of mRNAs in cultures transduced with L2HGDH shRNA was compared to the level in cultures transduced with Control TRC shRNA. **(B)** Relative mRNA levels were determined in primary RPT cells transfected twice with either L2HGDH Stealth siRNA or Scrambled siRNA. **(C)** Relative mRNA levels were determined in primary RPT cells transfected twice with either L2HGDH Silencer Select siRNA or Negative Control siRNA, as described in Materials and Methods. In part A, (*) $p < 0.05$ relative to either the TRC Control (Part A), the Scrambled Control (Part B), or, the Negative Control (Part C).

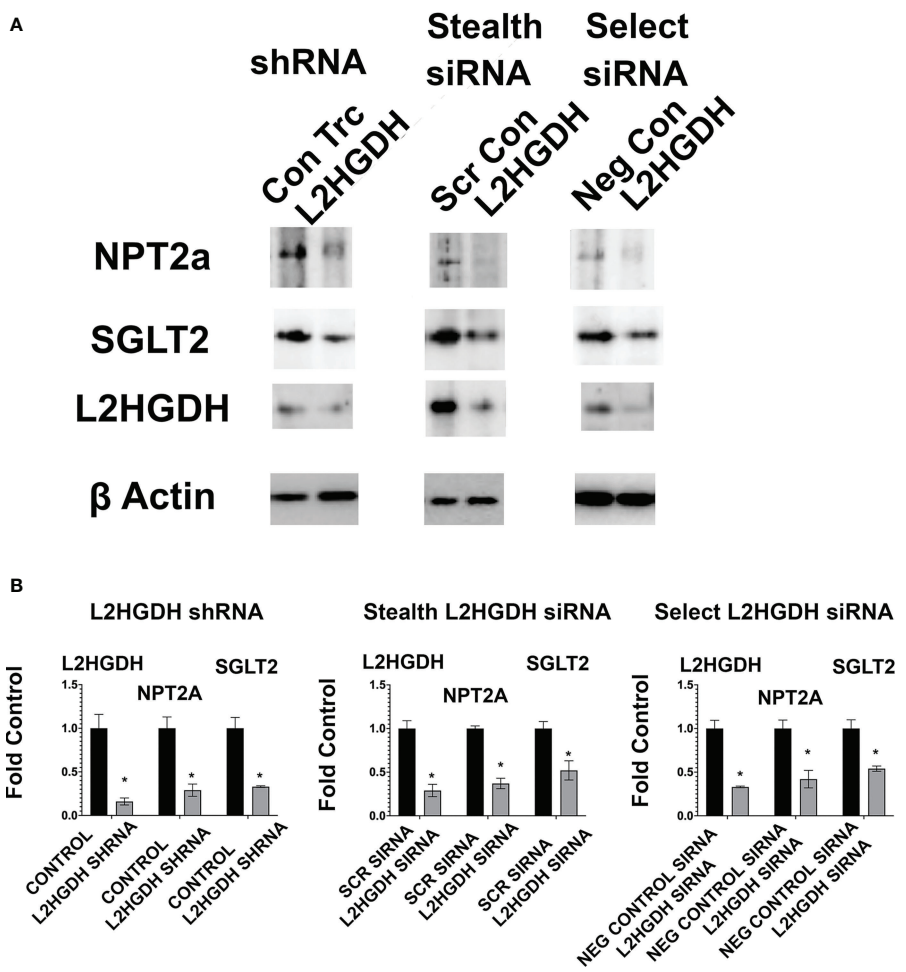


FIGURE 8
Effect of lentiviral L2HGDH shRNA and L2HGDH siRNA on the level of the NPT2a, SGLT2 and L2HGDH proteins in monolayer cultures. **(A)** Blots of shRNA and siRNA-treated primary cultures. Blots used in the study were prepared following Western transfers of SDS/PAGE gels, as described in Materials and Methods. The samples in the blots were derived from lysates of primary cultures treated either with (i) L2HGDH shRNA (or Control TRC shRNA), (ii) L2HGDH Stealth siRNA (or Scrambled siRNA), or (iii) L2HGDH Silencer Select siRNA (or Negative Control siRNA). **(B)** Relative Levels of NPT2a, SGLT2 and L2HGDH. The relative levels of NPT2a, SGLT2 and L2HGDH were determined using ImageLab software. Values are averages (+/- SEM) from duplicate bands for each sample. (*) $p < 0.05$ relative to the TRC Control (for L2HGDH shRNA), the Scr Control (for L2HGDH Stealth siRNA), and the Negative Control (for L2HGH Silencer Select siRNA).

as well as PI3K/Akt/mTOR (42, 43) and VHL/HIF (44). Both the EGF and Wnt mediated signaling pathways control kidney development and differentiation (45, 46). Thus, changes in these signaling pathways would be expected to alter the differentiated state, and to select for stemness. Indeed, recent studies indicate that these pathways are activated in renal cancer stem cells (41, 47).

In this report, evidence is presented that EGF-induced renal proximal tubulogenesis is controlled by the L2HG/L2HGDH axis. As we have previously reported, tubulogenesis by primary RPT cells in matrigel normally occurs in response to EGF (9). However, here we present evidence indicating that tubulogenesis in matrigel is inhibited when L2HGDH is knocked down by lentiviral L2HGDH shRNA. Consistent with the hypothesis that L2HG is involved in mediating the inhibitory effect of EGF on tubulogenesis, a) the L2HG level increased in cultures with an L2HGDH knockdown, b) L2HG octyl-ester inhibited tubulogenesis, and c) the glutaminase inhibitor CB-839 prevented the inhibitory effect of L2HGDH shRNA on tubulogenesis. This latter observation can be explained if CB-839 causes a decline in L2HG levels, similar to that reported by Shelar et al. (2) in RCC cells treated with CB-839. This latter observation can be explained as being the consequence of the inhibition of the glutamine (Gln) metabolic pathway leading to L2HG (the pathway being Gln → Glutamic (Glu)→ αKetoglutarate (αKG)→ L2HG) (2).

Our metabolomic studies indicate that an L2HGDH KD results in an increase in L2HG levels7, which is presumably

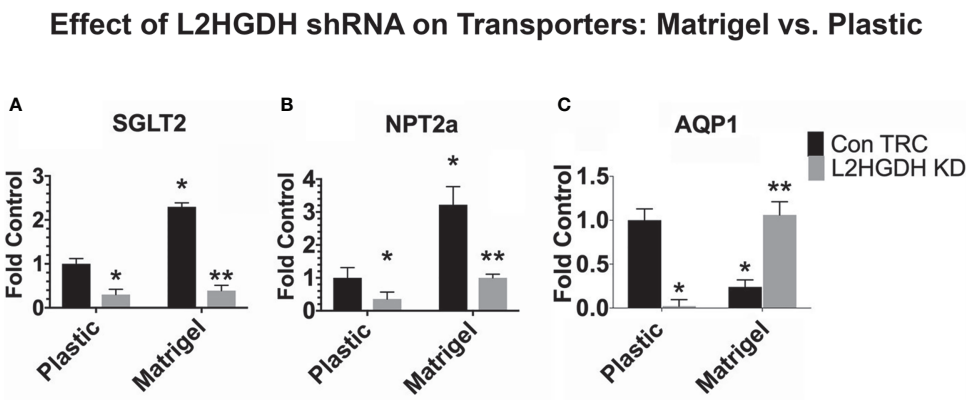


FIGURE 9
Effect of matrigel on transporter gene expression. Primary cultures of RPT cells were transduced with lentiviral L2HGDH shRNA or Control TRC shRNA. A portion of the cultures was transferred into either matrigel or onto plastic. The relative level of mRNA for **(A)** SGLT2, **(B)** NPT2a, and (C) AQP1 was determined as described in Materials and Methods. (*) $p < 0.05$ for the Con TRC Plastic Control; (**) $p < 0.05$ for the ConTRC Matrigel Control.

responsible for the inhibition of tubulogenesis. Both L- and D-2HG are competitive inhibitors of α-KG dependent dioxygenases. A considerable number of α-KG dependent dioxygenases are present in mammalian cells, including enzymes affecting metabolic processes, in addition to enzymes affecting the methylation of DNA and histones.

Our recent metabolomic studies (unpublished) also indicate that the level of a number of metabolites whose synthesis depends upon α-KG dependent dioxygenases is indeed altered in primary RPT cells treated with L2HGDH siRNA. For example, the level of saccharopine is reduced in cultures treated with L-2HGDH siRNA. Saccharopine is produced from lysine and α-KG by the lysine-ketoglutarate reductase domain of α-aminoadipate semialdehyde synthase (48). Similarly, the increased level of leucine can be explained by reduced leucine metabolism to 4-methyl-2-oxopentanoate by L-

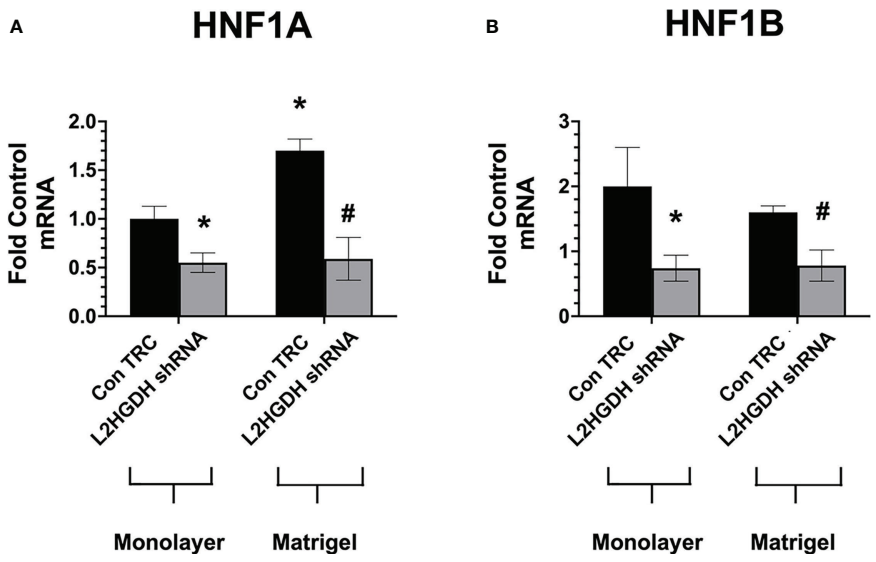


FIGURE 10
Effect of L2HGDH shRNA on expression of HNF1α and HNF1α. Primary RPT cells were transduced with lentiviral L2HGDH shRNA or Control TRC shRNA. A portion of the cultures was transferred either into matrigel or onto plastic. The relative level of mRNA for **(A)** HNF1α and **(B)** HNF1β was determined as described in Materials and Methods. (*) $p < 0.05$ relative to Con TRC Monolayers; (#) $p < 0.05$ relative to Con TRC Matrigel cultures.

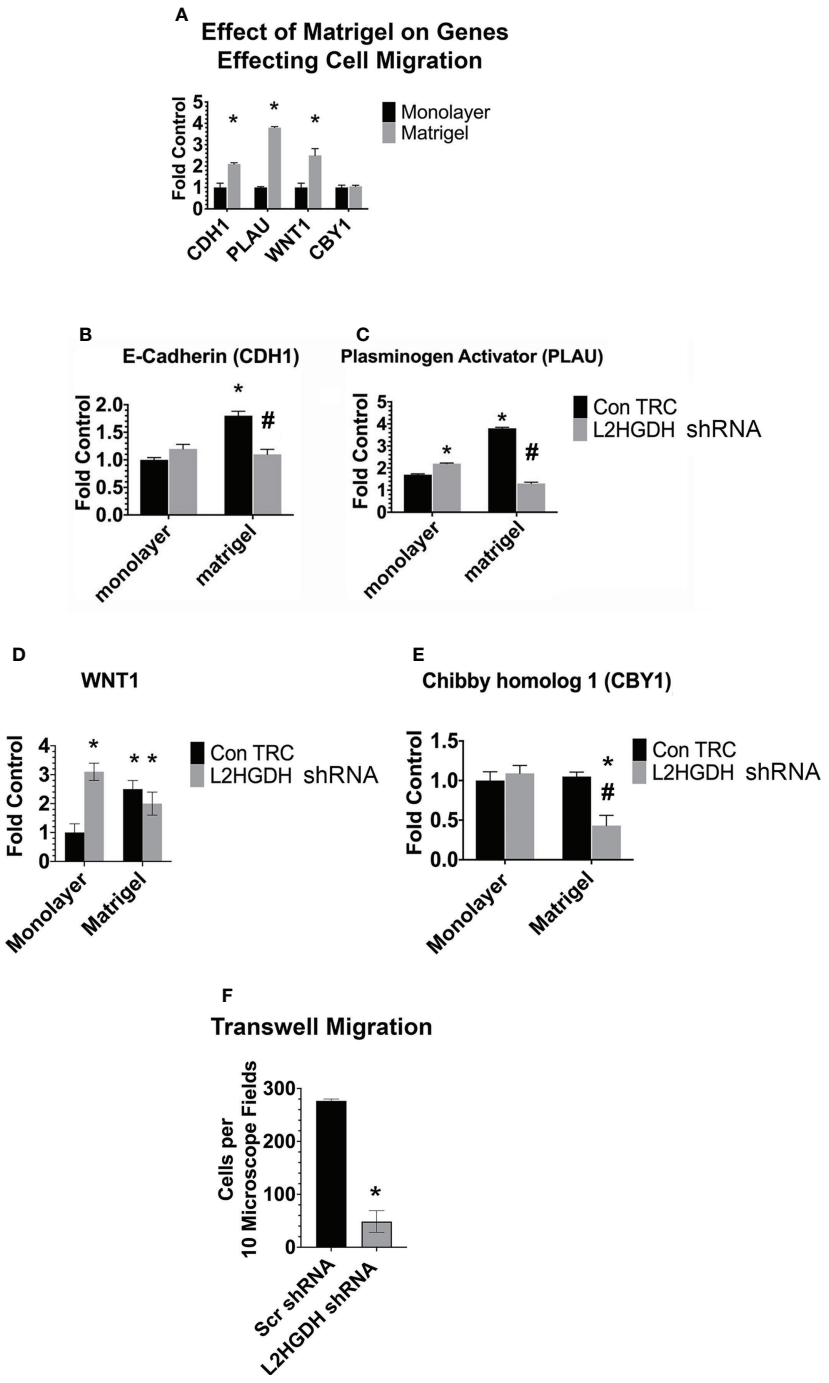


FIGURE 11
Expression of genes affecting tubulogenesis, and cell migration. **(A)**. The relative level of mRNA encoding for E-Cadherin (CDH1), tissue Plasminogen Activator (PLAU), Wnt1 and Chibby homolog 1 (CBY1) was determined in parallel monolayer and matrigel cultures. The relative level of the mRNA encoding for **(B)** E-Cadherin, **(C)** Plasminogen Activator, **(D)** WNT1, and **(E)** CBY1 (1) was determined in monolayer and matrigel cultures tranduced with lentiviral L2HGDH shRNA or Control TRC shRNA. **(F)** EGF-stimulated cell migration across PET membranes was determined in RPT cells following their transduction with lentiviral L2HGDH shRNA or Con TRC shRNA. (*) $p < 0.05$ relative to monolayer cultures in the same condition in panel **(A)**; In panels **(B–E)**, (*) $p < 0.05$ relative to the Con TRC monolayer culture condition; in 11F (*) $p < 0.05$ relative to the scr shRNA condition; (#) $p < 0.05$ relative to the Con TRC Matrigel Condition **(E)**.

leucine-2-oxoglutarate aminotransferase (α-KG receiving the NH2-group from leucine) (49). The contribution of these metabolic changes to the altered tubulogenesis observed in these studies is unclear.

However, it is likely that the L2HG-mediated changes in differentiation observed in these studies can be attributed to the inhibition of those DNA and histone demethylases which are included amongst the family of αKG-dependent dioxygenases. Included amongst the DNA demethylases which are αKG-dependent dioxygenases, and inhibited by 2HG, are TET1, TET2, and TET3 enzymes. The TETs sequentially remove the methyl group from 5methylcytosine, by a series of successive oxidations which include the initial formation of 5hmC. Our 5hmC slot blotting study indicates that the level of 5hmC increased in primary RPT cells treated with L2HGDH siRNA, consistent with the inhibition of TETs by L2HG in this condition. Thus, the inhibition of tubulogenesis by L2HG can possibly be explained by the inhibition of DNA demethylation. Consistent with this hypothesis, αKG octyl ester alleviated the inhibition of tubulogenesis caused by L2HG-octyl ester, presumably by preventing competitive inhibition of TET enzymes by L2HG.

Our experimental results show a generalized increase in the level of methylated histones in primary RPT cell cultures treated with L2HGDH siRNA. A similar observation was made in 3T3-L1 cells with a 2HG-producing IDH2 mutation (5). In the 3T3-L1 cells expressing this IDH2 mutation, the increase in histone methylation (as well as DNA methylation) was associated with a block which prevented their differentiation into adipocytes (5). The increased histone methylation observed in these 3T3-L1 cells was attributed to the inhibition of JHDMs by 2HG (5, 50). Consistent with this hypothesis, Lu et al. (5) observed that differentiation of 3T3L1 cells into adipocytes was similarly impaired when the histone demethylase KDM4C was inhibited. KDM4C is a H3K9me3 demethylating JHDM. These latter studies suggest that the block in tubulogenesis observed in our RPT cultures transduced with lentiviral L2HGDH shRNA is also a consequence of repressive H3K9 trimethylation. Consistent with this hypothesis, H3K9me3 is downregulated in nascent nephrons during kidney development (51).

The effect of an L2HGDH knockdown on the expression of mRNAs encoding for RPT transporters was also examined in our studies. The level of Npt2a and Sglt2 mRNA was substantially reduced in matrigel cultures which had been transduced with lentiviral L2HGDH shRNA (as observed in monolayer cultures). These observations can be explained if the expression of the genes encoding for Npt2a and Sglt2 (SLC34A1 and SLC5a2, respectively) is repressed due to increased methylation of CpG islands present within their promoters and/or the promoters of HNF transcription factors (presumably due to the inhibition of TET enzymes). Consistent with the hypothesis of gene repression due to promoter methylation is the observation that the SLC5a2 gene is present within a differentially methylated region (DMR) of genomic DNA, that is hypomethylated in the RPT, unlike other tissues (52).

Consistent with the hypothesis that HNF transcription factors are involved are studies indicating that the expression of SLC34A1 and SLC5a2 is dependent upon the binding of HNF1α and HNF4α to their promoters (13). Similarly, the expression of renal OATs depends upon the binding of HNF1α and HNF1β to the promoter region, which in turn is controlled by the DNA methylation status (13, 53). The expression of the genes encoding for the HNF family of transcription factors themselves can also be suppressed by methylation of their promoters. For example, the methylation of 4 CpG sites in the HNF1A promoter results in the silencing of the HNF1A gene, and its downstream targets, such as GnT-4a glycosyltransferase in pancreatic β cells (54). The HNF4A gene is similarly silenced by DNA promoter methylation (5mC), as exemplified by liver progenitors, whose differentiation depends upon TET mediated formation of 5hmC, resulting in the expression of HNF4A, and the initiation of a hepatocyte developmental program (55).

Although DNA 5mC hypermethylation is a characteristic of cells that overproduce 2HG, increased histone methylation is also observed in cells which overproduce 2HG. Indeed, Schvartzman et al. (4) and Lu et al. (5) have proposed that the block in adipocyte differentiation and myocyte differentiation caused by 2HG is a consequence of increased histone H3K9 methylation rather than a rise in DNA methylation. However, in nephron progenitors, the Polycomb proteins EZH1 and EZH2 maintain stemness by stimulating H3K27 trimethylation. EZH2 reportedly suppresses expression of HNF1β (56), as well as HNF1α (57), presumably by stimulating H3K27 trimethylation. The increased level of H3K27me3 in primary RPTs treated with L2HGDH siRNA may similarly be responsible for the inhibition of tubulogenesis in matrigel (58). However, we cannot rule out the involvement of other trimethylated histones, and/or methylated CpG islands, given that similar results were obtained with the EHMT1/2 inhibitor Unc0638 and the DNA methylase inhibitor 5AzaC (unpublished).

A consequence of increased DNA and histone methylation may very well include reduced expression of transcription factors such as HNF1α and HNF1β. Indeed, epigenetic silencing of both the HNF1A and HNF1B genes has been reported and has been attributed to the methylation of CpG islands in the promoters of these genes (54, 59). Thus, reduced expression of HNF1α, and HNF1β may contribute to the inhibition of tubulogenesis caused by L2HGDH shRNA. Both HNF1α and HNF1β play significant, but distinct roles in kidney development (37, 60, 61). HNF1β appears when the nephrogenic mesenchyme is induced to form a polarized epithelium (which involves Wnt signaling) (37, 62, 63). HNF1β is also involved in segmentation of the developing nephrons, which involves Notch signaling (64). HNF1α appears after HNF1β, playing a role in

formation of renal proximal tubules, including the expression of differentiated RPT transporters (e.g., Npt2a, SGLT2 and OAT1) (13, 65).

In our experimental studies, we examined the effects of an L2HGDH KD on the expression of a number of mRNAs and their respective proteins, expressed in the RPT, unlike other nephron segments. Consistent with our observations, are the results of high-throughput technologies employed to quantitatively analyze the transcriptomes, proteomes, and genomes of mammalian cells (66). A central conclusion that has come from this work is that protein levels at steady state are primarily determined by mRNA levels (66). Admittedly, this relationship between protein and mRNA levels is not necessarily maintained during "dynamic" adaptation processes, and during short-term temporal adaptions post-transcriptional processes are important (66). However, our studies with primary RPT cells treated with lentiviral shRNA were conducted more than 10 days after lentiviral transduction. Although the effects of an L2HGDH knockdown on mRNA levels were also examined following transfection with L2HGDH siRNA, the results obtained were very similar to those obtained with an L2HGDH KD obtained with lentiviral L2HGDH shRNA. A very significant aspect of our studies was the observation that the expression of a number of the mRNAs encoding for differentiated RPT transporters was higher in matrigel cultures, as opposed to monolayer cultures. However, the mRNA levels were examined after 1 week in matrigel, and under these conditions, a reduction in the level of SGLT2 and NPT2a mRNAs was still observed, similar to results observed in parallel cultures maintained on a plastic substratum.

Recently, cancer stem cells (CSCs) with activated Wnt and Notch signaling, have been isolated from clear cell RCCs (41). The activation of Wnt signaling (observed in RPT monolayers with an L2HGH KD) occurs when the level of the Wnt antagonist DKK1 declines, an event which may result from the hypermethylation of the DKK1 promoter, the trimethylation of H3K27, and the recruitment of the Polycomb complex, as observed in lung cancers (67). In contrast, the activation of Notch signaling may occur when STRAP (serine-threonine kinase associated protein) interacts with the Polycomb complex, so as to inhibit H3K27 methylation, which as a consequence increases the expression of the Notch effectors HES1 and HES5, as observed in colorectal CSCs (68). Unlike the case with Wnt and Notch signaling, HNF1α- and HNF4α-mediated signaling is reduced in RCCs (69, 70), resulting in reduced expression of distinctive RPT genes, such as SLC34A1 (NaPi2a) and SLC22A6 (OAT1) (71). In contrast, the expression of AQP1 often increases in RCCs (72, 73), similar to the increased expression of AQP1 mRNA in RPT matrigel cultures with an L2HGDH KD.

To summarize, this report has evaluated the effects of an L2HGDH KD on the differentiation of normal RPT cells, the cell of origin of ccRCCs. Evidence is presented that EGF-induced tubulogenesis is inhibited by L2HG itself, as well as an L2HGH KD, which frequently occurs in ccRCCs. We have conducted metabolomic studies which indicate that the L2HGDH KD results in a significant increase in L-2-hydroxygutarate. The inhibition of tubulogenesis caused by an L2HGDH KD was associated with reduced expression of a number of mRNAs encoding for differentiated transporters expressed in the RPT, as well as reduced expression of mRNAs encoding for transcription factors which regulate the expression of these transporter mRNAs. The reduced expression of these mRNAs can be attributed to the increased DNA and histone methylation which occurred as a consequence of an L2HGDH KD. In addition, our studies indicate that EGF-induced cell migration was impaired as a consequence of an L2HGDH KD, which could be explained by reduced expression of mRNAs encoding for such proteins as plasminogen activator. Thus, the reduction in tubulogenesis observed in normal cells with elevated 2HG can be attributed to the impairment of functions required for the process of tubulogenesis, as well as dedifferentiation.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University at Buffalo.

## Author contributions

MT has directed and conducted studies with primary cultures of renal proximal tubule cells both as monolayer and matrigel cultures, in addition to preparing the manuscript. NM conducted studies which led to the measurement of L- and D-2Hydroxyglutarate, in addition to interpreting the results. JT directed the GC/Mass Spec studies, assessing the results and making appropriate scientific changes that led to the final definitive results. SS determined the direction of the overall studies, and made arrangements for appropriate experiments to be conducted in cases where the experimental direction required the involvement of others, such as JT and NM. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Co-administration of MDR1 and BCRP or EGFR/PI3K inhibitors overcomes lenvatinib resistance in hepatocellular carcinoma

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Lenvatinib is the first-line treatment for hepatocellular carcinoma (HCC), the most common type of primary liver cancer; however, some patients become refractory to lenvatinib. The underlying mechanism of lenvatinib resistance (LR) in patients with advanced HCC remains unclear. We focused on exploring the potential mechanism of LR and novel treatments of lenvatinib-resistant HCC. In particular, we established a Huh7 LR cell line and performed *in vitro*, bioinformatic, and biochemical assays. Additionally, we used a Huh7-LR cell-derived xenograft mouse model to confirm the results *in vivo*. Following LR induction, multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) transporters were markedly upregulated, and the epidermal growth factor receptor (EGFR), MEK/ERK, and PI3K/AKT pathways were activated. *In vitro*, the co-administration of elacridar, a dual MDR1 and BCRP inhibitor, with lenvatinib inhibited proliferation and induced apoptosis of LR cells. These effects might be due to inhibiting cancer stem-like cells (CSCs) properties, by decreasing colony formation and downregulating CD133, EpCAM, SOX-9, and c-Myc expression. Moreover, the co-administration of gefitinib, an EGFR inhibitor, with lenvatinib retarded proliferation and induced apoptosis of LR cells. These similar effects might be caused by the inhibition of EGFR-mediated MEK/ERK and PI3K/AKT pathway activation. *In vivo*, co-administration of lenvatinib with elacridar or gefitinib suppressed tumour growth and angiogenesis. Therefore, inhibiting MDR1 and BCRP transporters or targeting the EGFR/PI3K pathway might overcome LR in HCC. Notably, lenvatinib should be used to treat HCC after LR induction owing to its role in inhibiting tumour proliferation and angiogenesis. Our findings could help develop novel and effective treatment strategies for HCC.

KEYWORDS

**hepatocellular carcinoma (HCC), lenvatinib resistance (LR), multidrug resistance protein 1 (MDR1), breast cancer resistance protein (BCRP), epidermal growth factor receptor (EGFR), elacridar, gefitinib, copanlisib**

# 1 Introduction

Primary liver cancer (PLC) poses a global health challenge. According to GLOBOCAN 2020, PLC ranks sixth in cancer incidence and second in cancer-related mortality, with approximately 906,000 new cases and 830,000 deaths worldwide in 2020 (1). Unfortunately, these numbers will continue to rise, as over one million individuals will be diagnosed with HCC annually by 2025 (2). Hepatocellular carcinoma (HCC) is the most common form of PLC, accounting for 75–85% of PLC cases (1). Marked improvements have been achieved in the early detection and subsequent treatment of HCC; however, the reality of HCC management remains poor. Presently, only 44% of HCC cases are diagnosed at the localised stage, and 27% and 18% of HCC cases are diagnosed at the regional and distant stages, respectively (3). Consequently, the five-year survival rate of all HCC stages is barely 20%, and this rate decreases to as low as 3% for distant stage HCC (3). The mainstay treatments for localised stage HCC include resection, transplantation, and ablation. However, the presence of underlying diseases (e.g., liver cirrhosis) often complicates surgical management, as liver transplantation is not always available due to the scarcity of donor organs, and local ablation is sometimes not amenable in cases of knotty tumours.

Nevertheless, systemic therapies, such as tyrosine kinase inhibitors (TKIs), provide hope for patients with unresectable HCC and increase overall survival and improve the quality of life of this population (2). Lenvatinib, an oral inhibitor of multiple receptor tyrosine kinases (RTKs), exerts its antitumour effect by inhibiting vascular endothelial growth factor receptors 1–3 (VEGFR1–3), platelet-derived growth factor receptor α (PDGFRα), fibroblast growth factor receptors 1–4 (FGFR1–4), c-KIT, and RET (4). In patients with unresectable HCC, lenvatinib showed non-inferiority in improving survival outcomes compared with sorafenib (5). In the past decade, sorafenib has become the only effective therapeutic choice for patients with advanced HCC, and lenvatinib has been approved as the first-line drug and is used worldwide (6).

Numerous clinical trials have verified the therapeutic efficacy of lenvatinib in patients with HCC. However, the clinical benefits of lenvatinib administration are limited, as some HCCs become refractory to lenvatinib treatment. Hence, substantial interest has focused on the mechanisms of lenvatinib resistance (LR). Particularly, LR is mediated by hepatocyte growth factor/c-MET axis-associated mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathway activation (7), upregulated interferon regulatory factor 2 (IRF2) and β-catenin expression (8), FGFR1 overexpression and downstream AKT/mTOR and ERK signalling activation (9), and upregulated VEGFR2 expression and downstream RAS/MEK/ERK pathway activation (10). However, we could hardly find studies on the underlying mechanism of LR following long-term exposure to lenvatinib. Notably, a well-designed combined therapy might successfully inhibit compensatory signalling activation following LR induction; however, a feasible drug combination that could overcome LR has not yet been established.

In this study, we aimed to establish a Huh7 LR cell line to elucidate the underlying mechanism of LR and explore novel drugs that could be used to overcome LR in HCC.

# 2 Materials and methods

## 2.1 Reagents and antibodies

Lenvatinib (*HY-10981*), gefitinib (*HY-50895*), and copanlisib (*HY-15346A*) were purchased from MedChemExpress (Shanghai, China), and elacridar (*S7772*) was purchased from Selleck Chemicals (Shanghai, China). Stock solutions of 20 mM lenvatinib, 100 mM elacridar, and 20 mM gefitinib were dissolved in 100% dimethyl sulfoxide (DMSO), and the stock solution of 10 mM copanlisib was dissolved in Milli-Q water. Antibodies against total epidermal growth factor receptor (EGFR; A11577, ABclonal), phospho-EGFR (AP0820, ABclonal), total PI3K (ab32089, Abcam), phospho-PI3K (4228, CST), total AKT (9272, CST), phospho-AKT (4060, CST), total MEK1/2 (A4868, ABclonal), phospho-MEK1/2 (AP0209, ABclonal), total ERK1/2 (4695, CST), phospho-ERK1/2 (4376, CST), caspase-3 (T40051, Abmart), Bcl-2-associated X (Bax; T40044, Abmart), multidrug resistance protein 1 (MDR1; 13978, CST), breast cancer resistance protein (BCRP; 130244, Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5174, CST) were used. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Beyotime Biotechnology (Shanghai, China). Alexa Fluor-conjugated goat anti-rabbit (647 nm) and goat anti-mouse (488 nm) antibodies were purchased from Invitrogen (Shanghai, China).

## 2.2 Cell line and cell culture

The Huh7 parental (Huh7 P) cell line was obtained from the Cell Bank of National Biomedicine Research (Beijing, China) and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotics (penicillin and streptomycin) at 37°C and 5% $CO_2$. To generate the Huh7 LR cell line, Huh7 P cells were exposed to lenvatinib at an initial dose of 1 μM. Thereafter, the stable cell line was exposed to a lenvatinib concentration that was gradually increased by 1.0–2.0 μM per week. Approximately 10 months later, the Huh7 LR cell line was established and maintained in culture medium containing 20 μM lenvatinib.

## 2.3 RNA sequencing assay

Total RNA was extracted using TRIzol from a 10 cm cell culture plate when the cells reached 70–80% confluence. Three independent samples from each group (Huh7 P and Huh7 LR) were used for RNA-seq by Biomarker Technologies (Beijing, China). $Log_2$ (mRNA fold change) was used to assess differentially expressed mRNAs, with the calculated value of < -1 or > 1 deemed statistically significant ($p < 0.001$). The online bioinformatics database (DAVID Bioinformatics Resources 6.8, NIAID/NIH; website, https://david.ncifcrf.gov/tools.jsp) was used to analyse the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and biological processes based on the RNA-seq results.

## 2.4 Cell proliferation assay

Cells were plated at a density of 4,000 cells per well in a 96-well plate and cultivated overnight. The cells were then exposed to drugs suspended in DMEM (10% FBS) for 96 h. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at a working concentration of 5 mg/mL was added to the culture medium. After 4 h of incubation, the upper medium was removed, and 100 μL of DMSO was added to dissolve the crystals formed in the lower medium. After 10 min of incubation and shaking, the absorbance was measured at a wavelength of 490 nm. A real-time cell analyser (RTCA) S16 (Celligence, China) was used to compare cell proliferation ability. During this process, 4,000 cells per well were seeded in a 16-well plate and cultivated in medium containing 20 μM lenvatinib. During the next 72 h, a detector connected to a computer constantly calculated and displayed relative cell proliferation by measuring the electrical resistance of the plate bottom.

## 2.5 Clonogenicity assay

To compare the clonogenicity of Huh7 P and Huh7 LR cell lines, 1,000 cells per well were seeded in a 6-well plate and continuously exposed to culture medium containing lenvatinib (20 μM) for two weeks. To assess the clonogenicity of the Huh7 LR cell line after different drug treatments, 2,000 cells were seeded per well in a 6-well plate, drug-containing media was removed from the cells after 72 h of exposure, and the medium without drugs was changed every 3 days for the next 11 days. After fixing in methyl alcohol for 15 min and staining with crystal violet for 20 min, the colonies were photographed using a camera and analysed using Image J Software.

## 2.6 Cell apoptosis assay

Cells were seeded at a density of $2\times10^5$ cells per well in a 6-well plate and cultivated overnight. The cells were then treated with the control or drug-containing media for 72 h. Subsequently, the cells were collected and stained with Annexin V-FITC and propidium iodide (Beyotime Biotechnology, China) for 20 min and then analysed using flow cytometry (Beckman Coulter, USA). At least $5\times10^4$ cells were analysed for each sample.

## 2.7 Quantitative real-time polymerase chain reaction

Total RNA was extracted from Huh7 P and Huh7 LR cells using TRIzol reagent, and cDNA synthesis was conducted using a reverse transcription kit (Toyobo FSQ 301, Japan) following the manufacturer's protocol. Subsequently, qRT-PCR was performed in a total volume of 20 μL, containing Milli-Q water (2 μL), c-DNA (6 μL), forward and reverse primers (2 μL), and q-PCR mix (10 μL; Toyobo QPS-201, Japan). The primers used in this study were manufactured by Ruibiotech (Beijing, China) with the following sequences: β-actin forward: 5′-ATCGTCCACCGCAAATGCT TCTA-3′ and reverse: 5′-AGCCATGCCAATCTCATCTTGTT-3′, MDR1 forward: 5′-GGGAGCTTAACACCCGACTTA-3′ and reverse: 5′-GCCAAAATCACAAGGGTTAGCTT-3′, and BCRP forward: 5′-GCCACAGAGATCATAGAGCCT-3′ and reverse: 5′-TCACCCCCGGAAAGTTGATG-3′. The results were normalised to β-actin expression and are presented as relative mRNA expression levels.

## 2.8 Immunofluorescence staining

Cells (Huh7 P and Huh7 LR) were seeded at a density of 30,000 cells per well in an 8-well plate (BD Falcon 354108, USA) overnight. The cells were then washed thrice with PBS, fixed with 4% paraformaldehyde (PFA) for 20 min, blocked with 10% goat serum, and incubated with primary antibodies MDR1 (Rabbit mAb #13978 CST) and BCRP (Mouse mAb #130244 Abcam) for another day. After washing thrice with PBS, the cells were incubated with conjugated secondary antibodies for two hours at room temperature. Subsequently, 4′,6-diamidino-2-phenylindole was added and incubated for 15 min, and images were captured using a VS200 SlideView (Olympus, Japan).

## 2.9 Western blotting analysis

After incubation with different drugs, the cells were collected and lysed using radioimmunoprecipitation assay buffer supplemented with a protease and phosphatase inhibitor cocktail (Beyotime Biotechnology, China). Equal amounts of protein from each sample were loaded on 8% or 10% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis and transferred onto an Immobilon®-P Transfer membrane (Merck Millipore Ltd.). After blocking with 5% non-fat milk, the labelled membrane was

incubated with relevant primary antibodies at 4°C overnight. The membranes were then incubated with HRP-conjugated secondary antibodies for two hours at room temperature. Finally, the membranes were incubated with enhanced chemiluminescence reagent (Applygen, China), and the bands were detected. GAPDH was used as an internal reference.

## 2.10 Xenograft tumour in nude mice

BALB/C nude mice (male, 6 weeks old) were obtained from Charles River (Beijing, China). Huh7 LR cells ($1.0\times10^7$ cells per mouse) were injected into the flanks of the mice. After tumour establishment, the mice were randomly assigned to six groups (five mice per group): the vehicle, lenvatinib (5 mg/kg), gefitinib (80 mg/kg), elacridar (80 mg/kg), lenvatinib (5 mg/kg) combined with elacridar (80 mg/kg), and lenvatinib (5 mg/kg) combined with gefitinib (80 mg/kg) groups. The drugs were suspended in 5‰ carboxymethylcellulose sodium (powder dissolved in Milli-Q water). In the lenvatinib and elacridar group, elacridar was administered two hours prior to lenvatinib. All indicated treatments were orally administered to the mice 5 days per week. Tumour length and width were measured using callipers, and their volumes were calculated using the following formula: tumour volume = ½ length × width$^2$. All animal experiments were conducted in accordance with the approved protocol from Charles River (No. P2021049).

## 2.11 Histological analysis

Harvested tumours were fixed in 4% PFA, dehydrated gradually, embedded in paraffin, and sliced into 4 μm thick sections. Some sections were subjected to haematoxylin-eosin (H&E) staining, whereas other sections were used for immunohistochemistry (IHC). After routine IHC procedures, the samples were incubated with primary antibodies against Ki67 (14-5698-80, Invitrogen) and proliferating cell nuclear antigen (PCNA; 13110, CST) at 4°C overnight. The samples were then incubated with secondary antibodies using the VECTASTAIN ® Elite ® ABC Universal Kit, Peroxidase (Horse Anti-Mouse/Rabbit IgG; PK-6200, Vector Laboratories, Inc., USA).

## 2.12 Statistical analysis

OriginPro 2021 software was used to perform data analysis. Data are presented as the mean ± standard deviation based on triplicate experiments, and the final results are representative of more than two independent experiments, excluding the xenograft tumour experiment. All $p$ values are denoted as significant at $p < 0.05$. Following the Chou-Talalay method (11), CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) was used to calculate combination index (CI) values. The CI values reflect the interaction between two drugs (CI < 1, synergism; CI = 1, additive effect; CI > 1, antagonism).

# 3 Results

## 3.1 Establishment of a lenvatinib resistant cell line

After continuous exposure to lenvatinib (1–20 μM) for approximately 10 months, the Huh 7 LR cell line was normally passaged and maintained in medium containing 20 μM lenvatinib (Figure 1A). In contrast to Huh7 P cells, Huh7 LR cells were smaller in size and grew aggressively (Figure 1B). The MTT results revealed that Huh7 LR cells exhibited a higher proliferation rate than Huh7 P cells did in cultivation medium containing different lenvatinib concentrations (1.25, 2.5, 5, 10, and 20 μM), and the half maximal inhibitory concentration ($IC_{50}$) of lenvatinib in Huh7 LR cells ($IC_{50}$ > 20 μM) was significantly higher than that in Huh7 P cells ($IC_{50}$ 5.34 ± 1.07 μM) (Figure 1C). Meanwhile, Huh7 LR cells exhibited a relatively higher proliferation rate (Figure 1D) and higher colony forming ability (Figure 1E) than Huh7 P cells did in medium containing 20 μM lenvatinib. Moreover, Huh7 LR cells exhibited a higher anti-apoptotic activity than Huh7 P cells did in cultivation medium containing 20 μM lenvatinib (Figure 1F). These results confirmed that the Huh7 LR cell line was resistant to lenvatinib.

## 3.2 Transcriptomic analysis results

RNA-seq results were obtained to assess differentially expressed mRNAs between the Huh7 P and Huh7 LR cell lines (Supplementary Table 1). Three independent samples were examined for each cell line (Figure 2A), and the Huh7 LR cell line exhibited 728 upregulated and 274 downregulated genes compared with those in the Huh7 P cell line (Figure 2B). KEGG pathway enrichment analysis revealed that pathways related to metabolism and ATP-binding cassette (ABC) transporters and the ERBB signalling pathway were enriched after LR induction (Figure 2C). Additionally, gene annotation analysis of biological processes demonstrated that cellular efflux and metabolic processes were increased after LR induction (Figure 2D).

## 3.3 MDR1 and BCRP overexpression and EGFR signalling pathway activation following LR induction

The expression of MDR1 and BCRP, important ATP-binding cassette (ABC) transporters, was upregulated according to the RNA-seq results (Supplementary Table 2). We used qRT-PCR,

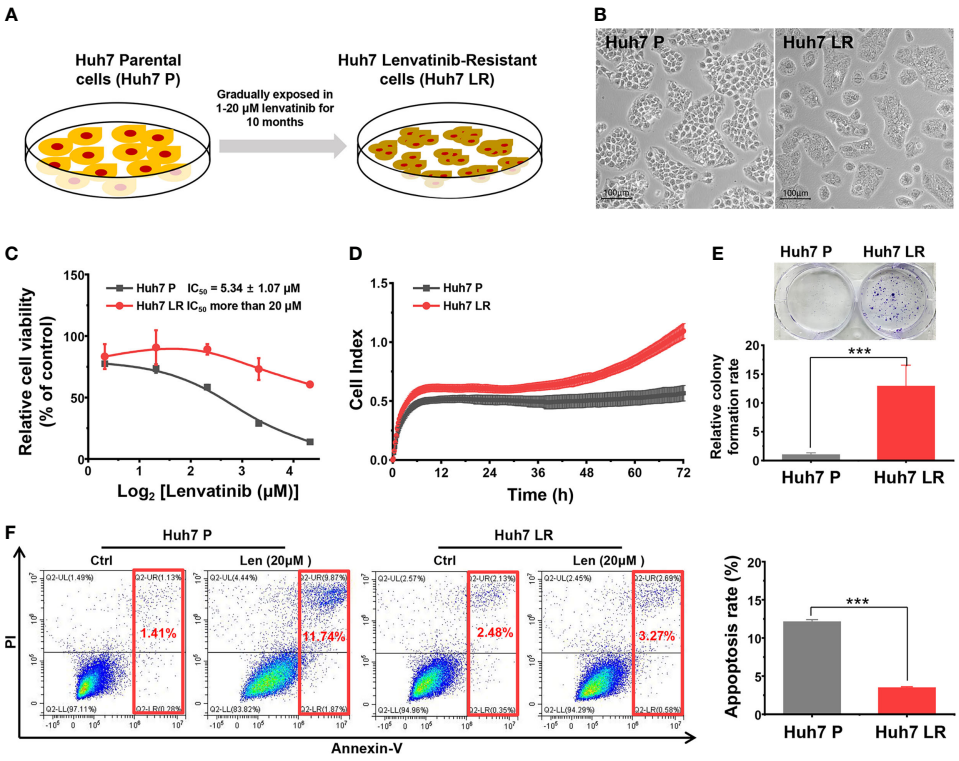


FIGURE 1
Successfully established Huh7 LR cell line. **(A)** The timeline and lenvatinib concentration. **(B)** The representative morphology of the Huh7 P and Huh7 LR cell lines. **(C)** The MTT assay during 96 h revealed that the Huh7 LR cell line had a substantially higher $IC_{50}$ than the Huh7 P cell line did. **(D)** RTCA revealed that Huh7 LR cells had a higher proliferation rate than Huh7 P cells did in medium containing 20 μM lenvatinib. **(E)** The Huh7 LR cell line exhibited higher colony forming ability than the Huh7 P cell line did in cultivation medium containing 20 μM lenvatinib. **(F)** Flow cytometry assay showed that Huh7 LR cells exhibited higher anti-apoptosis ability than Huh7 P cells did in cultivation medium containing 20 μM lenvatinib for 72 hours. ***$p < 0.001$.

western blotting, and immunofluorescence staining to further verify MDR1 and BCRP expression levels. First, the qRT-PCR results revealed that Huh7 LR cells exhibited significantly higher MDR1 and BCRP mRNA levels than Huh7 P cells did (Figure 3A). Second, western blotting demonstrated that Huh7 LR cells exhibited significantly higher MDR1 and BCRP levels than Huh7 P cells did (Figure 3B). Third, immunofluorescence staining revealed that MDR1 and BCRP were located in the cell membrane, and their expression was significantly higher in Huh7 LR cells than in Huh7 P cells (Figure 3C).

The EGFR signalling pathway is an important branch of the ERBB signalling pathway, and the activation of EGFR signalling pathway is a hallmark of human malignancies (12–14). Supplementary Table 3 demonstrates that the transcriptional levels of EGFR and the downstream RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways were normal or upregulated after LR induction. Importantly, among the upregulated mRNAs, PIK3R2, an oncogene involved in the physiological activation of PI3K (15), ranked first in fold-change ($Log_2FC = 5.71$) (Supplementary Tables 1, 2, Figure 2B). However, RNA-seq only reflects transcriptional level but cannot comprehensively reflect protein levels and functional alterations. Therefore, western blotting was performed to determine the levels of total and phosphorylated proteins involved in EGFR signalling and its downstream pathways. The western blot results revealed that phosphorylated EGFR, PI3K, AKT, MEK1/2, and ERK1/2 were significantly upregulated in Huh7 LR cells compared to those in Huh7 P cells (Figure 3D).

## 3.4 *In vitro* antitumour effect of combined treatments

### 3.4.1 Elacridar ameliorated LR by inhibiting MDR1 and BCRP

Both MDR1 and BCRP mediate drug efflux from tumour cells, which decreases the effective concentration of antitumour drugs and results in chemotherapeutic failure (16–18). Here, we speculated that elacridar, a dual MDR1 and BCRP inhibitor (19), could overcome LR in Huh7 LR cells by inhibiting MDR1 and BCRP (Figure 4A).

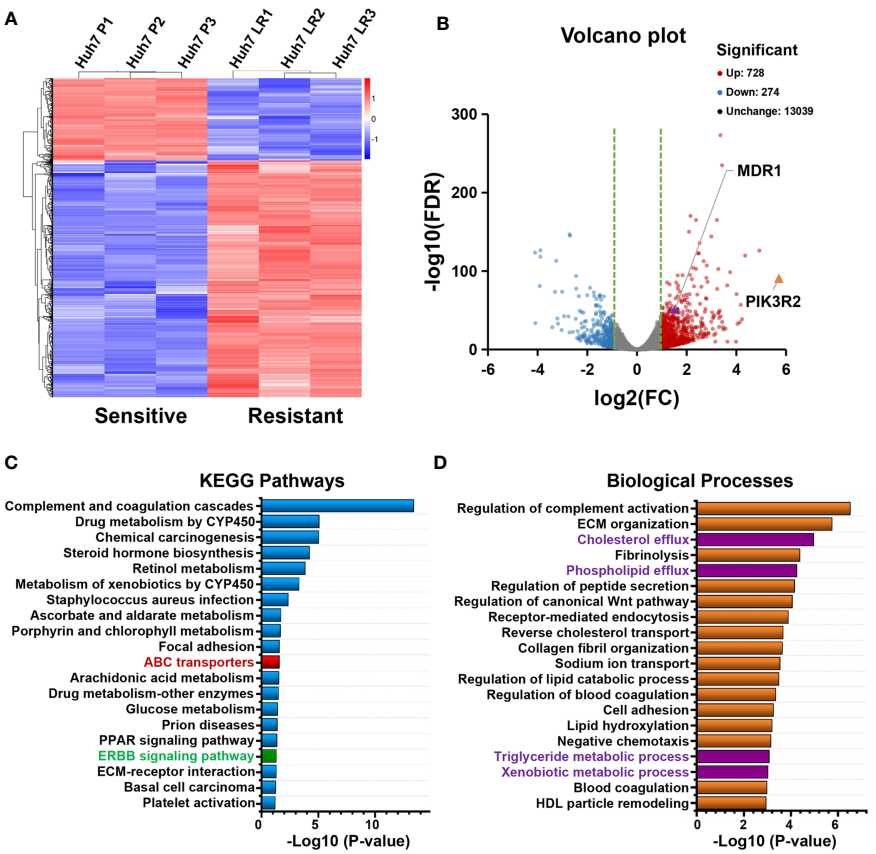


FIGURE 2
Transcriptomic analysis of Huh7 P and Huh7 LR cells based on RNA sequencing (three samples for each group). **(A, B)** Clustering heatmap and volcano plot show differentially expressed genes between the Huh7 P and Huh7 LR cell lines (> 2-fold change, p < 0.001). **(C, D)** KEGG pathways and biological processes associated with significantly upregulated genes in Huh7 LR cells (> 2-fold change, *p* < 0.001).

According to the MTT results, the relative cell viability of the 20 μM lenvatinib-treated group was 76.23%, whereas the relative cell viability of the 5 μM elacridar-treated group was 92.49%. However, the relative cell viability of the 20 μM lenvatinib- and 5 μM elacridar-treated group significantly decreased to as low as 39.67% (Figure 4B). The synergistic antitumour effect of lenvatinib and elacridar was further verified using CI values and the Chou-Talalay method. As shown in Figure 4C, the calculated CI values were < 1, indicating that elacridar synergised with lenvatinib to inhibit Huh7 LR cell proliferation. Thereafter, flow cytometry was performed to assess the pro-apoptotic effect of elacridar in Huh7 LR cells following treatment with a single drug or with co-administration of lenvatinib for 72 h (Figure 4D). According to the quantitative results (Figure 4E), lenvatinib (20 μM) in combination with elacridar (10 μM) significantly induced Huh7 LR cell apoptosis (apoptosis rate, 37.32%) compared with that of the control (1% DMSO), lenvatinib (20 μM), and elacridar (10 μM) groups (apoptosis rates, 2.14%, 3.79%, and 5.48%, respectively). Therefore, elacridar sensitised Huh7 LR cells to lenvatinib treatment.

Additionally, our *in vitro* results demonstrated that the combination of elacridar with lenvatinib significantly inhibited colony formation (Figure 4F) and decreased CD133, epithelial cellular adhesion molecule (EpCAM), SRY-box transcription factor 9 (SOX-9), and c-Myc expression (Figure 4G).

### 3.4.2 Gefitinib or copanlisib ameliorated LR by targeting the EGFR/PI3K pathway

EGFR and/or PI3K/AKT pathway activation is associated with chemotherapeutic resistance in human cancers (20, 21). Unfortunately, lenvatinib exerts antitumour effects by targeting multiple cell membrane RTKs, including VEGF1-3, PDGFR, FGFR1-4, c-KIT, and RET rather than EGFR (4). Here, we speculated that EGFR signalling pathway activation might be associated with LR; therefore, we investigated whether the addition of TKIs targeting the EGFR/PI3K/AKT pathway could overcome LR. We selected and tested FDA-approved clinical drugs, including gefitinib (targeting EGFR) and copanlisib (targeting PI3K) (Figure 5A).

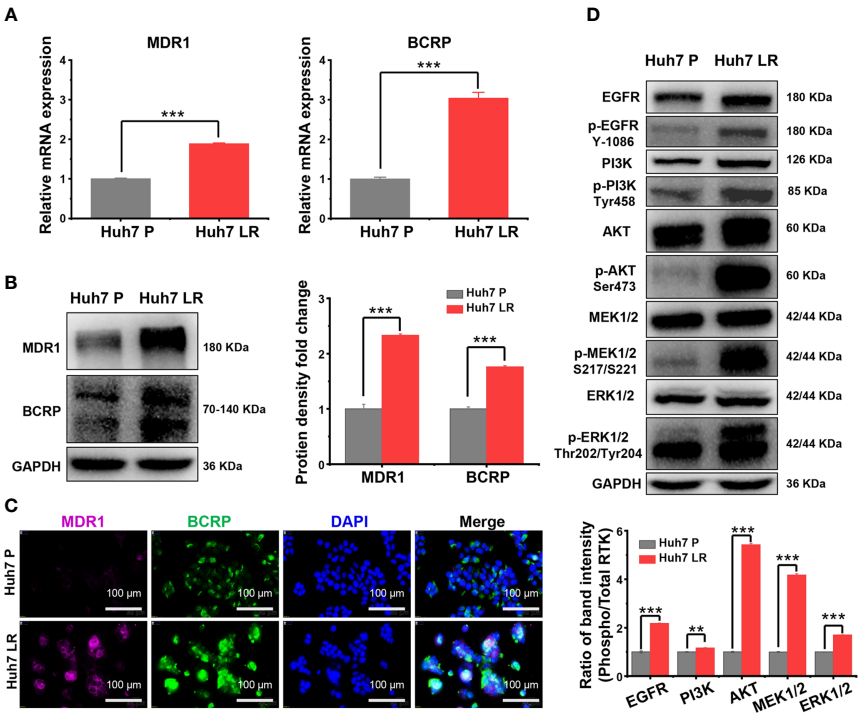


FIGURE 3
MDR1 and BCRP overexpression and EGFR signalling pathway activation following LR induction. **(A–C)** qRT-PCR, western blotting, and immunofluorescence analysis demonstrated that MDR1 and BCRP expression was upregulated following LR induction. Scale bars, 100 μm. **(D)** Western blotting revealed that EGFR and its downstream MEK/ERK and PI3K/AKT pathways were markedly activated following LR induction. $^{**}p < 0.01$. $^{***}p < 0.001$.

First, we performed an MTT assay to assess the effect of gefitinib or copanlisib on Huh7 LR cell proliferation following treatment with a single drug or with co-administration of lenvatinib. As shown in Figure 5B, the addition of gefitinib or copanlisib significantly enhanced the inhibitory effect of lenvatinib in Huh7 LR cells. We used the Chou-Talalay method and determined that lenvatinib and gefitinib or copanlisib synergistically inhibited cell proliferation, as the calculated CI values were < 1 (Figure 5C). Thereafter, we performed flow cytometry to assess the pro-apoptotic effect of gefitinib or copanlisib in Huh7 LR cells after drug treatment for 72 h (Figure 5D). According to the quantitative results (Figure 5E), the addition of gefitinib or copanlisib significantly enhanced the pro-apoptotic effect of lenvatinib in Huh7 LR cells. Therefore, gefitinib or copanlisib sensitised Huh7 LR cells to lenvatinib treatment.

Regarding the potential antitumour mechanism, western blotting revealed that co-treatment with gefitinib and lenvatinib significantly inhibited the phosphorylation of EGFR, PI3K, AKT, MEK1/2, and ERK1/2 (Figure 5F), whereas the combination of copanlisib and lenvatinib significantly inhibited the phosphorylation of PI3K and AKT (Figure 5G). Moreover, the addition of gefitinib or copanlisib increased the levels of apoptosis-associated proteins, including caspase-3 and Bax (Supplementary Figure 1). Here, we proposed that upon targeting cell membrane RTKs, including VEGFR, FGFR, RET, PDGFR, and c-KIT, with lenvatinib, EGFR was activated to compensate for LR. The downstream MEK/ERK and PI3K/AKT pathways were then activated in response to EGFR activation, which resulted in LR by promoting cell proliferation and survival. However, upon targeting EGFR with gefitinib or PI3K with copanlisib in combination with lenvatinib, compensatory activation of the EGFR signalling pathway or its downstream PI3K/AKT pathway, respectively, was inhibited (Figure 5H).

## 3.5 *In vivo* antitumour effect of combined treatments

The *in vivo* antitumour effects of lenvatinib in combination with elacridar or gefitinib were assessed using xenografts derived from the Huh7 LR cell line. One week after tumour cell injection, the average xenograft size reached approximately 6 mm in diameter, and therapeutic treatment was initiated accordingly. Subsequently, the tumour volume and mouse body weight were

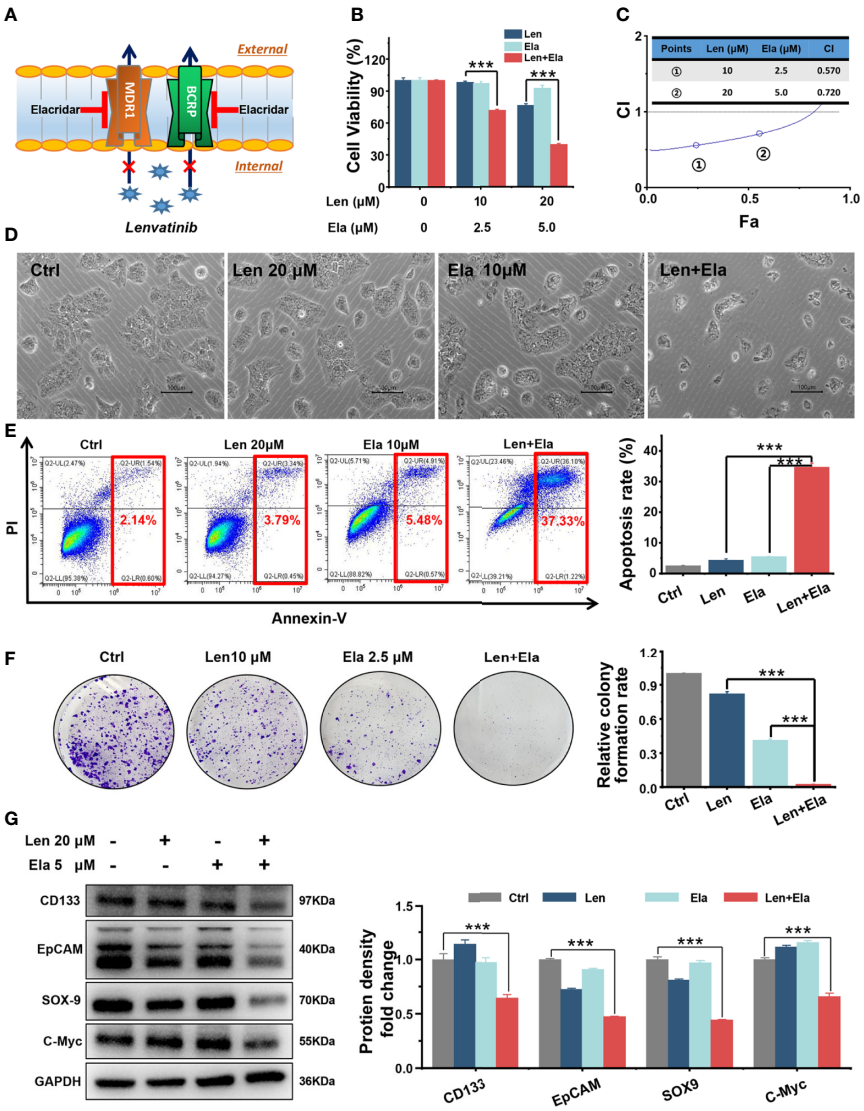


FIGURE 4
*In vitro* combined antitumour effect of lenvatinib and elacridar. **(A)** Schematic diagram indicates that elacridar dually inhibited MDR1 and BCRP. **(B, C)** The MTT results and CI plots confirmed that elacridar synergised with lenvatinib to inhibit Huh7 LR cell viability. **(D, E)** Co-treatment with elacridar and lenvatinib enhanced cell apoptosis, as shown in micrographs and flow cytometry plots. **(F, G)** Combined elacridar and lenvatinib treatment inhibited colony formation and downregulated CD133, EpCAM, SOX-9, and c-Myc expression. ***$p < 0.001$.

measured once every two to three days. The mice were orally administered with the drugs for two weeks and then sacrificed. The harvested tumours were imaged and their corresponding weights were measured. Compared with that in the vehicle group, neither elacridar nor gefitinib inhibited tumour growth, and lenvatinib-based co-treatments significantly suppressed tumour growth (Figures 6A–C). Intriguingly, lenvatinib-based co-treatments exerted a much better antitumour effect than lenvatinib treatment alone did. Particularly, the co-administration of lenvatinib with elacridar exhibited the most potent antitumour efficacy (Figures 6A–C). During the treatment, no significant side effects were observed, as the mouse body weights were comparable in different groups (Figure 6D).

Regarding the histological analysis, lenvatinib alone and lenvatinib-based co-administrations significantly inhibited tumour angiogenesis, which could be easily determined by observing the general shape of the harvested tumours (Figure 6B). Upon further analysis using pathological H&E staining, remaining tumour micro-vessels were observed in the group treated with lenvatinib alone, but not in the group treated with co-administration of gefitinib or co-administration of

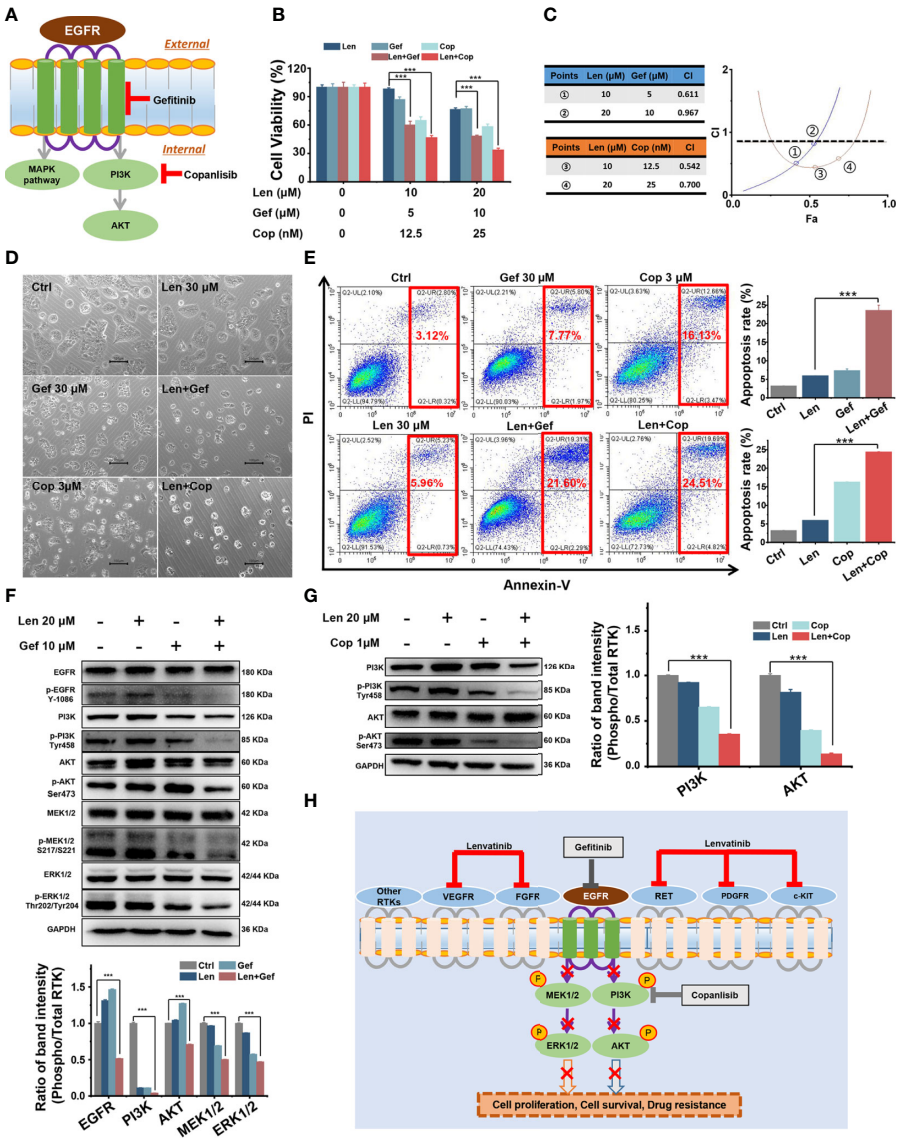


FIGURE 5
*In vitro* combined antitumour effect of lenvatinib and gefitinib or copanlisib. **(A)** Schematic diagram indicates that gefitinib and copanlisib targeted EGFR and PI3K, respectively. **(B, C)** The MTT results and CI plots confirmed that combined treatments synergistically inhibited cell viability. **(D, E)** The addition of gefitinib or copanlisib enhanced cell apoptosis, as shown in micrographs and flow cytometry plots. **(F, G)** The EGFR pathway was significantly inhibited by the addition of gefitinib, and the PI3K/AKT pathway was significantly inhibited by the addition of copanlisib. **(H)** Schematic diagram delineates the proposed mechanism of overcoming LR in HCC by targeting the EGFR/PI3K pathway. ***$p < 0.001$.

elacridar (Figure 7A). Lenvatinib alone could inhibit cell proliferation; however, lenvatinib-based co-administration enhanced the inhibition of cell proliferation, which was assessed using IHC for Ki67 and PCNA (Figures 7B, C).

## 4 Discussion

Systemic therapies for unresectable HCC are limited. In addition to sorafenib, lenvatinib is currently the first-line treatment for patients with advanced HCC worldwide (6). Recently, a meta-analysis comprising five clinical studies with 1,481 patients demonstrated that lenvatinib treatment significantly improved progression-free survival (PFS), objective response rate (ORR), and disease control rate compared with those of sorafenib treatment in patients with advanced HCC (22). However, a standard salvage treatment has not yet been established for patients with advanced HCC after lenvatinib therapy failure. Considering the dismal outcomes of patients with HCC after lenvatinib treatment failure, exploring

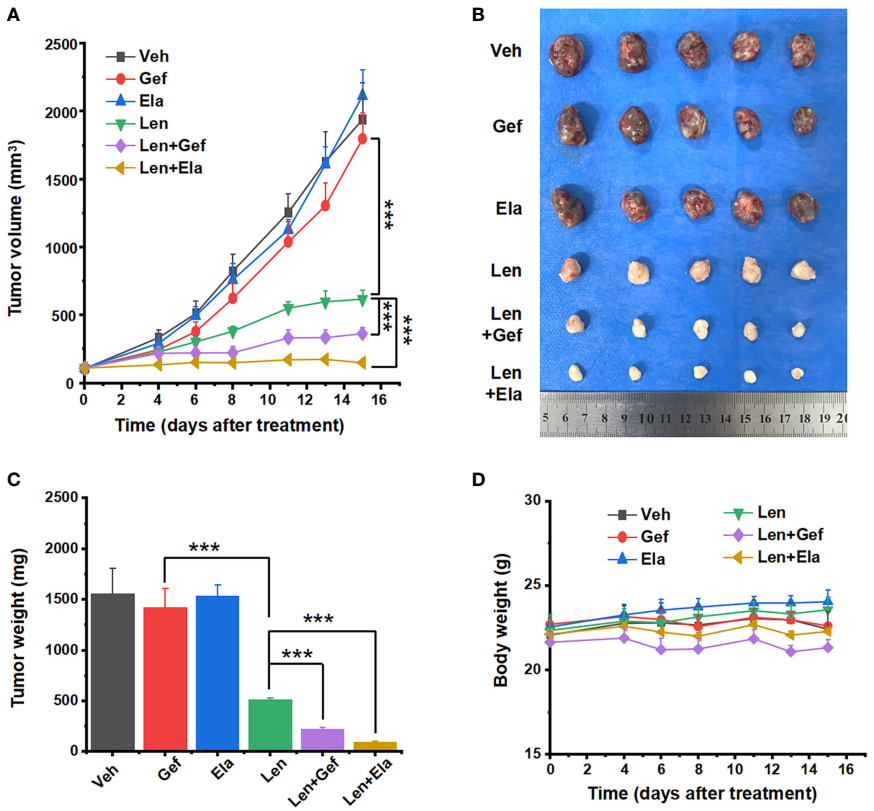


FIGURE 6
Lenvatinib in combination with elacridar or gefitinib suppressed HCC xenograft growth. **(A)** Xenograft response to treatment with vehicle, elacridar (80 mg/kg), gefitinib (80 mg/kg), lenvatinib (5 mg/kg), and drug combination (elacridar 80 mg/kg and lenvatinib 5 mg/kg or gefitinib 80 mg/kg and lenvatinib 5 mg/kg). **(B)** Harvested tumours are arranged according to the treatment group. **(C)** Tumour weights were measured after resection. **(D)** Mouse body weights were measured during the treatment. ***$p < 0.001$.

the underlying mechanism of LR and novel drugs to overcome LR is warranted.

To the best of our knowledge, this is the first study to reveal that ABC transporters and the EGFR signalling pathway are activated in HCC after long-term exposure to lenvatinib. MDR1 or P-glycoprotein and BCRP, important ABC transporters, have consistently been implicated in mediating multiple drug resistance by promoting drug efflux in various human cancers (16–18). Coincidentally, lenvatinib is a substrate for MDR1 (23, 24); however, the changes in MDR1 and BCRP transporters after LR induction have not yet been clarified. Moreover, EGFR, a pioneer member of the RTK family, is frequently overexpressed in human cancers (13, 25, 26), and its activation is crucial for essential cancer cell processes, including cell growth, survival, and drug resistance (25). Unfortunately, lenvatinib targets multiple cell membrane RTKs but EGFR (4). Recently, one study has revealed that blocking EGFR by gefitinib and lenvatinib exhibited a relatively potent antitumour efficacy in HCC (27), whereas the activation status of EGFR and its downstream pathways (MEK/ERK and PI3K/AKT) after LR induction in HCC has not been fully understood. Notably, our *in vitro* results revealed that MDR1 and BCRP transporters were significantly upregulated, and EGFR and the MEK/ERK and PI3K/AKT pathways were activated after LR induction.

Subsequently, considering that ABC transporters and EGFR signalling pathways were activated after LR induction, we utilised three drugs: elacridar, gefitinib, and copanlisib. Elacridar (GF12098) is a dual MDR1 and BCRP inhibitor (19). *In vitro*, preclinical, and clinical studies have demonstrated that co-administration of elacridar could reverse MDR1 and/or BCRP-mediated chemotherapeutic resistance and increase systemic exposure to antitumour drugs by inhibiting efflux pumps (19, 28, 29). Furthermore, gefitinib selectively inhibits EGFR and was first used to treat advanced non-small cell lung cancer after other treatments failed (30). As monotherapy or combination therapy, gefitinib is also used to treat other human malignancies (31). Moreover, gefitinib inhibits the growth and accelerates the apoptosis of human HCC cells and promotes cell cycle arrest in these cells (32). Blocking EGFR by gefitinib exerts antitumour effects by reducing HCC nodule formation in rats

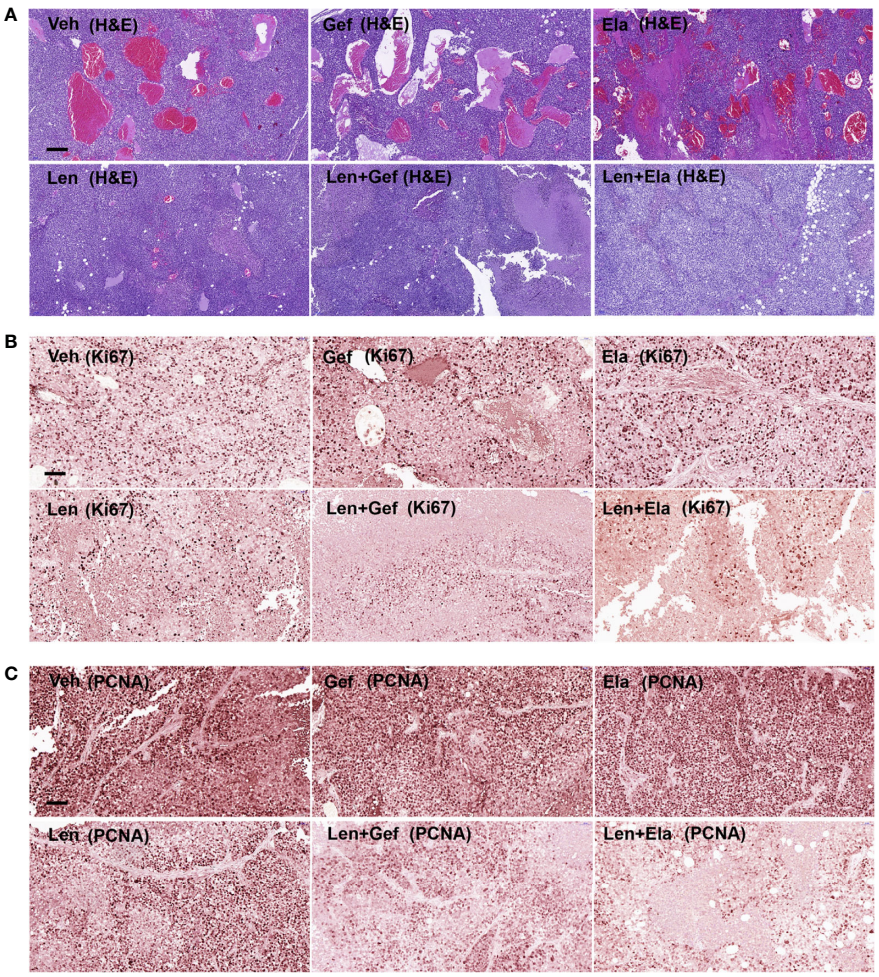


FIGURE 7
Lenvatinib in combination with elacridar or gefitinib inhibited tumour proliferation and angiogenesis. **(A)** Representative images of blood vessel density visualised by H&E staining. Scale bars, 200 μM. **(B, C)** IHC for Ki67 and PCNA expression. Scale bars, 100 μM.

(33). Lastly, copanlisib (BAY80-6946) has emerged as a newly developed pan-PI3K inhibitor (34, 35) and was first approved for treating relapsed follicular lymphoma (36). Subsequently, copanlisib has been used in patients with advanced or refractory solid tumours (37). *In vitro* studies have recently demonstrated that copanlisib synergises with sorafenib to promote cell death in HCC (38). However, the therapeutic role of these drugs in HCC after LR induction has not been reported.

Previous *in vivo* studies have demonstrated that lenvatinib is a substrate of MDR1, and inhibiting MDR1 using rifampicin or ketoconazole can significantly increase plasma lenvatinib concentrations in healthy adults (23, 24). Elacridar, a third-generation MDR1 inhibitor and a dual inhibitor of MDR1 and BCRP transporters (39), can improve therapeutic efficacy in various diseases by blocking drug efflux, according to previous *in vitro*, preclinical, and clinical studies (28). Theoretically, elacridar should also inhibit lenvatinib efflux by inhibiting MDR1 and BCRP efflux pumps. Additionally, cancer stem-like cells (CSCs) harbouring stem cell-like properties, including aberrant differentiation and self-renewal potential, are associated with chemotherapeutic resistance in cancers (40–42). Coincidentally, Sugano et al. found that inhibiting MDR1 using elacridar inhibits CSC properties (43). Parallelly, our *in vitro* experiments demonstrated that inhibiting lenvatinib efflux by inhibiting MDR1 and BCRP efflux pumps might represent the potential mechanism of synergism between elacridar and lenvatinib to overcome LR. Here, we attempted to summarise and explain the antitumour effect of combined treatment. Lenvatinib in combination with elacridar exerted a significantly synergistic antitumour effect *in vitro* and the most significant antitumour effect *in vivo*. Additionally, a combination of lenvatinib and elacridar significantly inhibited CSC properties by decreasing colony formation and downregulating CD133, EpCAM, SOX-9, and c-Myc expression. Moreover, lenvatinib

alone suppresses CSCs marked by CD133 and CD44 expression in HCC (44), and this inhibitory effect was presumably enhanced because elacridar could inhibit lenvatinib efflux by inhibiting MDR1 and BCRP transporters, which might account for the above findings. Furthermore, co-administration of lenvatinib and gefitinib significantly inhibited EGFR, MEK/ERK, and PI3K/AKT activation, and co-administration of lenvatinib with copanlisib significantly inhibited PI3K/AKT activation; both combinations exerted synergistic antitumour effects *in vitro*. Moreover, gefitinib in combination with lenvatinib exerted potent antitumour effects *in vivo*.

According to our literature review, other research groups are also attempting to develop salvage systemic treatment for patients with HCC after lenvatinib treatment failure. For example, one clinical study of 22 participants with failed lenvatinib therapy who received second-line regorafenib treatment revealed that the PFS and ORR were 3.2 (range, 1.5–4.9) months and 13.6%, respectively (45). Another clinical study involving 13 patients with unresectable HCC who were treated with sorafenib after lenvatinib treatment failure revealed that the PFS and ORR were 4.1 (range, 2.1–9.2) months and 15.3% (2/13), respectively (46). The survival outcomes were poor in patients who received second-line treatments after lenvatinib withdrawal. Coincidentally, our study found that xenografts grew faster and exhibited increased angiogenesis in the groups without lenvatinib treatment, including the gefitinib-treated group.

We proposed hypotheses regarding the dismal patient outcomes after stopping lenvatinib treatment and the increased xenograft growth observed in the groups without lenvatinib treatment. One hypothesis is that despite drug resistance, lenvatinib still blocked the intracellular signal transduction phosphorylation cascade by inhibiting ligand binding to cell membrane RTKs; in particular, the activation of VEGFR correlated with angiogenesis and the activation of PDGFR, FGFR, c-KIT, and RET correlated with cell proliferation (47, 48). Another hypothesis is that lenvatinib inhibited CSCs harbouring stem cell-like properties, including aberrant differentiation and self-renewal potential, which was verified by our *in vitro* experiments (lenvatinib inhibited colony formation) and the findings reported by Shigesawa et al. (lenvatinib inhibited CD133- and CD44-positive CSCs) (44). However, once lenvatinib is withdrawn, the underlying inhibition of intracellular signal transduction and of CSC-associated characteristics is reversed, which consequently accelerates tumour growth and angiogenesis. Therefore, we wondered whether patient outcomes would improve if lenvatinib was continuously administered in combination with second-line treatment after LR, which is merely our theoretical conjecture based on the xenograft experiment results. Certain issues, particularly the side effects and energy expenditure caused by combination treatment, remain to be seriously considered.

Finally, our study had certain limitations and provided scope for further research. First, our *in vitro* and *in vivo* results were based on a Huh7 LR cell line and the Huh7 LR cell line-derived xenografts. Thus, our findings and conclusions should be further replicated and verified in more cell lines, as well as in patients with HCC after LR induction, if possible. Second, some drugs (e.g., lenvatinib and gefitinib) used in this study are readily soluble in DMSO but not in cultivation media, which resulted in the parallelism of MTT results being lower than expected. Third, the antitumour effect of lenvatinib combined with copanlisib was not examined in the xenograft model. Therefore, the antitumour effects of copanlisib, an FDA-approved drug, alone or in combination with lenvatinib in HCC after LR induction should be investigated. Lastly, *in vivo* side effects of drug combinations, such as changes in organ function and/or microscopic structure, should also be assessed in the future.

## 5 Conclusions

In summary, inhibiting MDR1 and BCRP transporters or targeting the EGFR/PI3K pathway might overcome LR in HCC. Intriguingly, we observed the synergistic effects of lenvatinib and elacridar or gefitinib. Notably, lenvatinib should be used to treat HCC after LR because of its role in inhibiting tumour proliferation and angiogenesis. Importantly, our results and raised hypotheses should be further evaluated in patients with HCC following LR induction. Nevertheless, we provide a theoretical basis for the salvage treatment of HCC after LR induction.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found at https://www.ncbi.nlm.nih.gov/geo/, using the following accession numbers: GSE211850, GSM6503394, GSM6503395, GSM6503396, GSM6503397, GSM6503398, GSM6503399.

## Ethics statement

The animal study in this research was reviewed and approved by Charles River of Beijing (No. P2021049).

## Author contributions

JL, YW, and JD designed and supervised this research. DS performed the experiments, analysed the data, and wrote this paper. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.944537/full#supplementary-material

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# Hepatic macrophage mediated immune response in liver steatosis driven carcinogenesis

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Obesity confers an independent risk for carcinogenesis. Classically viewed as a genetic disease, owing to the discovery of tumor suppressors and oncogenes, genetic events alone are not sufficient to explain the progression and development of cancers. Tumor development is often associated with metabolic and immunological changes. In particular, obesity is found to significantly increase the mortality rate of liver cancer. As its role is not defined, a fundamental question is whether and how metabolic changes drive the development of cancer. In this review, we will dissect the current literature demonstrating that liver lipid dysfunction is a critical component driving the progression of cancer. We will discuss the involvement of inflammation in lipid dysfunction driven liver cancer development with a focus on the involvement of liver macrophages. We will first discuss the association of steatosis with liver cancer. This will be followed with a literature summary demonstrating the importance of inflammation and particularly macrophages in the progression of liver steatosis and highlighting the evidence that macrophages and macrophage produced inflammatory mediators are critical for liver cancer development. We will then discuss the specific inflammatory mediators and their roles in steatosis driven liver cancer development. Finally, we will summarize the molecular pattern (PAMP and DAMP) as well as lipid particle signals that are involved in the activation, infiltration and reprogramming of liver macrophages. We will also discuss some of the therapies that may interfere with lipid metabolism and also affect liver cancer development.

KEYWORDS

**macrophages, Kupffer Cells, steatosis, liver cancer, inflammation**

## Introduction

Metabolic disorders, particularly obesity increases the risk of a number of cancers, e.g. colon, mammary, pancreas, liver (1, 2), etc. Obesity, which occurs in half of the US population, is now recognized as a confounding factor for cancer-related death (3, 4). The contribution of lipid dysfunction to cancer is particularly high for liver cancer. The mortality risk for liver cancer is estimated to be 4.52-fold higher in men with >35 body mass index (BMI) compared with those with BMI <29 (1). Liver steatosis is a common comorbid disease for liver cancer and is associated with metabolic diseases including obesity, insulin resistance (IR), and diabetes as well as in other related disorders such as alcohol usage disorders (5). While hyperinsulinemia, hyperglycemia, and hyperlipidemia as a result of peripheral insulin resistance and metabolic disorder can directly contribute factors to promote tumorigenesis (6), the resulting development of liver steatosis due to these conditions directly establishes the microenvironment to promote tumor development. This review will focus on the local tumor microenvironment in liver steatosis for its role in promoting cancer development.

## Contribution of steatosis to liver cancer

In the liver, steatosis is defined when at least 5% of lipid droplets are accumulated among hepatocytes in the histopathological diagnosis (7, 8), and is classified as alcoholic or nonalcoholic forms due to etiology. Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) develop in patients with metabolic syndromes including obesity, IR and diabetes (9, 10) whereas alcoholic liver disease (ALD) and ASH (alcoholic steatohepatitis) are caused by excessive alcohol drinking which also contributes to lipid metabolic dysfunction (11, 12). While simple fatty liver is reversible by lifestyle changes, ASH and NASH can progress to more morbid forms of liver pathologies including fibrosis/cirrhosis and is highly associated with liver cancer (13).

Patients with varying degrees of steatosis are susceptible to hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), the two dominant forms of liver cancer. In particular, the NAFLD-HCC incidence ratio increased significantly (1.92-fold for men and 12.7-fold for women) in the last 20 years whereas it decreased or remain unchanged for many other major etiologies of HCC (14). This increase is concurrent with the increase of obesity epidemic particularly in women, suggesting a role of lipid dysfunction in liver carcinogenesis. Consistently, alcohol consumption and associated alcoholic liver disease was estimated to be an independent risk factor for poor disease-free survival, particularly in non-virus hepatitis associated HCC (15).

Earlier studies using chemical carcinogen to induce cancer formation found that high fat diet (HFD) feeding significantly induced cell proliferation in diethyl nitrosamine (DEN) induced HCC models (16). While this observation is supported by high fructose, high cholesterol and alcohol feeding studies (17–19), other experiments show that HFD protects against DEN induced liver injury, leading to reduced HCC (20, 21). Avoiding the complications of chemical induced injury, genetic models were used to explore liver cancer development. Liver cancer is highly heterogenous on the pathohistological levels as well as genetic landscape. In recent years, exome sequencing has led to the discovery of TERT, CTNNB1 and TP53 as the dominant mutations and PI3K/AKT/PTEN/mTOR together with MAPK pathway as the primary signaling pathways that promote liver cancer development together with Wnt/β-catenin signaling pathway (22, 23). Mutation of TERT1 promoter is found to be a primary characteristic of NAFLD associated liver cancer (24) and loss of telomerase promotes metabolic dysfunctions in hepatocytes (25). Activating mutation of CTNNB1 (encodes β-catenin) occurring in 37% of HCC is thought to support the growth and transformation of liver cancer stem cells (26–29). As such, activating mutation of CTNNB1 confers the oncogenic potential of β-catenin and promotes HCC development (26, 27). Interestingly, manipulation of neither TERT, CTNNB1 nor TP53 by themselves is sufficient to result in liver tumor development (25, 26, 30, 31). Activation of PI3K signaling pathway, however unequivocally resulted in the development of HCC and CCA. Activating PI3K/AKT signal *via* deletion of Pten showed spontaneous tumor development following steatosis and fibrosis (32–35). The PI3K/AKT signal upregulation results in increased lipid anabolic metabolism in addition to acting as a pro-growth and pro-survival signal (35–45). In the Pten deletion model, inhibiting steatosis attenuates or abolishes tumor development, suggesting that steatosis is required for liver tumor development (32, 33), whereas short term feeding of HFD accelerates the development of tumors (46). The PI3K/AKT signal is necessary for driving the steatosis phenotypes in the liver (35, 37). As such, introduction of activated AKT delivered through hydrodynamic injection of myristylated AKT is necessary to drive the development of HCC and CCA for a number of signals including Notch, YAP, Shp2, Hippo and others (28, 47–49). Consistent with this notion, combining other genetic models with non-genotoxic chemicals and diet manipulations demonstrated that liver injury and steatosis promotes the development of tumors (28, 31–33, 50–52). In several mouse models including those lacking p53 and Indian Hedgehog, consumption of a Western-style diet, or a high-fat/high-cholesterol diet to the point of developing hepatic steatosis was shown to promote higher liver tumor incidence than the control diet group (53, 54).

In these genetic models where HFD feeding accelerates/promotes tumorigenesis, liver injury is a main consequence associated with steatosis (33, 46, 55). In fact, the effect of p53

on hepatocytes apoptosis may have contributed to the lack of tumorigenic effects observed in p53 deletion mice (31, 51) as p53 deficiency protected hepatocytes from undergoing apoptosis in response to HFD feeding and subsequent liver injury (56). Similarly, while activated β-catenin mutation is capable of promoting hepatocyte regeneration, the genotoxic effect requires steatosis and/or liver injury to promote liver cancer development (26, 28, 30). In fact, the function of β-catenin in sustaining normal hepatocyte function explains how β-catenin loss also promotes a protumor environment (57, 58). Deletion of β-catenin leads to loss of liver zonation, resulting in spontaneous repopulation of β-catenin+ cells due to hepatocyte death associated with loss of zonation. The death of hepatocytes also leads to cancer development from the β-catenin+ cells when genotoxic chemicals are introduced (29, 58, 59). Similar to chemical induced injury, steatotic injury has been shown to induce Wnt signal in the liver and elsewhere (60–63). Together, these studies suggest that liver steatosis establishes a microenvironment that promotes the growth of liver cancer cells and permits the expansion of any initial genotoxic events to develop into tumors (Figure 1).

## Macrophage response to steatotic liver injury: A double-edged sword in liver carcinogenesis

The liver is known as an immunosuppressive organ as illustrated by the lower dose of immunosuppressive therapy needed for liver transplantation as compared with other organ transplantations (64, 65). Liver macrophages play a critical role in this process. There are 2 basic populations of macrophages in the liver, the local proliferating Kupffer cells and the infiltrating monocyte derived macrophages (66). Kupffer cells are located within the liver sinusoids and play surveillance functions by monitoring pathogens coming into the liver. Being the largest tissue resident macrophage population in the body, Kupffer cells are the first responders in liver immune system. Unlike monocyte derived macrophages, Kupffer cells are highly effective in binding and clearing *Escherichia coli (E. coli)* brought in *via* the portal circulation (67).

During homeostasis, Kupffer cells, but not monocyte derived macrophages, present antigens to induce immune tolerance through expansion of select regulatory T-cells and inhibition of T cytotoxic lymphocytes and to induce apoptosis in other T-cells (64, 68). In response to inflammation that cannot be cleared by Kupffer cells alone including those induced by pathogens and injury, inflammatory mediators released by Kupffer cells also recruit other inflammatory cells including monocytes-derived macrophages and neutrophils in addition to subsets of CD4+ and CD8+ T lymphocytes and NK/NKT cells (Figure 2). In particular, neutrophils, being the most abundant leukocytes in circulation are the first responders to acute inflammation to clear pathogens and damaged/dying cells. Similar to macrophages, neutrophils are highly enriched in the steatotic livers and depletion of neutrophils protects mice from experimentally induced steatohepatitis (69). Together, these infiltrating immune cells crosstalk with macrophages to clear pathogens

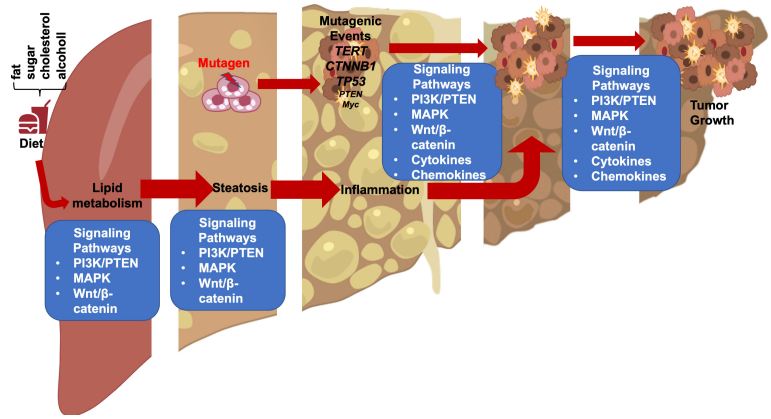


FIGURE 1
Steatotic Liver Damage Establishes a Tumor Microenvironment. The primary functions of the liver are metabolism and detoxication. Nutrients from the gut are metabolized in the liver involving the insulin regulated PI3K/PTEN pathway. Wnt/β-catenin signaling also plays important role in regulating the metabolic and detoxicating functions of the liver as it regulates liver structure and zonation. Following a diet containing high fat, sugar, cholesterol, or alcohol, activation of these signals results steatosis. The consequence cell death due to steatosis and loss of liver structure leads to inflammatory cell infiltration. Inflammatory mediators produced due to liver inflammation propagate any genotoxic events as the induce the proliferation of tumor initiating cells that carry mutations of *TERT, CTNNB1, TP53* and to a lesser extend *PTEN* and *MYC* as well as others. The Wnt/β-catenin, PI3K/PTEN and MAPK signaling pathways as well as cytokine and chemokine are all implicated in the proliferation of the tumor cells and play roles in propagating the initial mutagenic events.

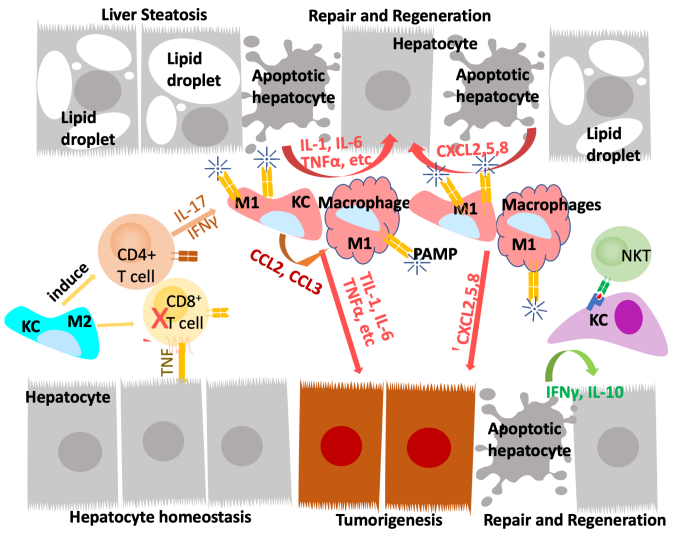


FIGURE 2
Innate immune system regulate liver repair and tumorigenesis due to steatosis. The liver developed a unique immune response system that tolerate gut bacterial-induced inflammation while eliminating them at the same time. During hepatocyte homeostasis (grey cells), Kupffer cells (KC) binds to and eliminate gut bacterial while producing anti-inflammatory cytokines to inhibit CD8+ cytotoxic T cells and induce their apoptosis. At the same time, Kupffer cells also induce antigen specific CD4+ Treg cells to assume tissue repair functions. In response to chronic injury presented in NAFLD and liver steatosis (grey cells with lipid droplets), Interleukine-17 (IL-17) and interferon g (IFNγ) produce from Tregs recruit monocyte derived macrophages as well as activating a M1 proinflammatory program in Kupffer cells. The proinflammatory cytokines produced by these M1 macrophages/Kupffer cells including IL-1, IL-6, TNFα, etc induces hepatocyte proliferation to repair the damaged tissues and replace apoptotic hepatocytes due to steatosis. These proinflammatory cytokines also establishes a pro-tumor microenvironment as they propagate any genotoxic events that are present in the tumor initiating cells (orange cells). The M1 macrophage/Kupffer cells also produces chemokines to promote hepatocyte and tumor cell proliferation, leading to tissue repair and/or tumorigenesis.

and damaged tissues. In chronic injury conditions such as those presented by ALD and NAFLD, these inflammatory cells establish an environment that is pro-tissue repair and returning to homeostasis on the one hand; and pro-tumor growth when genotoxic events are present on the other. Recognition of damaged hepatocyte-released molecules by macrophages is important in the propagation of the signals and the sustained inflammatory response (see later section). Interaction of macrophages with other cell types such as cholangiocytes and hepatic stellate cells are also important in the disease progression and the establishment of the tumor microenvironment. This review focuses on the role of macrophages and macrophage produced inflammatory mediators. The interactions of macrophages with other inflammatory cells and their function in the tumor immune environment is also important for liver cancer development (70).

## Steatotic liver damage establishes a pro-inflammatory tissue microenvironment

Early studies showed that administration of liver toxicants such as carbon tetrachloride (CCL4) and 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) provoke the growth and infiltration of macrophages in the liver (71, 72). In patient samples, macrophages have been observed to be recruited to the NASH livers (73). These macrophages play roles in the disease progression of steatosis by producing inflammatory factors that sustain injury (6, 65, 74). In B6 mice fed HFD to induce NAFLD, infiltration of immature macrophages that are $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ are observed. These macrophages are more readily able to produce proinflammatory cytokines than those from the lean mice controls (75). In mice fed methionine-choline deficient (MCD) diet to induce NASH, induction of macrophage proinflammatory genes is found to associate with more progressive fibrosis (76). Depletion of macrophages using liposomes to deliver clodronate led to reduced expression of proinflammatory genes and attenuated the progression to NASH and fibrosis in mouse models (32, 77). In genetic models where cytokine signals are manipulated, infiltration of macrophages are also found to prolong liver injury (78). Deletion of *Ccl2* (C-C motif chemokine ligands 2), a chemokine that recruits monocytes to the liver, results in reduced liver damage and fibrosis (76). Treatment with a dual antagonist for CCR2 and CCR5, receptors for CCL2 and CCL5, significantly reduced macrophages and protected rats from liver injury in a diet induced NASH model (79) and has shown promising effects

for NASH in phase II clinical trial (80). Inhibiting activation of Kupffer cells and infiltration of monocytes by deletion of proinflammatory receptor Trem-1 also significantly attenuated liver inflammation, injury and liver fibrosis induced by CCL4 treatment (81). Adoptive transfer of Trem1-sufficient Kupffer cells led to reactivated inflammation and injury, suggesting that the presence of Trem1-sufficient Kupffer cells can sustain chronic inflammation. In both CCL4 induced liver injury and MCD feeding induced NASH mice, pharmacological inhibition, or deficiency of monocyte chemoattractant protein (MCP-1 or CCL2) led to reduced liver injury and inflammation (76, 82). Together, inflammation is thought to be a crucial phase for the disease progression of NAFLD and the role of macrophages appear to be important in this progression.

## Steatosis induced inflammation establishes a pro-tumor microenvironment

Chronic injury and the associated inflammatory responses are a major link between liver steatosis and cancer development. The development of liver cancer is a slow process that evolves from premalignant lesions developed within chronically damaged livers (83). In chemical induced hepatocarcinogenesis, HFD feeding promotes hepatic inflammation and exacerbates tumor development (84). In HCC mice induced by transgenic expression of hepatitis C virus core protein, HFD feeding to induce liver steatosis significantly increased tumor incidence (85). In these mice, the toll-like receptor (TLR) signal involved in innate immune response was found to promote the transformation of liver tumor initiating cells (86). In mice lacking p53 and concurrent expression of c-Myc, T cell mediated immune surveillance was found to reduce tumor formation and increase survival. This tumor surveillance is overcome when the β-catenin pathway is induced by exogenous expression of active β-catenin, further confirming that β-catenin signal sustains tumor growth (87). In the *Pten* deletion model, steatosis is required for tumor growth and is accompanied by inflammation and induction of β-catenin (33, 88). It was discovered that depletion of macrophages reduces Wnt/β-catenin signals and attenuates tumor growth (32, 89). Together, these studies suggest steatosis establishes an inflammatory environment that is pro-tumor growth.

Infiltration and reprogramming of macrophages are observed in essentially all experimental models and HCC patients. In HFD fed mice where tumors are initiated by DEN treatment, macrophage recruitment accompanied chronic liver injury and liver cancer development (84). In genetic models of NAFLD-NASH-liver cancer, macrophages also play a dominant role in promoting liver cancer development. Depletion of macrophages resulted in reduced tumor incidence in the *Pten* deletion mice (32) and this was thought to involve TLR signaling (90). Together, this evidence suggests that while macrophages can produce pro-repair cytokines, sustained presence of macrophages can prolong liver injury and result in further liver damage.

During liver repair in response to injury, liver macrophages, particularly Kupffer cells are credited in producing pro-mitogenic cytokines to induce the growth of liver progenitor cells and promote liver regeneration (91–93). Depletion of macrophages attenuates tissue repair and resulted in exacerbated fibrogenic phenotype (92) and also led to delayed recovery of metabolic functions performed by the liver (94). When inflammation is not resolved, the signals produced by macrophages exacerbate liver injury and lead to chronic inflammatory conditions and sustain the production of proinflammatory cytokines (6, 65). During liver tumorigenesis, the chronic inflammatory condition and proinflammatory cytokines promote tumorigenesis by providing the tumor microenvironment as well as signaling the growth and promoting the proliferation of tumor initiating cells (91). High fat diet feeding induces macrophage production of a number of inflammatory factors and cytokines including interleukins, C-C ligands (CCLs), interferon γ (IFNγ) and tumor necrosis factor α (TNFα) to facilitate hepatocyte proliferation (84). Cytokines produced by these resident as well as infiltrating macrophages such as TNFα, transforming growth factor β (TGF-β), interleukin 6 (IL-6) and 18 (IL-18) are highly associated with the development and progression of hepatocellular carcinoma (HCC). In a mouse tumor model established by subcutaneous transfer of DEN-initiated liver tumor initiating cells, depletion of macrophages attenuated the progenitor cell properties and reduced tumor development (95). The presence of macrophage-produced TNFα also triggers chromosomal instability in liver tumor initiating cells, permitting propagation of genotoxic events leading to tumorigenesis (95). TNFα produced by macrophages are also proposed to promote the proliferation of liver cancer initiating cells (96). These tumor initiating cells were found to display similar transcriptome profiles as the ov-6 positive liver progenitors that express LIN-28 (97). The expression of LIN-28 allows these cells to respond to the interleukin-6 (IL-6) signal to proliferate. In MCD diet fed mice, macrophage reprograming also contributed to the proliferation of liver progenitors and promoted HCC proliferation (98). In tumors induced by expression of Myc and deletion of TP53, upregulation of β-catenin promoted immune escape of the tumors involving defective recruitment of myeloid lineage cells that include macrophages (87). As a potential driver mutation gene, activation of β-catenin is associated with liver tumor initiation (27, 48). In the *Pten* deleted NAFLD-NASH-Tumor mice, β-catenin was found necessary to sustain the growth of liver tumor initiating cells as deletion of β-catenin attenuated their growth (32, 33, 88). Depletion of macrophages suppressed Wnt/β-catenin signal and led to reduced tumor burden in these mice. TLR4 was found to

play a role in the macrophage-promoted proliferation of tumor initiating cells and tumorigenesis in these mice (90). Together, these data suggest that macrophages may be necessary to both sustain tumor initiating cell proliferation as well as establishing the liver injury environment that allows the tumors to grow.

## Cytokines in steatosis driven HCC

Inflammatory cytokines play key roles in the communication between macrophages with surrounding cell types and also reprograming macrophages to different spectrums of polarizations under given stimulatory conditions, resulting in high heterogeneity of liver macrophages (99). Beyond proliferation of resident Kupffer cells and infiltration of monocyte-derived macrophages, hepatic macrophages are also stimulated or "reprogrammed" to produce a variety of pro- and anti-inflammatory cytokines that classify them on the spectrums of M1 vs. M2 polarization (Figure 3). During steatosis driven liver cancer development, a complex interaction of anti- and pro-inflammatory cytokines promotes cell proliferation and activation of HCC progenitor cells and results in cancer promotion (95, 97). Like macrophages themselves, these cytokines play dual roles in liver cancer development by 1) promoting proliferation of cancer cells and 2) exacerbating liver injury to produce a protumor microenvironment.

## Proinflammatory cytokines

Several proinflammatory cytokines appear to be induced during the development and progression from steatosis to liver injury to cancer (71, 75, 90, 100–106). In patients with chronic inflammatory and fibrotic liver diseases, analysis of classical $CD14^{++}CD16^{-}$ monocytes in the liver found that they express both macrophage and dendritic cell markers with a high capacity for phagocytosis, antigen presentation, and regulatory T cell proliferation (103). They also secrete proinflammatory cytokines including TNFα, IL-6, IL-8 as well as IL-1 consistent with a role in the wound healing response where proinflammatory cytokines induce hepatocyte proliferation for tissue repair. In mice fed a Western diet, tumor progression is associated with a predominant M1 proinflammatory cytokine vs. the M2 pattern (83). In ALD, severe liver damage is also accompanied by significantly elevated M1 proinflammatory macrophage marker expression in C57Bl/6 mice, whereas less damage is observed in Balb/c mice where no change of M1 markers is

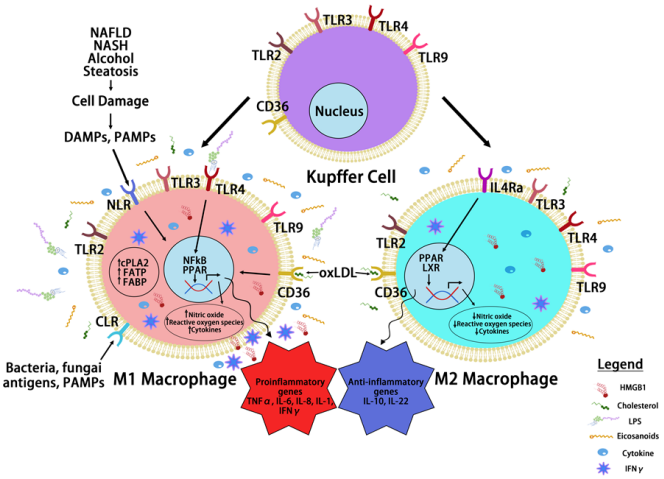


FIGURE 3
Macrophage Reprogramming in Steatosis Driven HCC. During liver inflammation, Kupffer cells and macrophages express scavenger receptors (SR) and pattern recognition receptors (PRR) to respond to pathogens and liver damages. Activation of PRR receptors by pathogen activated molecular pattern (PAMP) and damage activated molecular pattern (DAMP) molecules reprograms hepatic macrophages to produce inflammatory cytokines/chemokines. The binding of PRRs and SRs to steatotic induced PAMP and DAMPs reprograms hepatocyte macrophages. The reprogramed M1 macrophages produce a proinflammatory cytokines where the reprogrammed M2 macrophages produce anti-inflammatory cytokines to mediate the progression of steatosis to cancer. Toll like receptor (TLRs) and NOD-like receptors (NLRs) are two common PRRs used by PAMP and DAMP to induce macrophage reprogramming. Cluster of differentiation 36 (CD36) belongs to SR family of receptors and binds to oxidized LDL. Other PRR receptors include the C-type lectin receptors (CLRs) is also expressed on the reprogrammed macrophages. Binding of these receptors to their ligands such as lipopolysaccharide (LPS), high mobility group box 1 (HMGB1) and oxidized low density lipoprotein (oxLDL) activates the innate immune response and produce cytokines and chemokines that play important roles in tumorigenesis. It also activates nuclear factor kappa B (NF-κB) and proliferator-activated receptor (PPAR) and other liver nuclear receptors such as liver X receptor (LXR) regulates transcriptional reprograming of these macrophages.

found (104). In morbidly obese patients with NAFLD, reduced liver M2 anti-inflammatory macrophage marker expression (increased M1/M2 ratio) is associated with more severe steatosis. This reduced M2 macrophage phenotype also correlated with increased hepatocyte cell death and elevated serum levels of alanine aminotransferase (ALT), a clinical index of liver injury (107). Together, the proinflammatory cytokines secreted by macrophages in steatotic liver establishes a pro-inflammatory tissue microenvironment that can promote further liver damage and sustained inflammation.

One of the proinflammatory cytokines produced with steatosis, TNFα, plays a key role in liver carcinogenesis (16, 108). TNFα is a pleiotropic cytokine produced by many cell types with monocyte lineage cells being the primary source. In the liver, both Kupffer cells and infiltrating monocytes can produce TNFα in response to stimulation. TNFα produced by macrophages was found to promote cancer cell sphere formation *in vitro* (95). In this study, TNFα enhances the self-renewal abilities of the cancer cells. Consistently, in MUP-uPA mice fed with HFD, development of NASH and HCC are dependent on macrophage secreted TNFα. Knocking down TNFα Receptor 1 (TNFR1) significantly reduced liver damage and tumor formation (109). NFκB signal is implicated in this TNFR1 mediated hepatocyte death as deletion of IKKβ or NEMO, two NFκB signal modulators resulted in spontaneous progression of TNFα mediated hepatitis to cancer (110). In a DEN induced tumor model, deletion, or inhibition of TNFα resulted in reduced tumor incidence accompanied by suppressed activation and proliferation of hepatic progenitors *via* the TNFR2-STAT3 pathway (111). Consistent with a role of TNFα in liver regeneration, hepatocyte growth is also inhibited, resulting in a shorter lifespan even though tumor burden was reduced. In CCA, this effect of TNFα signal in chronic liver injury was shown to be mediated by JNK signaling and involves mitochondrial reactive oxygen species (ROS) production (112).

It was determined that hepatic IL-6 expression is significantly increased in the livers of patients with NASH (113). IL-6 signals through two pathways on target cell: classical signaling involves IL-6 binding to its receptor IL-6R on target cells. In the absence of IL-6R, IL-6 trans-signaling is induced, which involves an IL-6 binding to cleaved and soluble IL-6R provided by surrounding cells (114). During hepatocellular carcinogenesis, IL-6 trans-signaling pathway, rather than the IL-6 classic signaling contributes to the development of tumors by enhancing tumor proliferation through STAT3 and β-catenin activation and stimulating endothelial cell proliferation to promote tumor angiogenesis (115). Furthermore, IL-6 induces pre-cancerous progenitor cell proliferation and transformation into tumor initiating cells (97). IL-6 treatment *in vitro* led to early S phase entry in H4IIE HCC cells as shown by the reduced G0/G1 phase after treatment (116). IL-6 also contributes to the drastically different HCC incidence in male vs female mice treated with DEN (117). Recruitment of tumor-associated macrophages by the Yes-associated protein YAP, an oncogene overexpressed in a subset of HCC patients, also involves IL-6 signaling (118). Similar to the role of TNFα, IL-6 signals through STAT3 protect from chronic liver injury. However, the role of IL-6 in liver injury and tumorigenesis is also context dependent as IL-6 also protects from liver injury by promoting hepatocyte regeneration. In the multidrug-resistant gene 2 knockout ($Mdr2^{-/-}$) mice where 50% of the mice develop tumors after chronic injury, IL-6 signal deficiency led to more severe steatosis and inflammation presumably due to the inability of hepatocyte regeneration/increased hepatocyte apoptosis after injury (101). Regardless, the resulting infiltration of macrophages promoted tumor growth and led to increased tumor burden (101, 119).

Other proinflammatory cytokines including IL-1, IL-8, IL-17, IL-18 and IFNγ may also be produced by macrophages to play similar roles in liver regeneration and sustain tumor cell growth. Like IL-6 and TNFα, IL-1 is commonly induced in steatotic livers when macrophage proliferation and infiltration are induced (17, 77, 84, 120) and is necessary for the whole spectrum of pathologies associated with steatosis, injury and cancer (104, 106). The expression of C-X-C receptor 2 (CXCR2), a receptor for IL-8, is upregulated in both HCC and iCCA. Targeting inhibition of CXCR2 results in reduced proliferation in Huh7 and HepG2 cells (121). The induction of liver IL-8 also provides signals for breast cancer cells to escape dormancy when they metastasize to the liver, suggesting that IL-8 indeed establishes a protumor environment in the liver (122). Blockade of IL-17 was shown to protect from liver injury including injuries induced due to steatosis (123). While macrophages may or may not be the primary source of IL-17 (124, 125), IL-17 does induce hepatic macrophage production of IL-6 and TNFα (126, 127). IL-18 is produced by THP-1 macrophages together with IL-1 in cultures exposed to hepatitis C virus (128). In the liver, administration of recombinant IL-18 induces severe liver injury concurrent with induced IFNγ secretion from NK cells (129). Delivery of neutralizing antibody targeting IL-18 reduced serum ALT levels and liver inflammation. Together, the proinflammatory cytokines produced by Kupffer cells and infiltrating monocyte derived macrophages establishes a sustained inflammatory environment to promote the growth of hepatocytes. This proinflammatory environment also acts on tumor initiating cells to propagate the genotoxic events, leading to tumor development.

## Anti-inflammatory cytokines

Macrophage polarization was defined by IL-1β/iNOS producing macrophages as M1 and Arg-1/IL-10 expressing macrophages as M2 phenotypes. As the defining M2 cytokine, IL-10 is one of the best documented anti-inflammatory cytokines. In HFD feeding or alcohol induced liver injury, IL-

10 is also induced (84, 108). It was proposed that the Kupffer cell production of IL-10 is also pro-regeneration and pro-survival for the hepatocytes (130). During the initial stage of chronic liver damage, liver macrophages also express C-X-C Ligand16 (CXCL16) to recruit NKT cells (131). This results in the formation of NKT and Kupffer cell clusters during liver steatosis. Clustered NKT-Kupffer cells secrete IFNγ and IL-10 (23). Another IL-10 family of cytokine, IL-22 also plays a role in NASH driven hepatocarcinogenesis. IL-22 levels gradually increase 5 months after the start of DEN treatment. It was concluded that continuous activation of STAT3 and CyclinD1 sustained IL-22 promoted cell proliferation (132). More recently, metformin, the antidiabetic drug was found to promote cell apoptosis through activation of Hippo signaling and to inhibit IL-22 induced tumor cell proliferation and invasion (133).

## Chemokines

Chemokines are released by Kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells to recruit infiltrating immune cells (134). Chemokine levels and their receptors are elevated in tissue and blood samples from patients with NASH and HCC compared with healthy and non-tumor controls (135–142). Among the two primary groups of chemokines, CC chemokines (CCLs) are known for their ability to recruit monocytes and lymphocytes, while CXC chemokines (CXCLs) are potent neutrophil attractants and can promote angiogenesis (143).

Upon ligand binding, Kupffer cells release CCL2 to recruit monocytes (144). In *Ccl2* deletion mice, reduced inflammatory cell infiltration is observed (76). Inhibition of CCL2 with an RNA oligonucleotide that binds to CCL2 or neutralizing antibody for CCL2 led to reduced monocyte chemotaxis and reduced macrophage infiltration into the liver (82). These treatments resulted in reduced production of TNFα and IL-6, two macrophage produced cytokines. In NAFLD and NASH livers, macrophages also upregulate CCL3 and this induction of CCL3 facilitates macrophage infiltration and production of proinflammatory cytokines (135).

Kupffer cells also release CXCL1,CXCL2 and CXCL8 to recruit neutrophils (144). In HFD+Alcohol induced liver steatohepatitis, blockage of CXCL1 was found to reduce hepatic neutrophil infiltration and significantly inhibit liver injury (145). CXCL2 induction was shown to play a pivotal role in the recruitment of neutrophils in ConA induced hepatitis (146). In cholestatic patients, upregulation of CXCL8 and its receptors CXCR1/2 is associated with neutrophil infiltration whereas macrophage infiltration is associated with CXCL8 signal upregulation in non-cholestatic patients (147). This upregulation of CXCL8 signal plays important roles in the tumor microenvironment (122, 142). Furthermore, the macrophage derived CXCL9 and 10 are required for immune checkpoint therapy to block the infiltration of CD8+ T cells (148). The release of CXCL10 from macrophages is induced by steatosis (149) and deficiency of macrophage lipid receptor CD36 led to reduced release of CXCL10 in the liver (150). The role of CXCL5 in steatosis and liver cancer has drawn attention recently (151). Hepatic CXCL5 expression was higher in patients with severe fibrosis and cirrhosis (141). Multivariate Cox analysis of TCGA data identified that among 110 differentially expressed genes that were associated with HCC overall survival, CXCL5 and IL18RAP were the only 2 genes that predict the prognosis independently (142).

# Macrophage reprogramming in steatosis driven HCC

The crosstalk of macrophage with hepatocytes is crucial for sustaining inflammatory signals during liver injury. In normal livers, macrophages contribute to normal hepatocytes function by regulating glucocorticoid signals (152). During liver inflammation, Kupffer cells and infiltrating macrophages express scavenger (SR) and pattern recognition receptors (PRR) to readily respond to pathogens and liver damage (153). Activation of PRR receptors by pathogen activated molecular pattern (PAMP) and damage activated molecular pattern (DAMP) molecules produced primarily by hepatocytes reprogram hepatic macrophages to produce inflammatory cytokines/chemokines that reverse the immune-suppressive liver environment and facilitate tissue repair (154). Scavenger receptors (SRs) are defined as macrophage receptors for modified lipids in foam cell formation but can also bind to other bioactive ligands (155). While binding of PRRs to ligands induces the release of pro- and anti-inflammatory cytokines and chemokines, uptake of modified lipids *via* SRs also leads to removal of the pathogen/damaged cells that present the recognized molecular patterns in addition to releasing inflammatory mediators (Figure 4).

## Pattern recognition receptors

Macrophages possess a number of different receptors that recognize intracellular and extracellular PAMPs and DAMPs as well as membrane bound ligands. This includes the TLR family of membrane receptors that play key roles in both innate and adaptive immune response. A cytosolic nucleotide-binding domain and leucine-rich repeat containing receptors (NOD-like receptors, NLRs) is another super family of PRR that is responsible for inflammasome activation which is essential for a successful immune response. The C-type receptors (CLRs) at the cell membrane recognize foreign antigens including bacterial and fungal antigens. Other PRRs including the 5'-triphosphate-RNA and dsRNA RIG-I-like receptors, as well as several DNA cytosolic sensors are also expressed in the liver microenvironment.

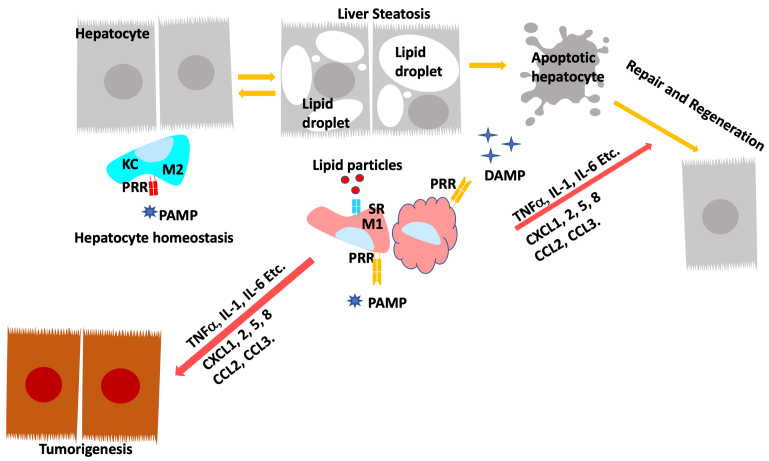


FIGURE 4
Programing of Hepatic Macrophages by PAMP and DAMP *via* PRR and SR. In normal, Kupffer cells recognize pathogen induced molecular patterns (PAMP) such as LPS coming through the portal vein. Kupffer cells clears these bacterial toxins without inducing inflammation to maintain hepatocyte homeostasis. During steatosis and steatotic injury, PRRs also bind to damage induced molecular patterns (DAMP) released by hepatocytes. The chronic injury induces proinflammatory responses from Kupffer cells as well as infiltrating macrophages. In addition, particles released by steatotic hepatocytes are also taken up by macrophages *via* scavenger receptors (SR). The binding of DAMP and lipid particles to PRR and SR induces the release of proinflammatory cytokines and chemokines including TNFα, IL-1, IL-6, CCL2 and 3 as well as CXCL1,2,5, and 8. These inflammatory mediators signals tissue repair and also promotes genotoxic events in liver cancer.

In NAFLD and ALD, steatosis induces chronic injury and hepatocyte damage. The damaged hepatocytes are the major source for DAMPs in the steatotic liver. For example, bile acid accumulation in hepatocytes triggers the assembly of NLR protein 3 inflammasome and the subsequent release of IL-1β that can bind to IL-1 receptors on macrophages. The TLR family members are high-affinity transmembrane receptors expressed on macrophages including Kupffer cells (156). The engagement of TLR4 with LPS triggers the sequential release of proinflammatory cytokines including TNF, IL-1, and IFN-β and other proinflammatory mediators such as the high mobility group box 1 (HMGB1) (157). During fatty liver diseases, free fatty acids also induce HMGB1 overexpression and secretion from hepatocytes. HMGB1 binds and activates TLR4 receptors on Kupffer cells and induce the release of proinflammatory cytokines such as TNFα and IL-6 (158). Similarly, during HFD feeding, hepatocytes release mitochondrial DNAs which stimulate Kupffer cell TLR9 receptors and subsequent TNFα secretion. Cholesterol laden lipid droplets formed within hepatocytes can also activate Kupffer cells through direct contact, this promotes IL-1β secretion in these Kupffer cells (159).

## Scavenger receptors

The distinct characteristic of steatotic liver injury is lipotoxicity. The accumulation of lipids in hepatocytes results in metabolic and oxidative stress that not only results in hepatocyte apoptosis but also directly signals inflammatory responses *via* macrophage cell surface receptors (55, 160, 161). During the pathogenesis of atherosclerosis, plaque formation is induced by the foam cells formed when macrophages scavenge modified low-density lipoproteins (LDL) and deposit them into the endothelial linings of blood vessels. Brown and Goldstein identified SR that are responsible for uptake of the modified LDLs (155). The family of scavenger receptors now are diverse and bind other DAMPs and PAMPs as well. In addition to modified and unmodified LDLs, other lipids such as cholesterols and phospholipids, bacterial pathogens, oxidative particles, and apoptotic cells are all scavenged by macrophages *via* these scavenger receptors (155). In NASH induced by feeding of Western diet, deletion of macrophage scavenger receptor MSR or type B1 scavenger receptor CD-36 led to reduced inflammation likely due to their effects on intracellular cholesterol trafficking in Kupffer cells (162, 163). In LDL receptor deficient (ldl4-/-) mice fed HFD, loss of CD36 or MSR resulted in reduced hepatic inflammation (162). In ConA induced liver injury, it was shown that CD36 sustains inflammation and expression of proinflammatory cytokines and is required for C-X-C ligand 10 induced apoptosis of hepatocytes (150).

Uptake of cholesterol *via* these SRs reprograms liver X receptor (LXR) regulated transcription in macrophages and attenuates the expression of anti-inflammatory genes (164, 165). In addition, the expression of macrophages CD36 and SR-B2 are also subjected to the transcriptional regulation by the orphan nuclear receptor peroxisomal proliferator-activated receptor (PPAR) (166–168). In THP-1 macrophages, it was shown that the downregulation of CD36 in macrophages likely resulted from reduced PPARγ regulated transcription when ratio of n-6/n-3 polyunsaturated fatty acids (PUFAs) is reduced (169). PPARγ has long been

recognized as a potential receptor for PUFA produced eicosanoids (170). These effects of PUFAs on CD36 expression and the function of macrophages to produce inflammatory metabolites are at least partially mediated through the activation of PPARs by the bioactive eicosanoids produced from PUFAs (171–173). In fact, cyclooxygenase 2 (COX-2), one of the enzymes metabolizing PUFAs to eicosanoids is only expressed in tissue and infiltrating macrophages in the healthy liver (174). Activation of PPARγ by eicosanoids was found to sustain the production of TNFα, IL-1 and IL-6 induced by LPS and induce IL-10 downregulation in macrophages (175). In HFD induced NAFLD, loss of CD47, an inhibitor for macrophage activation and phagocytosis, leads to increased production of proinflammatory cytokines involving activation of PPARα (176). In Kupffer cells, LPS treatment induced TNFα and IL-6 is attenuated by PPAR agonist rosiglitazone (177). Thus, *via* regulation of PPARs, macrophages scavenge lipid particles to produce both pro- and anti-inflammatory cytokines (160). These PUFA derivatives including prostanoids, leukotrienes, HETES, EETs and lipoxins have all been indicated to promote a protumor inflammatory environment (178). During hepatocarcinogenesis, inhibiting COX-2 and epoxide hydrolase led to reduced "cytokine and eicosanoid storm", resulting in cancer prevention (179). The treatment with lipoxin A4, a pro-resolving eicosanoid in inflammation, led to reduced HCC proliferation induced by activated macrophages (180). Together, macrophage engulfment of lipids *via* the scavenger receptors will result in increased production of PUFA derived eicosanoids. These eicosanoids can be inflammatory mediators on their own and induce the production of inflammatory cytokines/chemokines *via* the transcriptional activities of nuclear receptors such as PPAR and others. By producing the proinflammatory eicosanoids and cytokines, macrophages/Kupffer cells establish a pro-tumor microenvironment in the injured livers of NAFLD and NASH (181–183).

## The therapeutic potential of targeting steatosis for liver cancer treatment

Pathologically, 80% of liver cancer occurs in patients with underlying liver disease that displays lipid metabolic dysfunctions known as liver steatosis (184), a condition that develops in all obese individuals and is commonly associated with liver cancer (5). In a zebra fish model of HCC promoted by HFD, metformin the first line drug used for treatment in diabetes, reduced TNFα expressing pro-inflammatory macrophages leading to increase T-cell population in the livers, and inhibited cancer progression (185). In mouse HCC induced by DEN treatment, metformin treatment reduced the number of foci. This reduction was thought to be an effect of lowered hepatic expression of interleukin-22 and inhibition of YAP phosphorylation (133). The binding of metformin directly to the C-terminal of HMGB1 may also play roles in its anti-inflammatory and tumor suppressive functions (186). Statin, a cholesterol lowering drug has been proposed as treatment for chronic liver disease (187). NAFLD patients who take more than 600 cumulative daily doses of statin had a 70% reduction in hazards of developing HCC (HR, 0.30; 0.20-0.43) (188). Longer usage of more than 5 years and higher doses reduced the rate of NASH related HCC by 24-35% (189, 190). Both Metformin and Statin may target AMPK for their lipid reduction function (191). In a HFD model treated with DEN, AMPK activator reduced tumorigenesis and IL-6 signaling in the liver (192). Activation of AMPK also suppresses HCC progression and metastasis induced due to deficiency of FATP5 (fatty acid transporter protein 5) (193). Loss of the upstream kinase, LKB1 that phosphorylates and activates AMPK was also found to synergize with *Pten* loss to promote liver cancer development (194). Indeed, Sorafenib, the first line targeted therapy for HCC suppresses NASH through mechanisms involving alteration of mitochondrial uncoupling and subsequent activation of AMPK (195). These observations indicated that mitochondrial metabolism is an underexplored mechanism that may provide potential targets for HCC treatment as LKB-AMPK acts as primary cellular sensors of energy crisis to promote ATP production. Consistently, plasmas from NASH patients were found to contain high levels of mitochondrial DNA and these mitochondrial DNA signal through TLR9 to regulate hepatic inflammation, acting as a potential mechanism for how steatosis establishes the proinflammatory tumor microenvironment. In addition, targeting mitochondrial functions attenuates steatosis and inflammation in the liver (196, 197). Together, this evidence suggests that targeting steatosis *via* reducing lipid burden and/or altering mitochondrial function can impact liver cancer development.

The majority of liver cancer patients are diagnosed in the advanced stages of the disease, eliminating surgery or transplantation the only curative treatment for liver cancer. In patients with advanced disease, the combination of immune checkpoint (CPI) therapy such as anti PD-L1 antibody atezolizumab and the VEGF antibody bevacizumab has become the new standard of care. PD-L1 is highly expressed by liver macrophages in the tumor stroma (198). These macrophages repress the tumor-specific CD8 T-cell activity and induce their apoptosis through the Fas receptors to promote tumor growth (199, 200). Furthermore, Kupffer cells also stimulate the proliferation of antigen specific $CD4^+$ Tregs and their release of IL-10 to inhibit the activities of cytotoxic T lymphocyte (91, 92). Additionally, prostaglandins produced by Kupffer cells may inhibit T cell activation (201–203). Together, activation of hepatic macrophages and their expression of PD-L1 appears to promote tumor escape by inducing an immune tolerance and reduce immune surveillance.

Patients with NASH and ASH respond poorly (median survival 5.4 months) to CPIs compared to those without steatosis (Median survival 11 months) (204). Given that CPI blocks the ability of macrophage/Kupffer cells to induce immunosuppressive

TABLE 1 Current Therapy for HCC Treatment and Effects of Potential Lipid Modifying Therapy.

| Current Treatment | Advantage | Disadvantage |
|---|---|---|
| Resection | Potential curative | Many patients are diagnosed late |
| Sorafenib | Targeted | Poor response rate |
| CPI | Advanced patients | Steatosis interfers with response |
| Potential lipid metabolic targeting AMPK and mitochondrial function | | |
| Metformin | Promising mouse studies | |
| Statin (600 daily dose) | 70% reduction in hazards of developing HCC | |

environment in the liver, identifying the hepatic macrophage produced factors that allow the liver to escape this immune surveillance may be a key to future therapeutic development targeted at inflammatory tumor microenvironment associated with steatosis. As DAMP and PAMP that are present in the NAFLD/NASH livers, PRR and SR signals that controls the macrophage response to DAMP and PAMP are considered potential targets of intervention. A promising dietary intervention is n-3 fatty acids. Treatment with n-3 fatty acids was shown to inhibit both protein and mRNA levels of CD36 whereas n-6 fatty acids activate both (205, 206). In fat-1 transgenic mice fed STZ/HFD to induce NASH, ubiquitous expression of n-3 desaturase converts n-6 PUFAs to n-3 PUFAs and led to downregulation of CD36 and reduced liver damage (207). These dietary intervention studies suggest that targeting PRR and SR may be promising to reduce the tumor microenvironment and may work together with CPI to attenuate tumor growth in the liver.

One interesting discovery in CPI resistance is the role of the Wnt/β-catenin signal. The Wnt/β-catenin signaling pathway plays versatile roles in liver metabolism and tumorigenesis (32, 48, 208) due to its varied functions in different cell types in the liver. As such, upregulation of β-catenin allows tumors to escape CPI therapy and is one of the signals highly associated with CPI resistance together with steatosis (87). Interestingly, steatosis was found to induce macrophage expression of Wnt and the Wnt/β-catenin signaling mediates tumorigenesis in mouse models (32, 88). Thus, the induction of Wnt in macrophages by steatosis may play a role in the immune escape of these tumors. Further studies to elucidate how steatosis induces Wnt upregulation in macrophages is necessary to understand the resistance of steatosis associated liver cancer to CPI treatment.

Overall, liver cancer is the 6th most common type of cancer and the second leading cause of cancer deaths in the world with a median 10-year survival of just 11 months (209–211). In the liver, cancer development is highly associated with the development of steatosis and inflammation. Innate immune system and particularly Kupffer cells, the residence macrophages, act as the first responders following steatotic liver injuries. As such, targeting steatosis that show promising results in attenuating liver inflammation holds great potential in further therapeutic development as treatment of liver cancer (Table 1). Additionally, steatosis hinders CPI responses partially due to their effects on macrophages and macrophage production of inflammatory signals. Understanding how Kupffer cell are reprogramed to interact with innate immune system during the progression of steatosis is crucial for future therapeutic development targeted at overcoming resistance to current liver cancer therapy. Finally, identifying signals within tumor cells that respond to these protumor inflammatory signals produced by macrophages will result in novel therapeutic target that can overcome resistance to immunotherapy. In summary, targeting macrophages and macrophage interaction with tumor cells will provide therapeutic potential for steatosis-driven liver cancer treatment.

## Author contributions

TT organized the writing of this manuscript. BLS edited the final content of the manuscript. All other authors contributed to either the writing or the art work of this manuscript and are listed alphabetically.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# The magic bullet: Niclosamide

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The term 'magic bullet' is a scientific concept proposed by the German Nobel laureate Paul Ehrlich in 1907, describing a medicine that could specifically and efficiently target a disease without harming the body. Oncologists have been looking for a magic bullet for cancer therapy ever since. However, the current therapies for cancers—including chemotherapy, radiation therapy, hormone therapy, and targeted therapy—pose either pan-cytotoxicity or only single-target efficacy, precluding their ability to function as a magic bullet. Intriguingly, niclosamide, an FDA-approved drug for treating tapeworm infections with an excellent safety profile, displays broad anti-cancer activity in a variety of contexts. In particular, niclosamide inhibits multiple oncogenic pathways such as Wnt/β-catenin, Ras, Stat3, Notch, E2F-Myc, NF-κB, and mTOR and activates tumor suppressor signaling pathways such as p53, PP2A, and AMPK. Moreover, niclosamide potentially improves immunotherapy by modulating pathways such as PD-1/PDL-1. We recently discovered that niclosamide ethanolamine (NEN) reprograms cellular metabolism through its uncoupler function, consequently remodeling the cellular epigenetic landscape to promote differentiation. Inspired by the promising results from the pre-clinical studies, several clinical trials are ongoing to assess the therapeutic effect of niclosamide in cancer patients. This current review summarizes the functions, mechanism of action, and potential applications of niclosamide in cancer therapy as a magic bullet.

KEYWORDS

**niclosamide, mitochondrial uncoupler, metabolism, epigenetics, anti-tumor effect, oncogenic pathways, tumor suppressors, magic bullet**

## Introduction

In 1907, the German Nobel Laureate Paul Ehrlich conceived the pioneering concept of the "magic bullet," a medicine that specifically targets disease without causing harm to healthy tissues (1). Based on this theory, he identified salvarsan as the first "magic bullet" for syphilis in 1909. Likewise, oncologists have sought a magic bullet for cancer therapy, culminating in the discovery of chemotherapy (2). However, generations of oncologists interpreted the magic bullet as a compound that could target a single protein encoded by

a crucial oncogene, without proper consideration of the fact that cancer is a systemic disease that is not driven by a single driver/mutation (1). In fact, given the genetic heterogeneity of tumors, targeting the gene product(s) of any single mutation would lead to the selective outgrowth of a cancer cell population carrying other mutations, resulting in drug resistance and relapse (3). Thus, targeted therapy and other current cancer therapies that pose pan-cytotoxicity in patients, such as chemotherapy and radiation therapy, do not qualify as magic bullets. A true "magic bullet" for cancer treatment remains to be identified.

According to Otto Warburg, the inhibition of mitochondrial respiration leading to enhanced lactate production from glycolysis, namely the Warburg effect, is the primary cause of tumorigenesis (4, 5). The electron transport chain (ETC) coupled to ATP synthesis represents the core function of mitochondrial respiration. Based on Warburg's theory, we hypothesize that activating the ETC could reverse the Warburg effect and inhibit tumorigenesis. A potential candidate is the mitochondrial uncoupler niclosamide, an FDA-approved anthelmintic medicine that has been used to treat tapeworm infestations for nearly 50 years [6]. Recently, a number of studies and clinical trials have aimed to repurpose niclosamide for Covid-19 and cancer treatment (6, 7). Accumulating evidence indicates that niclosamide is a pleiotropic compound that targets multiple biological processes and signal pathways. Because niclosamide shuttles electrons across the mitochondrial inner membrane to activate the ETC, niclosamide reprograms intracellular metabolism (8), which can impact cellular epigenetic regulation at the transcriptional, translational, and post-translational levels (9, 10). Furthermore, the ability of niclosamide to modify the global epigenetic landscape through metabolic reprogramming (8) may explain its ability to simultaneously inhibit oncogenic signaling pathways and activate tumor suppressor signaling pathways. The fact that a modulator of metabolism, such as niclosamide, inhibits tumorigenesis through potentially pleiotropic mechanisms further validates Warburg's hypothesis: the primary cause of tumorigenesis is metabolic reprogramming.

## The discovery, nomenclature, formula and structure of niclosamide

Niclosamide, also known as Bayluscide, was first discovered in the Bayer chemotherapy research laboratories in 1958 (11) through screening chemical compounds against the aquatic pulmonated gastropod mollusk Biomphalaria glabrate, an intermediate host for the human parasitic trematode Schistosoma mansoni. As a secondary carboxamide that goes by the name of 5-Chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide in the IUPAC nomenclature system, niclosamide is a product formed through the condensation of the carboxy group of 5-chlorosalicylic acid with the amino group of 2-chloro-4-nitroaniline (Figure 1A). The molecular formulation of niclosamide is $C_{13}H_8C_{l2}N2O4$ with a molecular weight of 327.12 Dalton (Da). Niclosamide is considered thermally stable, with hydrolysis only happening by boiling in concentrated alkalis or acids (12).

## Applications of niclosamide as an anthelmintic drug

Niclosamide is a widely used anthelmintic drug in the treatment of parasitic infections. It was approved by the FDA in 1982 and listed in the World Health Organization's list of essential medicines (13, 14). It is generally taken at a 2g single

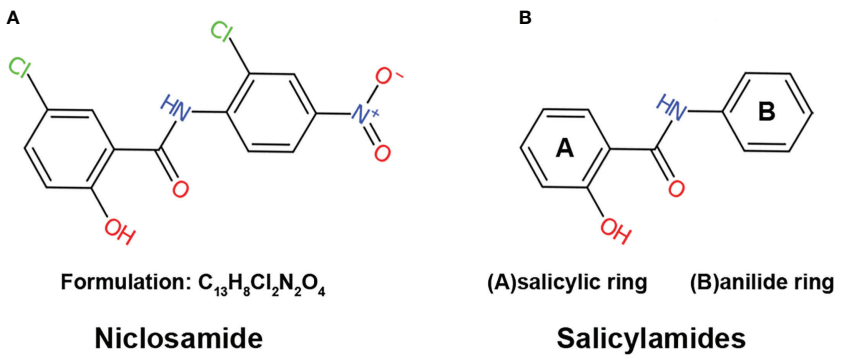


FIGURE 1
The structure of niclosamide **(A)** The structure and formulation of niclosamide. **(B)** The structure of salicylamides, which are weakly acidic phenolic compounds consisting of two basic chemical structures: a salicylic acid ring and an anilide ring.

dose for adults and 1-1.5g single dose for the children[4]. For *D. latum*, *T. saginata*, *D. caninum*, and *T. solium*, a single dose of niclosamide is effective. Because niclosamide is not effective against mature *H. nana* cysts, effective treatment regimens require repeated daily doses for 1 week to completely eradicate the infection (13). In humans and animals, niclosamide is partially absorbed in the intestinal canal and rapidly eliminated by the kidney (11). The original pharmacokinetics study showed that the maximal serum concentration can reach 0.25-6.0ug/ml (0.76-18.34 μM) following administration of a single 2g dose (11). The native form of niclosamide, along with its derivatives 2',5-dichloro-4'-aminosalicylanilide and 2',5-dichloro-4'-acetaminosalicylanilide, has been shown to be completely eliminated from the human body within 1-2 days (11). Overall, niclosamide shows a significant anthelmintic effect along with a strong safety profile and tolerability in humans.

## Mechanism of action: Mitochondrial uncoupling

Mitochondrial uncoupling is a process that dissipates the proton gradient across the inner mitochondrial membrane, inhibiting ATP synthesis and activating the ETC to promote NADH oxidation (15, 16). Niclosamide is a derivative of salicylamides, a class of potent mitochondrial uncouplers (17–20). Salicylamides are weakly acidic phenolic compounds consisting of two basic chemical structures: a salicylic acid ring and an anilide ring (Figure 1B). In general, drugs with uncoupling properties possess three characteristics: an acid dissociable group, a bulky hydrophobic moiety, and strong electron-withdrawing group (21). In the case of salicylamides, the salicylic acid ring and anilide ring serve as the acid-dissociable group and bulky hydrophobic moiety, respectively, while the amide group is the electron-withdrawing group (22).

Structural studies have determined that the formation of a six-membered hydrophobic ring between a -NH in the aniline moiety and a phenolic -OH in the salicylic acid moiety by intramolecular hydrogen bonding contributes to the high hydrophobicity and structural stability important for uncoupler activity (21, 22). These chemical structures are absolutely essential for the mitochondrial uncoupling activity of salicylamides (22–25). For example, replacing the phenolic hydroxyl (-OH) group to a methyl ($-CH_3$) of niclosamide is thought to abolish its mitochondrial uncoupling activity, resulting in a loss of anti-growth effect in both wild-type or p53-null cancer cells, suggesting that the antitumor effect of niclosamide relies on its uncoupling function (20). A signaling mechanism by which this effect is thought to be mediated involves niclosamide decreasing the mitochondrial potential to inhibit ATP synthesis (Figure 2A), leading to the activation of AMPK and the induction of either cell cycle arrest or apoptosis (15, 18–20). Nonetheless, a potential downside exists; namely, the hydrophobic properties of mitochondrial uncouplers may limit their bio-availability as drugs.

A potential solution to the aforementioned challenge is niclosamide ethanolamine (NEN), a salt form of niclosamide that also functions as a mitochondrial uncoupler with a superior safety profile and enhanced bioavailability (11, 26). Alasadi et al. reported that NEN treatment enhances pyruvate entry into mitochondria, and reduces glucose flux to the pentose phosphate pathway, serine synthesis, and lactate production (15). Recently, we discovered that NEN activates the ETC to boost NADH oxidation, thereby leading to an increased intracellular $NAD^+$/NADH ratio and driving the TCA cycle forward. The $NAD^+$/NADH ratio dictates the equilibrium of pyruvate/lactate and α-ketoglutarate (α-KG)/L-2-hydroxyglutarate (L2-HG) (27–29). Excessive lactate production is a hallmark of the Warburg effect, and 2-HG is a competitive inhibitor of α-KG-dependent dioxygenases such as DNA demethylase ten eleven translocation enzymes (TET) (30, 31). NEN treatment increases the intracellular pyruvate/lactate ratio, the α-KG/2-HG ratio, and total intracellular α-KG levels, leading to a reversal of the Warburg effect and the induction of cellular differentiation (Figure 2A). Consistent with these observations, NEN treatment induces promoter CpG island demethylation and epigenetic landscape remodeling (Figure 2B) (8). In neuroblastoma cells, many genes activated by NEN treatment are involved in neurogenesis, nervous system development and neuron differentiation. The NEN-upregulated genes are enriched in the favorable prognosis gene signatures, while the NEN-downregulated genes are more enriched in unfavorable prognosis gene signatures. Consistent to the prognosis gene signatures changes, NEN treatment not only reduced the tumor growth but also prolonged the survival for tumor bearing mice (8). In vivo, NEN treatment also effectively increased the NAD+/NADH ratio and reduced lactate and 2-HG levels in xenograft tumors (8).

Together, these data suggest that when the ETC is inhibited, a shift towards more Warburg-like metabolism leads to cell dedifferentiation, a consequence of global epigenetic remodeling rather than alterations within a single gene or a pathway. Thus, activating the ETC with mitochondrial uncouplers not only antagonizes the Warburg effect by promoting TCA cycling, but also redirects the cellular epigenome and transcriptome towards that of a differentiated state. This highlights the advantage mitochondrial uncouplers hold over other drugs: the ability to target many oncogenic pathways simultaneously.

## Common signaling targets of niclosamide

Multiple studies have now demonstrated the anti-cancer efficacy of niclosamide (6, 32). In this section, we summarize the

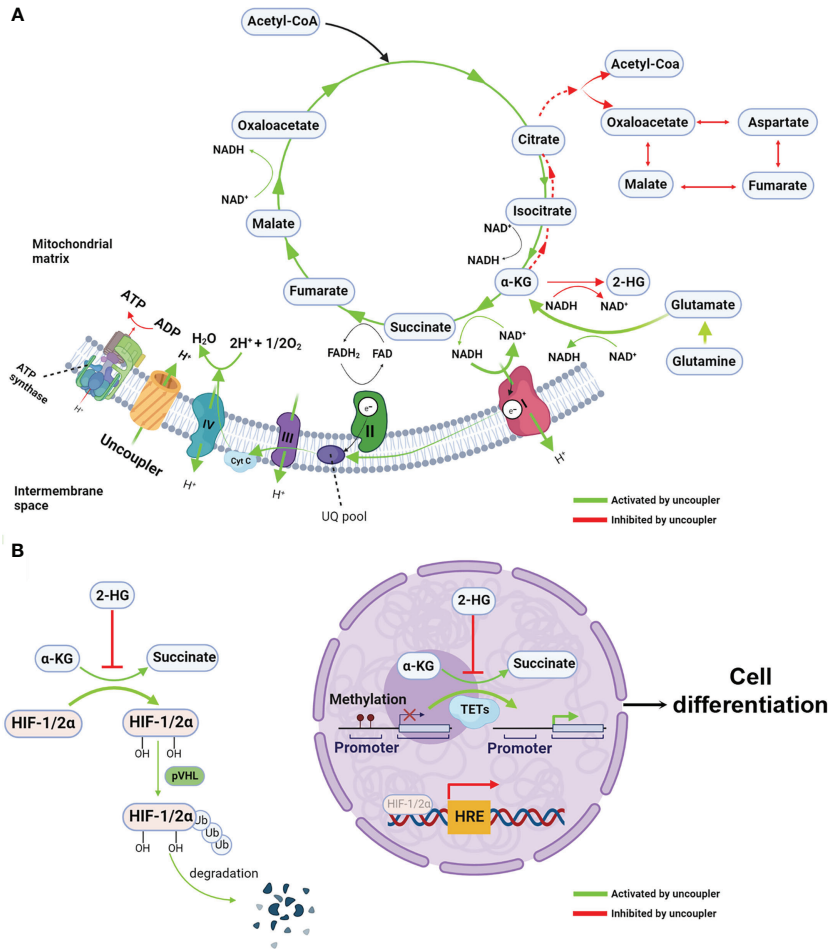


FIGURE 2
Mitochondrial uncoupling reprograms metabolism and epigenetic landscape **(A)** Mitochondrial uncouplers dissipate the proton gradients which are essential to ATP synthesis, resulting in reduction of ATP/ADP ratio. When proton gradient reduce, the electron transfer chain, particularly complex I, are activated, leading to increased intracellular redox $NAD^+$/NADH ratio. Given the $NAD^+$/NADH ratio is the major driving force for TCA cycle, the oxidative TCA cycle and glutaminolysis are accelerated. Because the chemical equilibrium of many metabolites pair such as α-KG/2-HG and pyruvate and lactate (not show in the figure) are dictating by $NAD^+$/NADH ratio. Thus, increased $NAD^+$/NADH mediated by mitochondrial uncoupler shift the equilibrium from 2-HG to α-KG, resulting in increased α-KG/2-HG ratio. In the other hand, opposite to the oxidative TCA cycle, the reductive TCA cycle particular reductive carboxylation is inhibited by mitochondrial uncoupler. **(B)** The increased α-KG/2-HG ratio activates the α-KG-dependent dioxygenases such as TET and PHD, leading to DNA demethylation and HIFs protein degradation. These epigenetic rewiring activate the expression of differentiation makers and repress the stemness genes, consequently, cell differentiation. Created with >BioRender.com.

major oncogenic and tumor suppressor signaling pathways that are modulated upon niclosamide treatment (Figure 3, Table 1).

## Oncogenic pathways

### Wnt/β-catenin

The Wnt/β-catenin pathway is a developmental signaling pathway that regulates multiple key cellular biological processes including proliferation, migration, genetic stability, polarity, apoptosis, differentiation, and stem cell renewal (70, 71). The Wnt/β-catenin pathway is commonly dysregulated in many cancer types, leading to research into the role of WNT signaling in tumorigenesis and the subsequent development of various Wnt signaling inhibitors for cancer therapies. In the absence of Wnt ligands, cytosolic β-catenin is sequestered by its destruction complex APC, axis inhibitor (AXIN), casein kinase 1α (CK1α), and glycogen synthase kinase 3β (GSK3β) (72). Subsequently, phosphorylation of β-catenin by both CK1α and GSK3β marks itself with ubiquitination by E3 ligases β-transducin repeat–containing protein (βTrCP), resulting in proteasomal degradation (71, 72). Conversely, when extracellular Wnt protein binds to a heterodimeric complex of Frizzled receptors (FZD) and coreceptors low-density

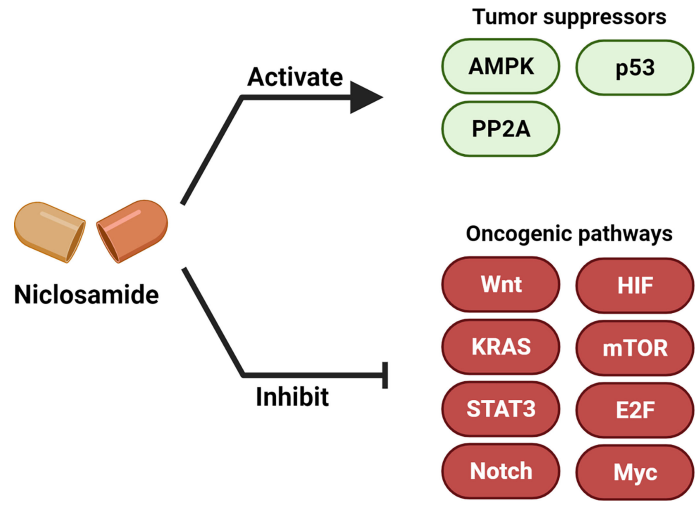


FIGURE 3
Niclosamide activates tumor suppressors and inhibits oncogenic pathways. Niclosamide has anti-tumor effect through inhibiting multiple oncogenic pathways such as Wnt/β-catenin, Ras, Stat3, Notch, E2F-Myc, NF-κB and mTOR, and activating tumor suppressor signaling such as p53, PP2A and AMPK. Created with BioRender.com.

lipoprotein receptor-related proteins 5 and 6 (LRP5/6), the cytoplasmic tail of LRPs is phosphorylated, recruiting axis inhibition (AXIN) and the destruction complex to the cell membrane and activating dishevelled (DVL). The activated DVL represses the destruction complex of β-catenin, allowing the cytoplasmic accumulation and nuclear translocation of β-catenin. Subsequently, nuclear β-catenin interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF) to induce the expression of specific target genes (71, 72).

Niclosamide inhibits Wnt/β-catenin signaling at multiple levels. Employing a primary imaged-based GFP fluorescence assay that uses Frizzled1 endocytosis as the readout to perform a high-throughput screen, Chen et al. reported that niclosamide downregulates Dishevelled-2 protein levels, antagonizing the Wnt3A-mediated induction of β-catenin and its downstream transcriptional activity (33). Ensuing studies have reported on the efficacy of niclosamide in targeting Wnt/β-catenin pathway in a wide spectrum of cancer types including prostate (34), ovarian (39), breast (34, 35), colorectal (36–38), pancreatic (41), and neuroblastoma (8). Niclosamide-driven Dishevelled-2 and Frizzled 1 degradation may also rely on the induction of autophagosomes (36, 38). Autophagosomes are double-membrane sequestering vesicles, originating from phagophores that engulf parts of the cytoplasm, eventually fusing with lysosomes to initiate substrate degradation (73). In support of this model, Frizzled 1 or β-catenin co-localizes with LC3, an autophagosome marker, in niclosamide-treated cells. Furthermore, niclosamide-mediated inhibition of Wnt/β-catenin signaling is rescued by the autophagosome inhibitor 3-MA and is attenuated in autophagy-deficient ATG5$^{-/-}$ MEF cells (38). At the signaling receptor level, niclosamide suppresses LRP6 expression and phosphorylation, leading to a block in β-catenin stabilization induced by Wnt3A without affecting the expression level of Dishevelled-2 (34, 39). Niclosamide was also reported to bind GSK3 directly, resulting in disruption of the Axin-GSK3 complex and attenuation of canonical Wnt activity (37). A recent study reported that niclosamide increases GSK-3β phosphorylation to promote the ubiquitin-mediated degradation of β-catenin (41). The mechanism of Wnt pathway inhibition by niclosamide is summarized in Figure 4.

## K-Ras

*KRAS* is the major mutated isoform of the Ras gene in cancers, including in ~85% of all cancers (74, 75). Cancers driven by mutant KRAS proteins are considered refractory to most therapies. Given the "undruggable" tertiary structures of Ras, a potent and selective Ras inhibitor remained elusive for clinical use until Sotorasib was approved by the US Food and Drug Administration in May 2021 to target the growth of tumors caused by *KRAS* G12C mutation (42, 76). Nonetheless, more options are needed to target KRAS mutation-driven malignant transformation.

Surprisingly, niclosamide activated GSK-3 through disruption of the Axin-GSK3 complex (37), leading to Pan-Ras or K-Ras protein degradation (42). Ras degradation can be rescued by pharmacological GSK-3 inhibition with the GSK-3 inhibitor BIO, suggesting that niclosamide inhibits Ras signaling in a GSK-3 dependent manner. In addition, niclosamide suppresses Ras activity at various levels in colon cancer cells regardless of mutational status and inhibits G12V mutant K-Ras-induced transformation (42).

TABLE 1 Niclosamide activates tumor suppressor and inhibit oncogenic pathways.

| | Target pathway | Effect of niclosamide | Cancer type | Reference |
|---|---|---|---|---|
| Oncogenic pathways | Wnt/β-catenin | Inhibition | sarcoma | (33) |
| | | | prostate | (34) |
| | | | breast | (34, 35) |
| | | | colon | (36–38) |
| | | | ovarian | (39) |
| | | | pancreas | (40, 41) |
| | KRAS | Inhibition | colon | (42) |
| | | | liver | (43) |
| | | | ovarian | (44) |
| | STAT3 | Inhibition | prostate | (45, 46) |
| | | | lung | (47, 48) |
| | | | colon | (49, 50) |
| | | | breast | (51, 52) |
| | | | liver | (53) |
| | Notch | Inhibition | colon | (54) |
| | | | liver | (55) |
| | E2F | Inhibition | neuroblastoma | (8) |
| | N-myc | Inhibition | neuroblastoma | (8) |
| | c-Myc | | ovarian | (56) |
| | NF-kB | Inhibition | ovarian | (44, 56) |
| | | | leukemia | (57, 58) |
| | mTOR | Inhibition | breast | (59) |
| | | | cervix | (60, 61) |
| | | | lung | (62, 63) |
| | | | ovarian | (64) |
| | HIF1α | Inhibition | colon | (65, 66) |
| | | | lung | (67) |
| | | | neuroblastoma | (8) |
| Tumor suppressors | p53 | Activation | ovarian | (20) |
| | | | neuroblastoma | (8) |
| | AMPK | Activation | liver | (18, 68) |
| | PP2A | Activation | lung | (69) |

## Stat3

Hyperactive STAT3 drives cancer progression by promoting cell proliferation, angiogenesis, migration, invasion, and immune invasion (77, 78). Hyper-activated growth factor signaling and overexpression of stimulatory receptor-ligand pairs contribute to constitutive STAT3 activation, characterized by phosphorylation of Y705 and nuclear translocation of STAT3. Specific inhibitors like 'Stattic' have been developed to target Y705 for STAT3 inhibition (79). However, recent studies showed that STAT3 is activated by Y727 phosphorylation independent of Y705 status in trible negative breast cancer, thereby attenuating the effect of STAT3 inhibitor 'Stattic' (80).

Niclosamide was identified as a potent inhibitor of STAT3 after screening 1500 clinical-approved compounds in a STAT3 reporter system (45). Niclosamide treatment inhibits phosphorylation and nuclear translocation of STAT3, leading to the repression of STAT3 transcriptional activity. Moreover, STAT3 dephosphorylation induces cell cycle arrest and apoptosis in Du145 cells expressing constitutively active STAT3 (45). More importantly, Pranay et. showed the niclosamide not only reduces the phosphorylation of the canonical site Y705 but also the phosphorylation of the non-canonical site Y727 (81). Aberrant activation of STAT3 by chemotherapeutic drugs or radiotherapy causes therapy resistance, which can be overcome by niclosamide-mediated STAT3 inhibition (46, 47, 49–51). Beyond affecting cancer cell-intrinsic signaling, niclosamide can regulate signals communicated from other cell types in the tumor microenvironment such as adipocyte-mediated epithelial to

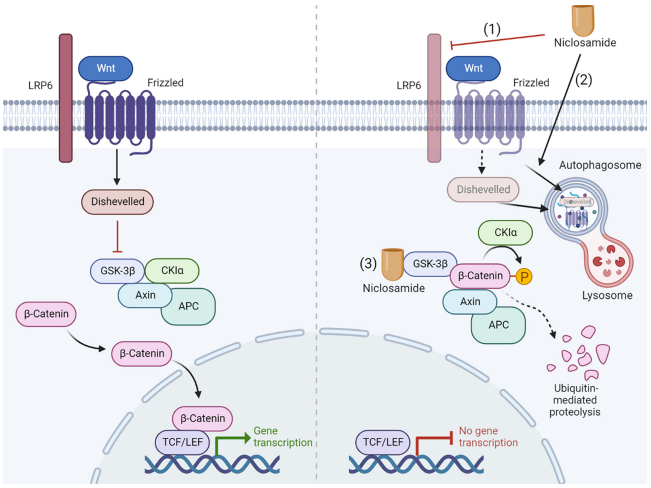


FIGURE 4
Niclosamide inhibits Wnt pathway through multiple mechanism. The Wnt pathway inhibition by niclosamide depends on multiple ways of action: (1) Niclosamide suppresses LRP6 expression. (2) Niclosamide promotes the degradation of Frizzled 1 and Dishevelled-2 through autophagy. (3) Niclosamide binds GSK3 directly, resulting in disruption of the Axin-GSK3 complex and attenuation of canonical Wnt activity. Created with BioRender.com.

mesenchymal transition through inhibition of the interleukin-6/STAT3 signaling axis (52).

## Notch

Like the Wnt/β-catenin pathway, Notch is also a developmental signaling pathway dysregulated in cancer that can promote cell proliferation, angiogenesis, invasion and migration, and immune evasion (82, 83). When cognate Notch ligands bind to Notch receptors, the Notch receptor is cleaved and released from the cell membrane. Subsequently, the released Notch Intracellular Domain translocates into the nucleus and regulates expression of Hes and Hey family genes such as p27cip1/waf1, p21.cyclin D1, c-Myc, Survivin, slug, and Nanog (84).

It was reported that niclosamide decreases the protein expression of Notch1, Notch2, and Notch3 in colon cancers and is associated with the inhibition of cell proliferation, repression of cell migration, and induction of apoptosis (54). Another study employed niclosamide-loaded pluronic nanoparticles (NIC-NPs) to treat thioacetamide-induced hepatocellular carcinoma (HCC) in rats (55). The researchers found that NIC-NPs treatment restores liver integrity, reduces alpha-fetoprotein (AFP) levels, and inhibits Notch signaling by reducing *notch1* mRNA levels.

## E2F and Myc

E2Fs are the ultimate effectors of the cyclin-dependent kinase (CDK)–RB–E2F axis, the central transcriptional pathway driving cell cycle progression. Dysregulation of one or more components of this axis such as CDKs, cyclins, the CDK negative regulator, and/or the RB family of proteins is common in all cancers, leading to hyperactive oncogenic E2F activity and unrestrained proliferation (85–87). The *MYC* oncogene plays an important role in the tumorigenesis of many cancer types, is deregulated in >50% of human cancers, and is generally associated with unfavorable patient prognosis (88–91). Reported cellular functions of *MYC* include amplifying transcription of already existing gene expression programs, promoting DNA replication, increasing protein synthesis, and reprograming metabolism to support cell proliferation (90–92). Additionally, *MYC* is essential for maintaining stemness and for rewiring the tumor microenvironment to evade the immune system (91). Given the "undruggable" protein structure of the Myc protein, targeting Myc directly in cancer treatment has been a challenge for decades (89, 91).

Multiple levels of crosstalk exist between E2Fs and Myc. E2F1, E2F2 and E2F3 were shown to bind the promoter region and activate the transcription of the *MYCN* gene in *MYCN*-amplified neuroblastoma (93). Furthermore, overexpression of the Cdk-inhibitor $p16^{INK4A}$ inhibits E2F activity, resulting in *MYCN* repression. However, overexpression of E2Fs fails to activate *MYCN* transcription in *MYCN* non-amplified neuroblastoma, indicating that E2Fs are necessary but not sufficient regulators of *MYCN* (94). In addition, *MYCN* overexpression induces E2F5 expression and promotes cell proliferation in neuroblastoma (95).

Due to the known crosstalk between E2F and Myc, we wondered whether E2F and Myc can be simultaneously targeted with a single intervention. We recently observed that a salt form of niclosamide, niclosamide ethanolamine (NEN), reduces the mRNA and protein expression of MYCN *in vitro*

and *in vivo*. In line with the reduction of MYCN, MYCN target genes are globally deregulated by NEN treatment (8). NEN also reduces expression of E2F target genes. Notably, our findings are supported by another study that utilizded a secreted *Gaussia* luciferase reporter system (56) to show that niclosamide treatment reduces *MYCN* transcription.

### NF-kB

The transcriptional factor NF-kB contributes to cancer initiation and progression, metastasis, and therapeutic resistance in human cancers (96–98). Constitutive activation of NF-kB activity caused by the inflammatory microenvironment and various oncogenic mutations are observed in many cancer types. NF-kB activation promotes cancer cell proliferation, suppresses cell apoptosis, and activates epithelial–mesenchymal transition to initiate metastasis (96, 97). Inhibition of NF-kB in tumor cells prevents tumor progression, making the NF-kB pathway an attractive therapeutic target (97). Under basal conditions, the inactive NF-κB complex (IKK, p65 and p50) is retained in the cytosol. Upon stimulation by factors such as TNFα, IκB is phosphorylated and degraded by ubiquitinylation *via* a multi-step process. The remaining NF-kB complex (p65 and p50) is then translocated into the nucleus to activate target gene transcription (99).

Niclosamide was reported to suppress NF-kB signaling and tumor growth in acute myelogenous leukemia (AML) (57, 58) and ovarian cancer (56). Mechanistically, niclosamide inhibits TNFα-mediated phosphorylation and degradation of iκbα, thereby inhibiting the phosphorylation and translocation of p65 to the nucleus (57, 100, 101). In line with the reduction of nuclear NF-kB, niclosamide represses NF-κB–mediated gene transcription as determined by luciferase reporter assays (56, 57).

### mTOR

Mammalian/mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that senses nutrients, growth factors, and environmental cues to regulate various fundamental cellular processes such as protein synthesis, autophagy, growth, metabolism, aging, and regeneration (102, 103). The mTOR pathway is frequently dysregulated in human cancers, rewiring cancer cell metabolism and the tumor microenvironment to promote tumor progression (102, 103).

Niclosamide was reported to inhibit mTOR signaling in lung cancer, ovarian cancer, cervical cancer, and the diabetic mouse kidney (61–64, 104). Accumulating evidence suggests that niclosamide-mediated mTOR inhibition may be accomplished through at least two distinct mechanisms. First, as a mitochondrial uncoupler, dissipating the mitochondrial proton gradient leads to a reduction in intracellular ATP and increase in the AMP/ATP ratio, resulting in the activation of AMP-activated protein kinase (AMPK) (8, 15, 18). AMPK activation inhibits mTOR directly through inhibitory phosphorylation of the mTORC1 subunit Raptor at Ser-792 or indirectly through disrupting the TSC2-Rheb axis (102). Second, Bruno et *al.* showed that niclosamide does not interact with or inhibit neither upstream PI3K/AKT signaling nor mTORC1 itself (59). Instead, the protonophoric activity of niclosamide is essential for dissipating protons (down their concentration gradient) from lysosomes to the cytosol and effectively lowering cytoplasmic pH, resulting in mTOR inhibition. Therefore, by suppressing mTOR signaling, niclosamide can also induce autophagy by inhibiting autophagic degradation (60).

### HIF

Hypoxia is a common tumor microenvironment stress that induces DNA methylation (105) and generation of the oncometabolite 2-hydroxyglutarate (2-HG) (27, 29) and is associated with poor prognosis and therapeutic resistance (106).

By using a hypoxia inducible factor 1 subunit alpha (HIF1α)-based luciferase reporter system as the read-out for high-throughput screening, niclosamide was identified as an inhibitor of HIF1α signaling with an approximate IC50 of 1.59 μM (65). Niclosamide inhibits HIF1α signaling to enhance the effects of radiation in non-small cell lung cancer (67) and blocks EGF-induced HIF1α signaling to repress tumorigenesis and invasion in colorectal cancer (66). Recently, we found that NEN represses both HIF1α and HIF2α protein and HIF target genes such as PDK1, PDK3, PGK1 and LDHA in both normoxia and hypoxia (8). Because HIF-1α and HIF-2α degradation relies on α-KG-dependent prolyl hydroxylases (PHDs), which can also be inhibited by 2-HG (31), we reasoned that niclosimide-mediated HIF1α/HIF2α inhibition could result from diminished generation of 2-HG from α-KG (8).

## Tumor suppressors

In addition to inhibiting oncogenic pathways, niclosamide was also reported to activate or restore tumor suppressor signaling (Figure 3A, Table 1)

### p53

Often referred to as "the guardian of human genome," the p53 protein is crucial for modulating DNA repair, cell division, survival, and metabolism (107–109). Following DNA damage, p53 plays a critical role in determining whether the cell initiates the DNA repair process or induces programmed cell death to eliminate damaged DNA. By preventing cells harboring mutated or damaged genes from dividing, p53 prevents tissues from acquiring cancer fitness-promoting genomic alterations (109). While loss of wild-type p53 is common in cancer, tumor-

associated p53 missense mutations can actually provide gain of function rather than simply loss of wild-type tumor-suppressing function. Mutant p53 proteins switch from a tumor suppressor to an oncogenic protein, promoting proliferation, cell survival, invasion, and metastasis (107, 108, 110).

A chemical library screen revealed that the mitochondrial uncoupling function of niclosamide selectively kills p53-deficient cells by triggering intracellular calcium flux leading to the release of arachidonic acid, a fatty acid normally detoxified by the p53 targets *ALOX5* and *ALOX12B* in wild-type cells (20). One could envision that the synthetic lethality between mitochondrial uncoupling and p53 loss would confer niclosamide tumor-suppressor functions by establishing a metabolic environment favoring the outgrowth of p53 wild-type cells. Moreover, niclosamide increases the expression of p53 at both the mRNA and protein level (8, 20). In adult cancers, TP53 is often mutated, yet in pediatric cancers such as neuroblastoma, TP53 mutations are very rare (111). Instead, p53 is typically silenced epigenetically through promoter methylation (111). Both NEN and 5-AZA treatment increase p53 protein levels in NB16 and SK-N-BE(2) cells, suggesting that mitochondrial uncoupling can upregulate p53 in NB cells through DNA demethylation.

### AMPK

AMPK is a highly conserved central energy sensor that coordinates energy status with intracellular metabolism during cell growth, development, and adaption to stress (112). AMPK is an essential downstream effector of the tumor suppressor LKB1, which signals to COX-2 (cancer progression), ULK1/2 (autophagy), ACC1/2 (Fatty acid metabolism), mTOR (cell growth and protein synthesis), and p53 (apoptosis) (113–115).

As described before, niclosamide dissipates the mitochondrial proton gradient requisite for ATP synthesis, leading to the reduction of intracellular ATP and an increased AMP/ATP ratio, culminating in the activation of AMP-activated protein kinase (AMPK) (8, 15, 18). Additionally, niclosamide may activate AMPK through a mechanism independent of the increased AMP/ATP ratio, namely through the AMPK β2 subunit (68).

### PP2A

Protein phosphatase 2A (PP2A) represents a family of ubiquitously expressed serine–threonine phosphatases that maintain cellular homoeostasis through regulating many important kinase-driven intracellular signaling pathways such as Akt, p53, c-Myc, and β-catenin (116, 117). The protein phosphatase 2A (PP2A) has a well-established role as a regulator of the cell cycle, signal transduction, and apoptosis. Loss of activity due to mutation in some of its subunits or the PP2A phosphatase activator (PTRA) is frequently observed in many cancer types, leading to neoplastic transformation (118, 119). In addition, CIP2A, an endogenous inhibitor of PP2A, is upregulated in many cancer cells, including non-small cell lung cancer (NSCLC) cells (120).

High-throughput screening identified niclosamide as a potent inhibitor of cancerous inhibitor of protein phosphatase 2A (CIP2A), leading to the activation of PP2A (69). The inhibitory effect of niclosamide on CIP2A depends on the reduction of CIP2A transcription, leading to lower CIP2A mRNA and protein levels and increased PP2A activity (69).

## Niclosamide regulates cellular epigenetics

DNA methylation is controlled by *de novo* methylation by DNA methyltransferases (DNMTs) and/or demethylation by DNA demethylases (121). Ten-eleven translocation (TET) DNA demethylase uses α-ketoglutarate (αKG) as the substrate to convert 5mC to 5-hydroxymethylcytosine (5hmC), followed by further reactions to remove methylation (122, 123). The two enantiomers of 2-hydroxyglutarate (2-HG) exert similar effects on TET and other α-KG-dependent dioxygenases but are generated under different conditions. The D-enantiomer (D-2-HG) is produced through gain-of-function point mutations in isocitrate dehydrogenases (IDH1/2) (124). In hypoxic tumor cells, including NB cells, the relatively lower $NAD^+$/NADH ratio favors the conversion of αKG to the L-enantiomer (L-2-HG) (27, 29). Recent reports have shown that α-KG promotes pancreatic cancer and colon cancer cell differentiation through reduced DNA methylation (125, 126). However, because the hypoxic tumor microenvironment promotes the conversion of α-KG to 2-HG, preventing this metabolic reaction presents a major challenge in cancer therapy.

Although inhibitors of mutant IDH enzymes exist and are being evaluated in the clinic (some has been approved by FDA, find it out and specify), an effective therapeutic strategy to inhibit L-2-HG production remains elusive. L-2-HG is a more potent inhibitor of a-KG dependent dioxygenases (31, 127). Tumor hypoxia develops when tumor growth exceeds the ability of available vasculature to supply tumor cells with oxygen and nutrients. Clinically, tumor hypoxia is a significant obstacle to treatment because hypoxic tumor cells are more resistant to radiation therapy (128, 129) and chemotherapy (130–132). It was reported recently that DNMT inhibitor (DNMTi) treatment overcomes hypoxia-induced chemoresistance (133), suggesting that DNA hypermethylation under hypoxia can cause chemoresistance. DNA hypermethylation is reinforced through hypoxia-mediated repression of TET activity (105). Due to their

similar chemical structures, 2-HG inhibits α-KG-dependent enzymes, including TET and Jumonji C domain-containing proteins (JMJDs) (31, 134), leading to hypermethylation of DNA and histones that blocks cellular differentiation. Therefore, under the low $NAD^+$/NADH ratios observed in solid tumors, the potential to use α-KG as a cancer demethylation agent is limited. In addition, both D-2-HG and L-2-HG inhibit other α-KG-dependent dioxygenases such as prolyl hydroxylase domain (PHD) proteins to stabilize hypoxia inducible factor (HIF) α subunits and activate HIF signaling (27, 31) (Figure 1).

The signaling and metabolic alterations caused by niclosamide can potentially reprogram the global epigenetic landscape in multiple ways. On one hand, as we discovered, NEN treatment increases the intracellular $NAD^+$/NADH ratio, inhibiting 2-HG generation from α-KG, leading to an increased intracellular α-KG/2-HG ratio to promote TET2 activity and DNA demethylation (8). Unlike DNMT inhibitors such 5-azacytidine, NEN treatment remodeled the DNA methylation landscape rather than simply reducing the global methylation level. The cancer epigenome is characterized with promoter CpG island hypermethylation but gene body hypomethylation. NEN treatment reversed this epigenetic remodeling pattern, reducing methylation in promoter CpG Island but increasing methylation in gene body region. This epigenetic remodeling strategy could be more effective and precise than DNMTi treatment (8). On the other hand, NEN treatment dramatically elevates ADP and AMP levels while lowering ATP levels (8). AMPK activation phosphorylates TET2 at serine 99, thereby stabilizing the tumor suppressor to promote DNA demethylation (135). Thus, it is possible that NEN treatment also increases TET activity through activating AMPK.

The fact that NEN treatment alters the cellular transcriptional profile is consistent with the theory that NEN treatment reprograms the epigenome. The number of upregulated genes is more than two-fold higher than the number of downregulated genes induced by NEN treatment, indicating that NEN treatment has a major role in activating gene expression (8). The top pathways upregulated by NEN treatment includes pathways related to neurogenesis, nervous system development, and neuron differentiation. The top downregulated pathways are involved in DNA replication and cell cycle progression. Importantly, while almost all the NEN-upregulated genes are enriched in gene signatures that indicate favorable prognosis, all the NEN-downregulated genes are enriched in gene signatures that indicate unfavorable prognosis (8). These data indicate that mitochondrial uncoupling rewires the global transcriptome in a way that leads to cell differentiation and proliferation arrest, rather than targeting one specific signaling pathway that may fail to trigger such broad-scale changes.

# Combination of niclosamide with other therapies

## Radiation

Radiotherapy is an effective cancer treatment for up to 50% of cancer patients. However, one significant challenge during radiotherapy is the buildup of acquired radioresistance (136). Thus, it is important to identify strategies that improve the efficiency of and overcome the resistance to radiotherapy.

Niclosamide was reported to enhanced the radiation sensitivity of many cancer types such as lung cancers (62, 67, 137), triple-negative breast cancer (35), nasopharyngeal carcinoma (138), and colorectal cancer (139). Synergism between niclosamide and radiotherapy may occur in part through the ability of niclosamide to inhibit multiple adaptive pathways upregulated during or following radiation. Niclosamide pretreatment induces C-Jun expression and phosphorylation, promoting apoptosis in cells that failed to control radiation-induced reactive oxygen species (ROS) (137). STAT3 was also reported to protect cells following radiation. As a potent inhibitor of STAT3, niclosamide reduces STAT3 nuclear translocation to restore radiation sensitivity (62). Niclosamide inhibits the hypoxic induction of Wnt/β-catenin and HIF1α signaling, leading to tumor radiosensitivty (35, 67). Niclosamide downregulates the expression of Ku70/80, inhibiting DNA double-strand break repair to sensitize the cancers to radiation (138, 139).

## Chemotherapy

Chemoresistance is a common obstacle to cancer treatment involving multiple resistance mechanisms (140–142). Identifying therapeutic strategies to enhance chemotherapy efficiency and overcome acquired resistance hold immense interest in the cancer biology field.

Niclosamide has shown synergistic anti-tumor effects with a broad spectrum of chemotherapy drugs. Niclosamide's potential functions as a chemotherapy enhance are summarized in Table 2.

## Immunotherapy

Discoveries from the last decade have shown that immunotherapy, unleashing power from the patient's own immune system to recognize and eliminate cancer cells, is a

promising approach for cancer treatment. The immune receptor/ligand pair PD-1/PD-L1 constitutes a key inhibitory immune checkpoint system hijacked by cancer to escape destruction by the immune system, thereby highlighting its importance as a target for cancer immunotherapy (152). Niclosamide is reported to disrupt PD-1/PD-L1 interactions in non-small cell lung cancer (153), metastatic lung adenocarcinoma (154), and pancreatic cancer (41, 155) primarily through PD-L1 ligand downregulation in cancer cells. Importantly, several studies observed that niclosamide potentiates PD-1/PD-L1 blockade in preclinical cancer models (41, 153–155). At the molecular level, this reduction of PD-L1 expression by niclosamide may rely on the suppression of STAT3 phosphorylation and transcription factor binding to the PD-L1 promoter in the nucleus (153).

## Clinical trials

The plethora of preclinical studies demonstrating impressive antiviral and anticancer effects of niclosamide have led to a series of clinical trials. There are currently 31 records of clinical trials involving niclosamide, as published on the clinicaltrials.gov database. Among these, 16 trials relate to Covid-19 treatment and 8 trials relate to cancer treatment. We summarize the cancer-relevant clinical trials in Table 3. Despite the promising data generated in preclinical models, proof of efficacy and safety is still required. These properties are associated with diverse biopharmaceutical challenges such as the relationship between physicochemical properties and oral absorption of the drug with clinical outcomes (156). Published data regarding the pharmacokinetics (PK) of niclosamide suggest that it has poor oral bioavailability (11), potentially limiting its application as a cancer drug, consistent with observations made in clinical trial NCT02532114 (156). In this trial, either 500mg or 1000mg niclosamide was given three times daily to patients. However, the maximal plasma concentration ranged from 35.7–82 ng/mL (0.1μM-0.25 μM), a range that failed to be consistently above the minimum effective concentration in preclinical studies (156). In contrast, the ongoing clinical trial NCT02807805 is administering 1200 mg of reformulated orally bioavailable niclosamide orally (PO) three times daily to patients, resulting in 0.21μM-0.723 plasma niclosamide concentrations exceeding the therapeutic threshold of > 0.2 μM. In prostate cancer patients, combination of niclosamide with abiraterone/prednisone induced a prostate-specific antigen (PSA) response in 5 of 8 evaluable patients (158). Overall, niclosamide displays an excellent safety profile across these clinical trials. However, the bio-availability and standalone anti-tumor effect of niclosamide are still major challenges. To overcome these limitations, new delivery strategies and rational combination therapies with other treatments need to be developed.

## Conclusion and future directions

Cancer is the second leading cause of death in the world after heart disease, accounting for 1 out of every 6 deaths in 2021 (159). An effective and low-risk cancer treatment has remained elusive for decades. Indeed, current treatments such as

TABLE 2 Niclosamide has synergetic effect with chemotherapy.

| Drugs | Cancer type | Potential mechanism | Reference |
|---|---|---|---|
| cytarabine | Acute Myelogenous Leukemia | NS | (57) |
| etoposide | | NS | |
| daunorubicin | | NS | |
| dasatinib | chronic myeloid leukemia | inhibiting Erk/Mnk1/eIF4E pathway | (143) |
| castration | prostate cancer | inhibition ofandrogen receptor variants | (144) |
| cisplatin | renal cellcarcinoma | NS | (145) |
| | lung cancer | Suppression of lung resistance–related protein and c–myc | (146) |
| | esophageal cancer | Inhibition of STAT3 pathway | (147) |
| | Hepatocellular Carcinoma | Inhibition of STAT3 pathway | (53) |
| Oxaliplatin | Colorectal Cancer | increased $H_2O_2$ production | (148) |
| 5-FU | esophageal cancer | Inhibition of STAT3 pathway | (147) |
| paclitaxel | triple negative breast cancer | NS | (149) |
| | esophageal cancer | Inhibition of STAT3 pathway | (147) |
| erlotinib | colorectal cancer | Inhibition of STAT3 pathway | (49) |
| SN38 | colorectal cancer | Inhibition of STAT3 pathway | (50) |
| Doxorubicin | Breast Cancer | downregulating the Wnt/β-catenin pathway | (150) |
| camptothecin | glioblastoma | NS | (151) |

NS, not sure.

TABLE 3 The clinical trials using niclosamide for cancer therapy.

| Ref | Cancer type | Potential target | Mechanism | Phase |
|---|---|---|---|---|
| NCT05188170 | Acute Myeloid Leukemia | CREB (58) | Inducing apoptosis and cell cycle arrest | Phase 1 |
| NCT04296851 | Familial adenomatous polyposis (FAP) | Axin-GSK3 (37) | inhibition of Wnt pathway and Snail-mediated EMT | Phase 2 |
| NCT03123978 | Metastatic/Recurrent Prostate Carcinoma | IL6-Stat3-AR pathway (46) | overcome enzalutamide resistance and inhibit migration and invasion | Phase 1 |
| NCT02807805 | Metastatic/Recurrent Prostate Carcinoma | androgen receptor variant 7 | synergizes with abiraterone | Phase 2 |
| NCT02687009 | Colon Cancer | Frizzled receptor (36) | Inhibition of Wnt/β-catenin pathway | Phase 1 |
| NCT02532114 | Castration-Resistant Prostate Carcinoma | | Inhibition of androgen receptor splice variants or Wnt/β-catenin pathway (156) | Phase 1 |
| NCT02519582 | Colorectal Cancer | Wnt/β-catenin pathway signaling (157) | restricting S100A4-driven metastasis | Phase 2 |

chemotherapy, radiation therapy, hormone therapy, and immunotherapy each have their own limitations as a "magic bullet" against cancer. Namely, their off-target effects stem from the fact that these therapies are aiming at the "passengers" but not the "drivers" in the cancer cell "bus."

A major metabolic hallmark of cancer is to divert glucose flux away from mitochondrial oxidation to cytosolic fermentation and lactate production, a process also known as the Warburg effect (160, 161). According to Warburg himself, the consequence of this metabolic reprogramming is to convert differentiated normal cells to undifferentiated cells, namely, cancer cells (4, 162). Hence, identifying compounds that can target the metabolic reprogramming of cancer should present substantial benefit for cancer treatment. Recently, we found that the mitochondrial uncoupler niclosamide could reverse this metabolic hallmark of cancer, leading to a rewiring of the global epigenetic landscape and the induction of cell differentiation (8). Thus, we propose the mitochondrial uncoupler niclosamide can serve as a compound to target cancer metabolic reprogramming.

Numerous oncogenic pathways or tumor suppressors have been reported to be influenced by niclosamide treatment. However, these alterations could be secondary effects resulting from inhibition of the primary target. What could this primary target be? Three major targets of niclosamide have been proposed. Firstly, as a mitochondrial uncoupler, niclosamide uncouples the mitochondrial membrane potential from ATP synthesis (11). Secondly, the protonophoric activity of niclosamide can dissipate protons from lysosomes (59). Thirdly, niclosamide directly binds to GSK3, resulting in disruption of the Axin-GSK3 complex and attenuation of canonical Wnt activity (37). Among these targets, the mitochondrial uncoupling function is reported to be essential for targeting both p53 wild-type and mutant cancers (20). Nonetheless, additional studies are needed to elucidate the primary target of niclosamide as an anti-tumor compound.

Clinically, the major challenge for niclosamide is poor oral bioavailability, potentially limiting its use as a cancer drug (11, 156). Efforts have been taken to improved its bioavailability, including: (1) reformulating niclosamide for better delivery and stability (158, 163–166) and (2) modifying the structure of niclosamide to generate derivatives with enhanced efficiency (167, 168) or pharmacokinetics (169, 170). Nonetheless, the process of identifying these derivatives involved screens with readouts of either cell apoptosis or oncogenic pathway inhibition, processes that may not reflect the primary property of niclosamide as anti-tumor compound, thereby reinforcing the need to identify the primary target of niclosamide to accelerate pharmacological development of new derivatives. Another area of important need is to improve the clinical potential of niclosamide; specifically, initiating studies that address the synthetic lethality of niclosamide in cancer to identify pathway dependencies or gene mutations sensitive to niclosamide treatment. Based on the results of clinical trials, it is likely that niclosamide treatment alone will not be enough to achieve a complete response in cancer patients. Therefore, further effort is needed to test combination therapies using niclosamide with other therapeutic agents.

## Author contributions

HJ, AL and JY conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

HJ and JY submitted a patent application related to this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# The "sweet" path to cancer: focus on cellular glucose metabolism

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The hypoxia-inducible factor-1α (HIF-1α), a key player in the adaptive regulation of energy metabolism, and the M2 isoform of the glycolytic enzyme pyruvate kinase (PKM2), a critical regulator of glucose consumption, are the main drivers of the metabolic rewiring in cancer cells. The use of glycolysis rather than oxidative phosphorylation, even in the presence of oxygen (i.e., Warburg effect or aerobic glycolysis), is a major metabolic hallmark of cancer. Aerobic glycolysis is also important for the immune system, which is involved in both metabolic disorders development and tumorigenesis. More recently, metabolic changes resembling the Warburg effect have been described in diabetes mellitus (DM). Scientists from different disciplines are looking for ways to interfere with these cellular metabolic rearrangements and reverse the pathological processes underlying their disease of interest. As cancer is overtaking cardiovascular disease as the leading cause of excess death in DM, and biological links between DM and cancer are incompletely understood, cellular glucose metabolism may be a promising field to explore in search of connections between cardiometabolic and cancer diseases. In this mini-review, we present the state-of-the-art on the role of the Warburg effect, HIF-1α, and PKM2 in cancer, inflammation, and DM to encourage multidisciplinary research to advance fundamental understanding in biology and pathways implicated in the link between DM and cancer.

KEYWORDS

**aerobic glycolysis, diabetes mellitus, hypoxia inducible factor (HIF)-1α, inflammation, methylglyoxal (MGO), oxidative phoshorylation, pyruvate kinase M isoform 2, Warburg effect**

## 1 Introduction

Recent epidemiological studies have reported a transition from cardiovascular diseases to cancer as the leading cause of excess death associated with diabetes mellitus (DM) (1, 2). Cancer mortality among people with DM, especially type 2 (T2) DM, is approximately 30%-50% higher than in the general population, particularly for pancreatic, liver,

colorectal, and endometrial cancers (3, 4). Clinical and preventive efforts must be directed at fighting DM-related risk factors for cancer to reduce the excess mortality risk in individuals with DM.

Possible mechanisms for a biological link between DM and cancer are hyperinsulinemia, inflammation, and hyperglycemia (5). Hyperglycemia is the distinctive feature of DM and the main cause of various life-threatening complications in both type 1 (T1) DM and T2DM (6, 7). A direct link between hyperglycemia and cancer comes from studies showing that, at high concentrations, glucose acts as DNA-damaging factor and impedes tumor suppressive functions, leading to genomic instability and eventually resulting in malignant transformation (8, 9). DM has been also associated with cancer promotion and progression (10–12); mechanisms involved in the regulation of cancer cell metabolism and the way cancer cells utilize glucose may mediate this association.

After briefly summarizing the molecular and biochemical characteristics of the Warburg effect in cancer cells, we will examine the updated evidence demonstrating similar metabolic and molecular changes in immune cells involved in inflammation and in target cells and tissues of chronic DM complications. In particular, the role of hypoxia inducible factor (HIF)-1α and M2 isoform of the glycolytic enzyme pyruvate kinase (PK) in driving the metabolic reprogramming of tumor, inflammatory, and diabetic cells will be discussed. Finally, to foster multidisciplinary investigation, the collected evidence will be illustrated in the context of a plausible hypothesis centered on changes in cellular glucose metabolism as mechanistic link between DM and cancer.

## 2 Warburg effect, HIF-1α, and PKM2 in cancer: when metabolism rhymes with opportunism

In cancer, a close relationship exists between the rate of glucose utilization and that of cell proliferation (13). In 1924, Otto Warburg identified the link between cancer and glucose by showing that tumor tissues consume and metabolize to lactate tremendous amounts of glucose relative to non-transformed tissues (14). While some cancer cells are oxidative and targeting mitochondrial oxidative phosphorylation (OXPHOS) may be a promising therapeutic target for oxidative carcinomas (15), most cancers cells exhibit suppressed mitochondrial respiration and a high rate of glucose uptake even in the presence of oxygen. This metabolic rewiring is known as both Warburg effect and aerobic glycolysis. Consistent with the importance of the Warburg effect for cancer cells, withdrawing glucose or inhibiting glycolysis is deleterious to tumorigenesis in experimental models of cancer (16, 17). How cancer cells take advantage from these metabolic changes and how glycolysis is related to cell proliferation is still not fully understood. Along with the proposal that the Warburg metabolism may be a way to produce ATP quickly (18), widely accepted hypothesis include: 1) expansion of the pool of glycolytic biosynthetic intermediates to support anabolic reactions and redox demand (19), 2) persistent NAD+ regeneration to sustain *de novo* lipogenesis (20), and 3) augmented lactate production to favor tumor growth and metastasis by affecting the tumor microenvironment (21). Along with changes in the tissue microenvironment, oncogenes and tumor suppressors that drives tumorigenesis contribute to the acquisition of the Warburg phenotype *via* activation of numerous transcription factors (including HIF-1α) regulating several genes encoding glycolytic proteins (including PKM2) (22).

HIF-1α is a master regulator of oxygen homeostasis playing a key role in the adaptive regulation of energy metabolism in mammalian tissues. By simultaneously increasing the expression of glycolytic enzymes and restraining mitochondrial function, HIF-1α can switch glucose metabolism from OXPHOS to glycolysis also in response to physiological and pathological stimuli other than hypoxia (23), including hyperglycemia-induced oxidative (24) and carbonyl (25) stress. In cancer cells, HIF-1α cooperates with the oncoprotein MYC to activate transcription of genes involved in glucose metabolism, including glucose transporters (e.g. GLUT1 and GLUT3) and glycolytic enzymes (e.g. lactate dehydrogenase A, hexokinase 2, PKM2, etc.) (26). In addition to stimulate glycolysis, HIF-1α actively represses mitochondrial respiration and biogenesis by inducing pyruvate dehydrogenase kinase 1 (27) and reducing peroxisome-proliferator-activated receptor γ co-activator-1α (28). Consistent with an important role in cancer cell biology, HIF-1α overexpression strongly correlates with poor prognosis for several solid cancers. Accordingly, pharmacological targeting of the HIF-1α signaling pathways has been recognized as a promising strategy for cancer therapy in the recent years (29).

The PKs are terminal enzymes of the glycolytic pathway that catalyze the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP and are important modulators of cellular glucose metabolism. The PKM1/M2 isoforms are encoded by the same gene (*PKM*) and are generated by the alternative splicing of *PKM* mRNA (30). While PKM1 only exists as a stable and highly active tetrameric form and is expressed in most adult tissues (31), PKM2 is highly expressed during embryonic development and is reactivated in tissue regeneration and tumor development, suggesting that it is critical for actively proliferating cells (32). Unlike the constitutionally active PKM1, PKM2 is in equilibrium among the dimeric and monomer forms, which are catalytically inactive, and the active tetrameric form. Therefore, the glycolytic activity of PKM2 is subject to allosteric control (33). This implies that, at the same protein level, PKM2 is much less effective than PKM1 in catalyzing the last step within glycolysis (31). Accordingly, high ratios of PKM2/PKM1 lead to accumulation of all upstream glycolytic intermediates and diversion of metabolic flux towards the glycolytic biosynthetic branches, including the pentose-phosphate pathway, the hexosamine pathway, and the glycerol synthesis (34). This process is exploited by cancer cells to sustain their high biosynthetic and redox demand (35).

PKM2 and HIF-1α regulate each other. In fact, PKM2 is a transcriptional target of HIF-1α and a key player in the Warburg effect of glycolytic cancer cells (26). In turn, as a dimer, PKM2 translocates into the nucleus, interacts with, and promotes the transcriptional activity of HIF-1α (36). Therefore, HIF-1α and PKM2 are recognized as major drivers of cancer metabolism participating in a positive feedback loop that enhances the Warburg effect and feeds the glycolysis branch pathways (23, 30).

## 3 Warburg effect, HIF-1α, and PKM2 in inflammation: a matter of polarization

It has been almost 50 years since the first demonstration of aerobic glycolysis during lymphocyte proliferation (37). In the 2000s, some observations on metabolic reprogramming were extended to other cells of innate and adaptive immunity. Since then, a growing interest in the role of metabolism in immune regulation has bloomed. The exciting advances in the field of immunometabolism have been recently reviewed (38, 39). We summarize here the role of aerobic glycolysis, HIF-1α, and PKM2 in immune cells involved in chronic inflammation, which participates in all stages of tumorigenesis as well as DM development and progression to complications (40, 41).

In dendritic cells and macrophages, pro-inflammatory stimuli induce the shift to aerobic glycolysis and the production of inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) (42). IL-1β and TNF-α are involved in insulin resistance (43, 44) and, together with other IL family members, promote tumorigenesis through complex mechanisms that involve direct growth stimulation and production of growth factors, recruitment of myeloid cells and immunosuppression, endothelial cell activation and promotion of angiogenesis (45, 46). In macrophages, the metabolic rewiring towards an enhanced glycolytic phenotype promotes polarization to the classically activated (or "M1") phenotype and the production of many inflammatory mediators (44). Consistently, the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) blocks (47), whereas GLUT1 overexpression enhances (48) M1 inflammatory functions. Conversely, OXPHOS is critical for the anti-inflammatory and tissue repair functions of alternatively activated (or "M2") macrophages (49, 50). The balance between glycolysis and mitochondrial respiration also differentially regulates the phenotype and function of various subsets of T cells. For instance, T regulatory cells (Tregs) rely on glycolysis only during initial activation and proliferation, after that they switch toward oxidative metabolism for their regulatory functions. Consistently, GLUT1 expression increases the number of Tregs, but reduces their immunosuppressive capacity (51). Vice versa, T helper (Th)17 cells - a distinct subset of CD4+ T cells that produce the highly pro-inflammatory IL-17 – can be converted into Tregs by blocking glycolysis with 2-DG (52). Like the pro-inflammatory CD4+ Th17 cells, CD8+ lymphocytes require a Warburg-like metabolism not only for their proliferative capacity, but also for their effector functions (53, 54).

It is by now long-established that HIF-1α stabilization in immune cells can occur in an oxygen-independent manner. Bacteria and their cell membrane component lipopolysaccharide (LPS), inflammatory mediators, and endogenous molecules, such as the tricarboxylic-acid cycle intermediate succinate (47), can induce HIF-1α protein accumulation in macrophages through transcriptional and post-translational mechanisms under normoxic conditions (55–58). By cross talking with the nuclear factor-κB pathway, HIF-1α modulates essential inflammatory functions in myeloid cells (59). In keeping with a critical role for HIF-1α in the pro-inflammatory response in macrophage and T cells, HIF-1α deletion induces defective macrophage response to LPS and inhibits Th17 cell generation in mice (44, 60).

In addition to induce HIF-1α accumulation, pro-inflammatory stimuli trigger the expression of PKM2, which is now recognized as a critical determinant of the Warburg effect in macrophages. In fact, stabilizing PKM2 tetramerization with the allosteric activator TEPP-46, thus favoring PKM2 glycolytic activity and the glycolytic flux toward pyruvate, restores OXPHOS and reduces LPS-induced production of IL-1β, while promoting macrophage M2 polarization (61). In addition, PKM2 over expression induces, whereas downregulation inhibits, the activation of several toll-like receptor pathways (62). PKM2 tetramerization by TEPP-46 also blocks PKM2 nuclear translocation and restrains pro-inflammatory polarization in T cells by inhibiting the Warburg metabolism and favoring OXPHOS (63).

Overall, upregulation of aerobic glycolysis supports inflammatory immune functions. Hindering HIF-1α accumulation and PKM2 expression or favoring glycolytic activity over transcriptional activity of PKM2 by allosteric activation, blocks the Warburg metabolism and curbs inflammatory responses by supporting regulatory and anti-inflammatory immune phenotypes.

## 4 Warburg effect, HIF-1α, and PKM2 in diabetes: team members or individual runners on the road to complications?

While confirming previous findings of increased levels of glycolytic intermediates, recent omics studies in DM and related target organ damage have provided evidence of impaired mitochondrial metabolism and biogenesis, along with other features of a metabolic rewiring resembling the Warburg effect (64–66). Several metabolic intermediates and glycolytic enzymes, including PKM2, have been proposed as potential triggers of aerobic glycolysis and diversion of glycolytic intermediates into branch pathways (64).

Diabetic complications arise in tissues that exhibit insulin-independent glucose uptake (67, 68). In the cells of these tissues, activation of aerobic glycolysis may be a consequence of increased glucose uptake from systemic circulation and an attempt to quickly metabolize excess cellular glucose (69) (Figure 1). The drawback of this process is the cellular accumulation of toxic glucose metabolites (66, 70). In fact, at variance with cancer cells, normal cells are not actively proliferating. Accordingly, the enhanced glucose uptake, buildup of glycolytic intermediates, and flux through the glycolytic branch pathways results in an accumulation of sorbitol, diacylglycerol, and advanced glycation end products (AGEs) leading to the activation of pro-inflammatory and -oxidative pathways (71). To our knowledge, only one study has attempted to establish a relationship between mitochondrial dysfunction, the Warburg-like metabolism, and accumulation of toxic glucose metabolites in DM. By showing that high glucose induces HIF-1α activity and a switch from oxidative metabolism to glycolysis and its principal branches, this study suggests that aerobic glycolysis may play an initiating role in glucotoxicity and diabetic complications (25).

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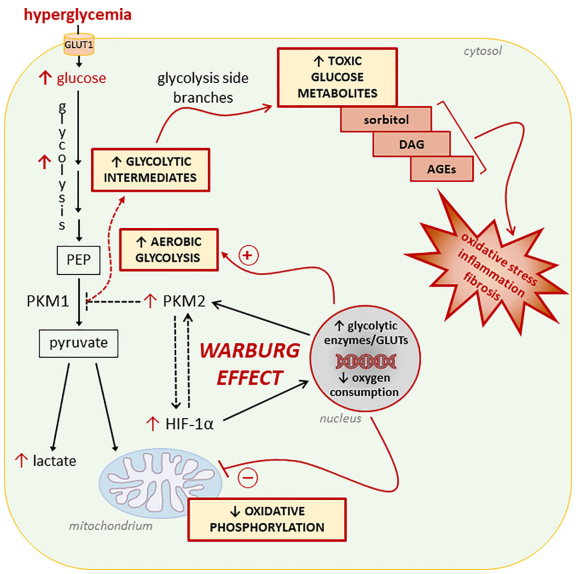


FIGURE 1
Glucose metabolic reprogramming induced by hyperglycemia in insulin-independent cells. Refer to the main text for detailed description and references. Dashed lines/arrows indicate well-established processes in cancer cells not yet confirmed in normal cells exposed to glucose concentrations in the diabetic range. In particular, the interaction between the hypoxia inducible factor 1-α (HIF-1α) and the M2 isoform of pyruvate kinase (PKM2), and the role of PKM2 in reducing the overall PK activity leading to accumulation of glycolytic intermediates need to be demonstrated. PEP, Phosphoenolpyruvate; GLUTs, glucose transporters; DAG, diacylglycerol; AGEs, advanced glycation end-products.

In addition to hypoxia, numerous metabolic stressors associated with DM, including hyperglycemia, affect HIF-1α stabilization and activity. Due to the heterogeneity of the findings, what is the net impact of DM-related factors on HIF-1α signaling is a matter of debate in literature (72). Concisely, hyperglycemia seems to inhibit HIF-1α expression induced by low-oxygen conditions (73, 74), suggesting a weaker HIF-1α-dependent response to hypoxia in DM. Conversely, high glucose concentrations promote HIF-1α nuclear translocation, transcriptional activity, and lactate accumulation in normoxic conditions (25, 72, 75–78). Ultimately, these discrepancies in the effect of DM on HIF-1α signaling are likely the result of different experimental conditions (e.g., hypoxia vs normoxia) used for modulating the levels and activity of HIF-1α. In this regard, it must be remembered that the Warburg effect occurs, by definition, in the presence of normal levels of oxygen.

Recently, proteomic studies conducted in T1DM patients have demonstrated increased circulating and renal levels of mitochondrial and glycolytic enzymes in those without diabetic nephropathy (DN). Curiously, elevated levels of the underactive glycolytic enzyme PKM2 were associated with reduced renal accumulation of toxic glucose metabolites and susceptibility to DN, suggesting a protective role of PKM2 by improving cellular glucose metabolism (66, 79). However, preclinical work by the same and other investigators has shown that TEPP-46 treatment reverses metabolic abnormalities, mitochondrial dysfunction, and kidney pathology of diabetic mice by enhancing PKM2 tetramer formation (i.e., glycolytic activity) and suppressing HIF-1α and lactate accumulation (66, 80, 81). These experimental findings suggest that also in DM, as in cancer and immune cells (61, 82), PKM2 overexpression may favor glucose metabolic reprogramming towards aerobic glycolysis, accumulation of glycolytic intermediates, and pro-inflammatory signaling. Collectively, these interesting findings could lead to greater understanding of DM complications if the functional complexity of PKM2 and its role as a key regulator of glucose metabolism is considered.

In general, the body of knowledge acquired in a century of research on cancer and immune metabolism has been overlooked in the interpretation of the data concerning several aspects of cellular glucose metabolism in DM, including aerobic glycolysis, HIF-1α induction, and PKM2 expression. For example, claims that HIF-1α activity should be enhanced in DM because HIF-1α signaling is submaximal for the degree of hypoxia in diabetic tissues (83, 84) overlook the role of this transcription factor in aerobic glycolysis and inflammation. Increased cellular glucose uptake, mitochondrial dysfunction, and pro-inflammatory signaling induced by HIF-1α activity (26–28, 44) would not be at all beneficial for tissues affected by DM complications (70). Rather, regarding the role of chronic hypoxia in DM complications, it might be more biologically sound to promote the activity of the HIF-2α isoform, which has different effects to HIF-1α on glucose metabolism and even opposite effects on redox state and inflammation (72, 85). Another naive conclusion, which is inconsistent with the mechanisms that regulate cellular glucose metabolism and drive hyperglycemia-induced cell damage, is that overall PKM2 expression should be increased to enhance PK activity and prevent DM complications (80, 86). Actually, to favor glycolytic flux to pyruvate and reduce the accumulation of toxic glucose metabolites, the expression of the constitutively active enzyme PKM1 should be preferred over that of PKM2. Favor the expression of the less active isoform M2 and then have to enhance PK activity with pharmacological agents does not seem the best strategy to improve cellular glucose metabolism and prevent DM-induced target organ damage. As in cancer (87), PKM2 activators are interesting drugs to consider for complications of DM, but the simplistic view that high PKM2 levels mean high levels of PK activity must be overcome. In fact, it is the exact opposite (31, 61).

Overall, more research is needed to fully characterize the changes in cellular glucose metabolism associated with DM complications and the role of HIF-1α and PKM2 as components of a molecular network that regulates metabolic reprograming towards the Warburg effect.

## 5 Discussion

Tight glucose control is important for reduction of cancer risk in T2DM (88). Substantial evidence supports a direct causal link between DM and carcinogenesis; hyperglycemia can in fact induce malignant transformation by causing DNA damage (9), oncogenic mutations (8), and loss of tumor suppressive functions (89). Besides this, cancer and DM share similar changes in some aspects of cellular energetics, particularly glucose metabolism. Hyperglycemia leads to excessive cellular glucose uptake and changes in glucose

metabolism resembling the Warburg effect, including accumulation of glycolytic intermediates (64–66). Interestingly, among the molecular mechanisms associated with the anti-tumor activity of the anti-diabetic drug metformin, suppression of the Warburg effect has also been proposed (90). The question whether the Warburg effect, besides being a consequence, might also play a causal role in carcinogenesis has been raised in the past without receiving much attention, mainly because of the lack of plausible pathomechanisms (91). However, there are numerous clues that lead us to consider the metabolic reprogramming induced by hyperglycemia as a possible field of investigation to unravel the connections between diabetes and cancer.

Tumorigenesis comprises multiple steps of mutations subjected to a natural selection (Figure 2). Environmental forces and cellular adaption mechanisms that provide the mutated cell clone with survival and proliferative advantages over the neighboring cells govern this process (92). As tumor microenvironment factors influence cancer metabolism (93), hyperglycemia may promote the acquisition of a Warburg metabolism in transforming cells. In turn, hyperglycemia-mediated glycolytic reprogramming may contribute to shape the metabolic features of the evolving tumor cells by increasing the activity and fostering mutations of oncogenes regulating cell metabolism (8, 93), thus playing an active role in cancer promotion and progression. For example, high glucose was recently shown to stabilize and induce aberrant transcriptional activity of N-MYC - a member of the MYC family - even in normal cells, leading to increased proliferation and functional impairment (94). Overall, by inducing a Warburg-like metabolism, hyperglycemia might favor tumorigenesis by both contributing to the selection of more malignant phenotypes and directly inducing, in normal cells, the transcriptional activity of oncogenes that regulate multiple aspects of tumor metabolism, eventually increasing the chances of malignant transformation.

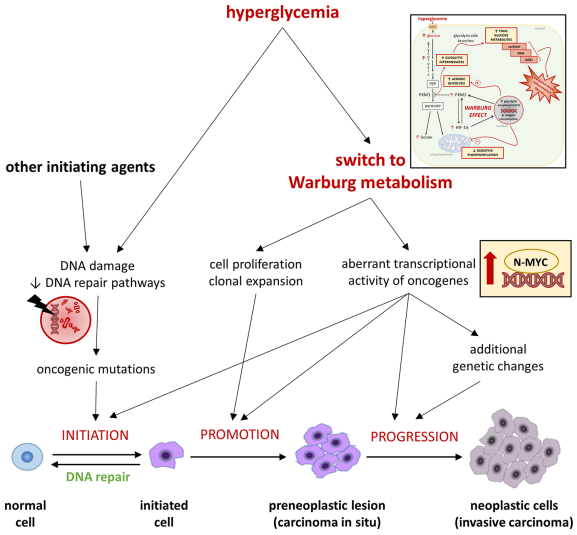


FIGURE 2
Hyperglycemia and the Warburg-like metabolism driven by excessive cellular glucose uptake in cancer initiation, promotion, and progression. Refer to the main text for detailed description and references.

The mechanisms by which aerobic glycolysis favors cell proliferation, regulates inflammatory immune functions, and induce cell damage in DM are not yet fully elucidated. The "anabolic" (34) and "energetic" (18) hypotheses explain how the Warburg metabolism provides building blocks and an increased rate of ATP to support the anabolic and energetic demand of proliferating cells. Regardless of the discussion of their validity (18), the current hypotheses do not address the question of the causal relationship and mechanistic link between aerobic glycolysis and cell proliferation in tumor and immune cells, or cell injury in DM. Studies in the fields of immunology and metabolism have identified interesting alternative (or complementary) mechanisms that may explain how glycolytic reprogramming benefits cancer and immune cells and promotes DM complications. These mechanisms rely on the signaling function of glycolytic intermediates and/or their spontaneous decomposition products, including the inevitable side-product of glycolysis methylglyoxal (MGO). This is a highly reactive dicarbonyl compound and major precursor of advanced glycation end-products (AGEs) (95). By acting at both transcriptional and post-translational levels, MGO plays important roles in the immune response to inflammatory stimuli (96–98) and, together with AGEs, is involved in the pathogenesis of DM complications (25, 95, 99–101) and in the onset and progression of many cancers (10, 11, 102–104).

In conclusion, oncology and immunology scientists have continued to build on the seminal work by biochemists to improve understanding of glucose metabolic rewiring in cancer and immune system biology and pathology. Researchers in endocrinology and metabolism have lagged behind in this process and are struggling to put the puzzle pieces together. A multidisciplinary approach could not only help to unravel the skein and effectively interpret data for a real progress in cardiometabolic research, it also may generate new knowledge on the mechanisms linking DM and cancer.

## Author contributions

Writing—original draft preparation, CI and SM; writing—review and editing, MV and GP; visualization, SM; supervision, GP; funding acquisition, SM. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Colorectal cancer and therapy response: a focus on the main mechanisms involved

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**Introduction:** The latest GLOBOCAN 2021 reports that colorectal cancer (CRC) is the second leading cause of cancer-related death worldwide. Most CRC cases are sporadic and associated with several risk factors, including lifestyle habits, gut dysbiosis, chronic inflammation, and oxidative stress.

**Aim:** To summarize the biology of CRC and discuss current therapeutic interventions designed to counteract CRC development and to overcome chemoresistance.

**Methods:** Literature searches were conducted using PubMed and focusing the attention on the keywords such as "Current treatment of CRC" or "chemoresistance and CRC" or "oxidative stress and CRC" or "novel drug delivery approaches in cancer" or "immunotherapy in CRC" or "gut microbiota in CRC" or "systematic review and meta-analysis of randomized controlled trials" or "CSCs and CRC". The citations included in the search ranged from September 1988 to December 2022. An additional search was carried out using the clinical trial database.

**Results:** Rounds of adjuvant therapies, including radiotherapy, chemotherapy, and immunotherapy are commonly planned to reduce cancer recurrence after surgery (stage II and stage III CRC patients) and to improve overall survival (stage IV). 5-fluorouracil-based chemotherapy in combination with other cytotoxic drugs, is the mainstay to treat CRC. However, the onset of the inherent or acquired resistance and the presence of chemoresistant cancer stem cells drastically reduce the efficacy. On the other hand, the genetic-molecular heterogeneity of CRC often precludes also the efficacy of new therapeutic approaches such as immunotherapies. Therefore, the CRC complexity made of natural or acquired multidrug resistance has made it necessary the search for new druggable targets and new delivery systems.

**Conclusion:** Further knowledge of the underlying CRC mechanisms and a comprehensive overview of current therapeutic opportunities can provide the basis for identifying pharmacological and biological barriers that render therapies ineffective and for identifying new potential biomarkers and therapeutic targets for advanced and aggressive CRC.

KEYWORDS

**CRC, adjuvant treatments, chemoresistance, CSCs, drug delivery system**

# 1 Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of death worldwide regardless of gender (1). Approximately 90% of CRCs are adenocarcinoma originating from epithelial cells of the colorectal mucosa, whilst the remaining 10% are represented by rare CRC types (i.e., squamous cell carcinoma, adenosquamous carcinoma, spindle cell carcinoma, and undifferentiated carcinoma) (2).

Most CRC cases are sporadically and associated with non-hereditary spontaneous mutations and epigenetic aberrations arising from several risk factors, including dysregulation of the gut microbiome, obesity, sedentary lifestyle, excess intake of meats, fats, starches, and sugars, folate deficiency, alcohol, cigarette smoking, and so on (3). However, a lower percentage of cases (about 30%) is represented by familial cases, of which approximately 5% present specific genetic signatures, penetrance, and transmission due to germline variants in CRC predisposing genes, e.g., adenomatous polyposis coli (APC), mismatch repair (MMR) genes, epithelial cell adhesion molecule (EPCAM), SMAD4/BMPR1A, and MUTYH (4–7).

Data report that the highest CRC incidence rates are recorded in developed countries and the incidence of early-onset CRC in individuals younger than 50 continues to rise (2, 4). Therefore, to facilitate diagnosis of CRC cancer in earlier stages, the recommended screening age was recently lowered to 45.

Early-stage colon cancer may be asymptomatic and often become symptomatic late in the disease. Indeed, about >25% of patients are diagnosed with advanced disease, i.e., extensive or metastatic colorectal cancer (mCRC), at the time of diagnosis, while more than 50% of patients with the initially localized disease develop metastases during or after therapies (8, 9). As known, metastasis poses a huge clinical challenge because only 20% of mCRC patients survive (10).

When neoadjuvant therapy is not included in the treatment plan, surgical resection is performed as the first curative intent in patients with localized and locoregional CRC (stages I, II, and III), as well as for those with resectable distant metastases (5, 11–13). However, the National Comprehensive Cancer Network (NCCN) guidelines recommend neoadjuvant oxaliplatin-based chemotherapy for patients with "bulky nodal disease or clinical T4b" colon cancer to decrease the size of the tumor before surgery (14). To reduce the risk of cancer recurrence and improve patient outcomes, an adjuvant postoperative chemotherapy regimen is routinely employed in stage III patients (i.e., localized tumor with lymph node invasion) and, in some cases, in stage II patients (i.e., localized tumor w/o lymph node invasion) (15, 16). Moreover, chemotherapy is the first-line therapy also for mCRC treatment.

The genetic variability of CRC makes necessary to identify the tumor subtypes (e.g., mismatch repair or microsatellite instability status, mutations in KRAS, NRAS, BRAF) to set the most suitable adjuvant therapy (i.e., systemic chemotherapy alone or with other FDA-approved drugs).

The CRC prognosis depends essentially on comorbid conditions, the frailty of patients, and drug resistance promoted by cancer stem cells and/or genetic mutations in key driver genes (e.g., KRAS, p53, BRAF) (17, 18). Therefore, this present review aims to summarize the mechanisms that characterize the stepwise nature of CRC, its genetic landscape, and the current and future approaches for CRC management.

# 2 Methods

The data in this present systematic review were collected using two different searches: PubMed (https://pubmed.ncbi.nlm.nih.gov/) and an online bioinformatic database (http://clinicaltrials.gov). The search of the references using PubMed identified a total of 144 hits from 1988 to 2022 relatively to keywords such as "Current treatment of CRC" or "immunotherapy in CRC" or "gut microbiota in CRC" or "chemoresistance and CRC" or "oxidative stress and CRC" or "novel drug delivery approaches in cancer" or "CSCs and CRC" or "systematic review and meta-analysis of randomized controlled trials". Thus, the combined information obtained from the two data sources has represented the basis for writing the review.

# 3 Results

## 3.1 Mechanisms of CRC initiation and progression

The stepwise nature of sporadic CRC is still poorly understood, even though several mechanisms have been described to be involved in its initiation and progression. Epidemiological studies have found a relationship between CRC and chronic exposure to environmental risk factors (see above section) with strong pro-inflammatory potential. Moreover, increasing evidence suggests that intestinal microbiota and its products (e.g., butyrate and bacterial toxins) play a pivotal role in all CRC steps (initiation, progression, and metastasis) (19–21) (Figure 1). CRC patients display a reduced bacterial diversity and richness compared to healthy individuals, reflecting a distinctive intestinal microbial dysbiosis (22). Dysbiosis causes alteration in gut mucosa integrity and permeability, due to alteration of intercellular tight junctions. This condition, enhancing the colocyte susceptibility to mutagenic/carcinogenic factors and pathogenic bacteria, also promotes the activation of Mucosal Associated Lymphatic Tissue. Moreover, Th2-derived cytokines induced by pathogens or autoantigens may result in myeloid cell recruitment (neutrophils and macrophages) and, consequently in Reactive Oxygen Species (ROS) production.

As known, inflammation and oxidative stress are tightly coupled; in fact, a chronic activation of inflammatory cells and production of pro-inflammatory mediators (e.g., cyclooxygenase 2, prostaglandin E2, tumor necrosis factor α, and transforming growth factor β) enhance ROS generation and dysregulate the activity of signal transduction pathways, including Transducer and Activator of Transcription 3, Nuclear Factor-kappa B (NF-kB), hypoxia-inducible factor-1α (HIF1α), NF-E2 related factor-2 (NRF2) (23–25). In addition, it has been reported that ROS overproduction may result in genetic/epigenetic changes, such as

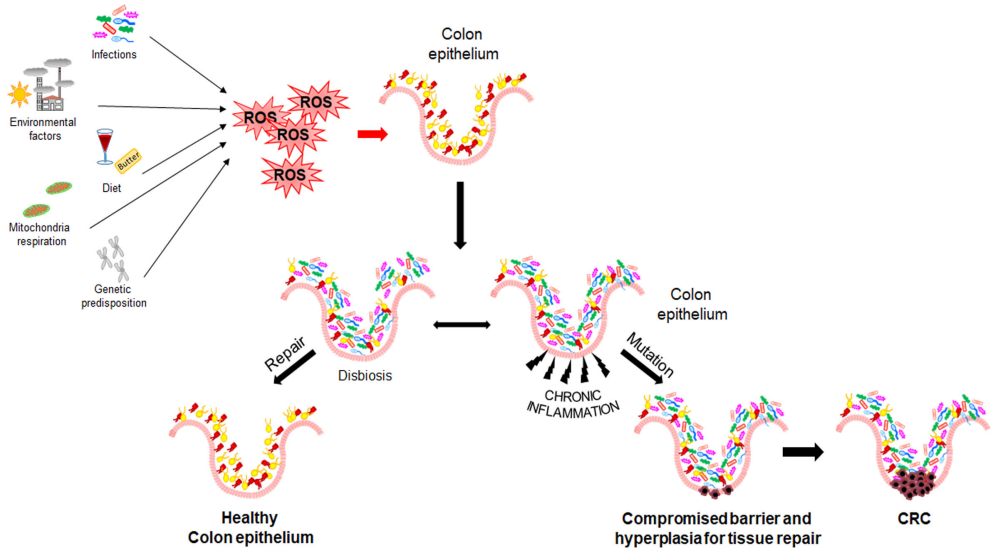


FIGURE 1
Mechanisms of CRC initiation and progression. Environmental and genetic risk factors promoting Reactive Oxygen Species production, dysbiosis, and inflammation.

single-strand cleavage, point mutations, miscoding, abnormal amplification, oncogene activation, and immune suppression, leading to possible precancerous lesions (i.e., adenomatous polyps) (26, 27).

Mutations providing a selective growth advantage to the cells within their microenvironment can potentially drive cancer development. Generally, two mutated gene drivers can lead to a net cell gain and detectable benign lesions, but over three gene mutations promote the invasion through the basement membrane, thereby leading to malignancy. In CRC, the first mutations usually concern the APC gene resulting in a proliferative advantage of epithelial cells promoting benign lesions (small adenomas) (Figure 2). APC mutated small adenomas have a slow growth rate, but further mutations of the KRAS gene can increase their proliferation. However, the mutational status may be worsen by sequential mutations of genes such as PIK3CA, SMAD4, and TP53 that promote the onset of malignant tumors capable of infiltrating surrounding tissue and metastasizing distant organs (Figure 2) (28). In addition, both colorectal adenomas and CRC are linked to epigenetic alterations such as aberrant DNA methylation in key tumor-suppressor and oncogenes and dysregulation of miRNA expression (29, 30).

Well-known examples of the carcinogenic role of ROS in CRC are missense mutations at p53 suppressor gene, activation of canonical Wnt signaling pathway (Wnt/β-catenin), which is involved in cancer stem cell renewal process, and PI3K/Akt signaling pathway, which regulates cell proliferation (31, 32). Moreover, the most frequent ROS-dependent pre-mutagenic DNA lesion is represented by 8-oxoguanine (8-oxoG) (33).

Notably, oxidative stress, due to either ROS overproduction or a reduced activity of the enzymatic and non-enzymatic antioxidant systems, is often involved in development and progression of several cancers via activation of redox-responsive signaling pathways leading to uncontrolled cell growth and oxidation of lipids, carbohydrates, and proteins (i.e., cancer initiation, promotion, and progression stages). Accordingly, oxidative stress is one of the main cancer research topics given its involvement in both genetic and metabolic cell damage (34–36). Increasing evidence showed that oxidative stress control biogenesis of cancer-associated microRNA (miRNA) via targeting various

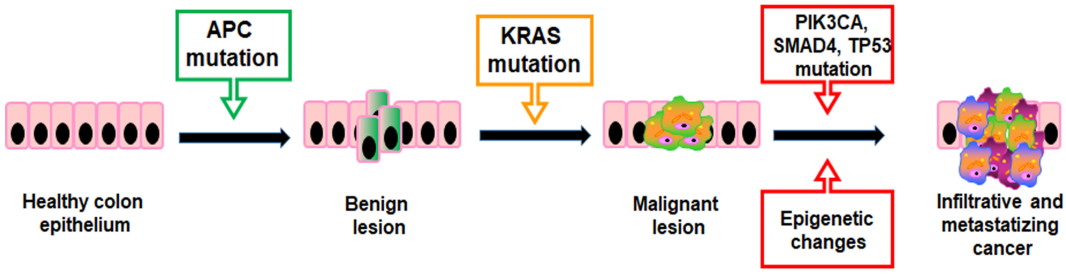


FIGURE 2
Stepwise molecular/genetic events that underlie the initiation and progression of CRC.

transcription and epigenetic factors. Recently, CRC-associated miRNAs (*e.g.*, miR-106b-5p, miR-335-5p, miR-193a-5p, miR-378a-3p and miR-423-5p) are becoming attractive biomarkers as they are expressed from the early stage of tumor development (37).

Moreover, either up-regulation or down-regulation of miRNAs also known as "onco-miRNAs" are involved in CRC progression and metastasis contributing to dysregulate several signaling pathways, including mitogen-activated protein kinases (e.g., miR-422a, miR-195), Wnt (e.g., miR-135a, miR-135b, miR-155, miR-17–5p, miR-224), transforming growth factor-β (e.g., miR-224, miR-20a-5p) and epithelial-to-mesenchymal transition (EMT) (e.g., miRNA-155, miR-34) (38–40).

Oncogene, oncosuppressor and metabolic gene mutations contribute to the profound metabolic alterations found in cancer cells, i.e., impaired respiration, increased fermentation and anabolism (41). In most cases, the metabolism of cancer cells favors aerobic glycolysis (the Warburg effect) rather than oxidative metabolism to fulfill their biosynthetic and bioenergetic demands of rapid and sustained proliferation. Mitochondrial Oxidative Phosphorylation System (OXPHOS) is not necessarily defective in tumorigenic cells, and it can take place proportionally to the oxygen supply. Indeed, it has been shown that cancer stem cells are able to revert glycolysis to TCA cycle to better satisfy their metabolic needs and overproduce ROS (42). The Warburg phenotype has been demonstrated to be driven by overexpression of oncogenes such as c-Myc and HIF-1α (43). The inhibition of pyruvate dehydrogenase activity and the increase of lactate dehydrogenase activity lead to the conversion of pyruvate to lactate following the mass action law (44). Moreover, lactate is also generated from catabolism of glutamine and it is considered a metabolite eliciting a broad spectrum of effects useful to sustain cancer progression and metastasis (45). Cancer cells are capable of adapting to metabolic-derived acidosis via monocarboxylate transporters (MCTs), which export lactate and favor intracellular alkalinization. Thus, the lactate exported by tumor cells can be imported by cells of tumor microenvironmental where it acts as important intracellular signaling for angiogenesis (46, 47).

Notably, cancer cells well adapt to ROS by triggering a powerful antioxidant response mainly driven by glutathione (GSH) and antioxidant enzymes, such as superoxide dismutase, catalase, peroxiredoxins, GSH peroxidases, and thioredoxins (48). Thus, the maintenance of the oxidative balance enables cancer cells to perform their biological functions such as proliferation, differentiation, and migration (49–51).

### 3.1.1 Genetic- molecular heterogeneity of CRC

CRC from a genetic-molecular standpoint is extremely diversified. In fact, there are four main mechanisms of gene alteration: (i) microsatellite instability (MSI), (ii) chromosomal instability (CIN), (iii) CpG island methylator phenotype (CIMP), (iv) and BRAF or KRAS mutations (52). Another aspect concerning the molecular and phenotypic differences is the tumor localization (i.e., the right or left side), which leads to different gene expression and mutation profiles. Right-sided CRC occurs mainly in patients with genetic predisposition and is characterized by hypermethylation, higher frequency of BRAF mutation, and, in some cases, MSI (53). Instead, left-sided CRC is characterized by CIN and the activation of the EGFR pathway (54). Moreover, differences in tumor microenvironment components (e.g., tumor epithelial cells, immune cells, and cancer-associated fibroblasts) play a critical role in defining CRC with a positive or poor prognosis and in maintaining immune surveillance (through the increase in tumor T-lymphocyte subset density) or in promoting immune escape (55). For example, a high density of specific tumor-infiltrating lymphocytes (i.e. cytotoxic and memory T-cells) in MSI-high CRC can be considered a favorable prognostic marker, because it counteracts the establishment of the "immunoediting" process and reduces the tumor spread (56–58). Furthermore, Canna et al. (59), found a relationship between systemic inflammatory response and local inflammatory response in patients undergoing resection for CRC, demonstrating that a high concentration of C-reactive protein and low tumor-infiltrating $CD4^+$ are predictive of poor cancer-specific survival.

The analysis of genetic profiles cannot be used for clinical purposes due to a discrepancy in results (e.g., sample preparation methods, use of different data processing and algorithms among different patient cohorts, gene expression platforms, and so on). However, the consensus molecular subtypes (CMS), which represent a transcriptome-based classification of CRC, include some superficial similarities useful for predictable CRC prognosis (60, 61). The first called CMS1 (MSI immune) is characterized by hypermutation, frequent BRAF mutation, MSI, and strong immune activation, the CMS2 (canonical) by CIN and marked WNT and MYC signaling, and the CM3 (metabolic) by evident metabolic dysregulation and KRAS-mutated tumors. Lastly, the CM4 (mesenchymal), includes tumors characterized by prominent TGFβ activation, epithelial-mesenchymal transition gene up-regulation, angiogenesis, and matrix remodeling.

## 3.2 Adjuvant treatments of CRC

Chemotherapy agents are usually used after a surgical excision as the treatment of choice to eradicate Minimal Residual Disease (MRD) in high-risk stage II and stage III patients and to increase the overall survival rate in stage IV patients (62–65). However, the only use of chemotherapy as standard-of-care (Table 1) can represent a limit due to the high systemic toxicity, unsatisfactory response rate, the onset of drug resistance, and the low tumor-specific selectivity. Therefore, massive investments have been earmarked to develop new approaches to improve patient outcomes. The identification of

TABLE 1 The main therapeutic approaches in the CRC treatment.

| | |
|---|---|
| Cytotoxic drug regimen | • Fluoropyrimidines;<br>• FOLFOX (5-FU/LV/Oxaliplatin);<br>• FOLFIRI (5-FU/LV/Irinotecan). |
| Targeted and immune-therapies | • EGFR inhibitors;<br>• Anti-angiogenesis therapies;<br>• BRAF inhibitors;<br>• Kinase inhibitor;<br>• Immunotherapeutics<br>• HER2 inhibitors;<br>• KRAS inhibitors. |

point mutations in specific oncogenes (KRAS, NRAS, and BRAF), amplification of human epidermal growth factor receptor 2 (HER2), the MSI status, the DNA mismatch repair status (deficiency or proficiency), has provided a framework for finding additional approaches, as well as new prognostic perspective (66). Up to today it is possible to hit the cancer more effectively, by administering the most suitable biological agents with the standard chemotherapy taking into account the genetic setting of patients (67, 68). In this regard, targeted therapies (i.e., antibodies and small molecules) and immunotherapies, which actively or passively target the patient's immune system, are widely used in combination with FOLFOX or FOLFIRI as a first-/second-line setting or alone as a third-line setting to improve the overall survival (OS) and progression-free survival (PFS) of advanced/metastatic cancer patients (69, 70). Moreover, antitumor immunity exerted by vaccines, specialized dendritic cells or new generation of cytotoxic T cells are currently under investigation in clinical trials (71, 72) (Table 2).

In patients with left-sided KRAS wild-type tumors, for instance, the administration of anti-EGFR (i.e., cetuximab or panitumumab) in combination with standard-of-care chemotherapy as a first-line setting shows improvement in both OS and progression-free survival (PFS) (73–75). Additionally, anti-EGFR can be used alone in chemo-refractory patients with advanced CRC (76).

Recently, in patients with BRAF-mutated mCRC, the use of anti-EGFR in combination with a selective inhibitor of BRAF kinase (encorafenib) and a reversible inhibitor of the kinase activity of mitogen-activated extracellular signal-regulated kinase 1 (MEK1) and MEK2 (binimetinib) has been proposed as the third line treatment to improve the prognosis (77, 78).

Among the first- and second line interventions for mCRC, also the VEGF inhibitors (i.e., Bevacizumab, Aflibercept) in combination with standard-of-care chemotherapy contribute to improve OS and PFS in patients (79, 80).

Another targeted therapy to treat advanced/metastatic CRC refractory to all standard treatments is represented by the diphenylurea-based multikinase inhibitor (i.e., Regorafenib). This anti-tumoral drug targeting multiple protein kinases regulating angiogenesis, proliferation, immunity, and metastases, increases OS of heavily pre-treated patients (79, 81–83).

KRAS-targeted drugs, such as sotorasib and adagrasib, are emerging for their anti-cancer activity in heavily pre-treated patients harboring the $KRAS^{G12C}$ mutation. These drugs are small molecules that keep KRAS in its inactive state, allowing apoptosis. However, their use in CRC treatment is still under investigation (84).

### 3.2.1 5- fluorouracil

5-FU is a fluorinated analogue of uracil that belongs to fluoropyrimidines. It was developed in 1957 and still today, it represents the mainstay of systemic combination chemotherapy for the treatment of CRC (85, 86). Although several 5-FU administration schedules were used (e.g., bolus intravenous, bolus *plus* intermittent intravenous infusion), today the standard of care is represented by continuous or intermittent intravenous infusion (87). Moreover, for around twenty years, also oral 5-FU prodrugs (*e.g.*, Capecitabine, Tegafur, 5′-deoxy-5-fluorouridine) are commonly used as part of combination regimens or as monotherapy (88, 89).

TABLE 2 Current clinical trials based on immunotherapy.

| Clinical trials N° | CRC stage | Treatment | Stage of trials | Status of trials |
|---|---|---|---|---|
| NCT01890213 | III | CEA (6D) VRP vaccine | I | Completed |
| NCT02466906 | III | RhGD-CSF | II | Unknown |
| NCT02912559 | III | Chemotherapy and Atezolizumab | III | Active |
| NCT02280278 | Post-therapy III | Cytokine-induced Killer cell Immunotherapy | III | Unknown |
| NCT03507699 | Metastatic | Nivolumab, Ipilimumab CMP-001 and radiosurgery | I | Completed |
| NCT04044430 | Metastatic | Encorafenib, Binimetinib and Nivolumab | I | Completed |
| NCT05130060 | Metastatic | Vaccine (PolyPEPI1018) and TAS-102 | I | Active |
| NCT03310008 | Metastatic | NKR-2 + Folfox | I | Unknown |
| NCT02834052 | Metastatic | Pembrolizumab and Poly-ICLC | I/II | Completed |
| NCT03377361 | Metastatic | Nivolumab, Trametinib with or without Ipilimumab | I/II | Active |
| NCT03436563 | Metastatic | Anti-PD-L1/TGFβII fusion protein M7824 | I/II | Active |
| NCT03711058 | Metastatic | Copanlisib and Anti-PD1 Nivolumab | I/II | Active |
| NCT04599140 | Metastatic | CXCR1/2 inhibitor (SX-682) and Nivolumab | I/II | Recruiting |
| NCT03993626 | Metastatic | CXD101 and Nivolumab | I/II | Unknown |
| NCT02981524 | Metastatic | GVAX (with CY) colon vaccine and Pembrolizumab | II | Completed |
| NCT04109924 | Pre-treated metastatic | TAS-102 Irinotecan and Bevacizumab | II | Active |
| NCT04362839 | Chemoresistant metastatic | Regorafenib, Ipilimumab and Nivolumab | I | Active |

5-FU is easily incorporated into DNA and RNA where it acts as an antimetabolite because shares a common structure with pyrimidines (90). After administration, 5-FU is converted via anabolic pathways into fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) (91). Stable complex between FdUMP and thymidylate synthase (TS) inhibits deoxythymidine mono-phosphate production, which consequently results in severe disruption of DNA synthesis and repair. Leucovorin (LV, Folinic acid), and Methotrexate (MTX, Folate analogue) are preferably used in combination with 5-FU to improve its antitumor activity (92, 93). Moreover, metabolites of 5-FU produce also alterations in the cellular membrane (89).

Approximately 80% of the total 5-FU dose is metabolized primarily in the liver by dihydropyrimidine dehydrogenase (DPD) (94), an enzyme that catalyzes the rate-limiting step in its metabolism.

Severe 5-FU-associated toxicity (e.g., leukopenia, neutropenia, thrombocytopenia, anemia, neuropathy, skin rash, hand-foot syndrome, and so on) is mainly due to a partial or complete DPD deficiency (95–99). In particular, different rare variants in the gene encoding DPD (DPYD) have been identified as validated risk variants for drug toxicity (86). Therefore, FDA-approved drug label prevents the use of 5-FU in individuals with absent DPD activity (88), while the Clinical Pharmacogenetics Implementation Consortium and the Dutch Pharmacogenetics Working Groups report dosing recommendations for 5-FU-based chemotherapy, based on DPYD genotype (88, 100, 101).

Although 5-FU-based chemotherapy combining with oxaliplatin or irinotecan has improved the response rate in patients with advanced CRC, primary or acquired chemoresistance is the leading cause of unsatisfactory outcomes in over 90% of patients with metastatic disease (93, 102). Indeed, intratumoral heterogeneity due to genetic mutations, tumor microenvironment (TME), and the presence of cancer stem cells, and the molecular complexity of CRC as well, making it necessary to develop other novel therapeutic strategies to overcome drug resistance and improve drug response rates.

## 3.3 The presence of cancer stem cells limits therapy efficacy against CRC

The main limiting factor for cancer patients is the onset of multi-drug resistance (MDR), which makes cancer cells tolerant to anti-cancer drugs. In fact, the combined chemotherapy and the development of different administration schedules are not always sufficient to avoid these issues due to the biological complexity of the tumor (103).

Tumor MDR is a highly-complex phenomenon that encompasses a plethora of molecular mechanisms involving not only cancer cells, but also infiltrating cells (e.g., endothelial, hematopoietic, and stromal cells) and the resulting tumor microenvironment (104). The constant interactions between tumor cells and their surrounding stroma result in alterations of many different cellular processes. Moreover, the presence of a sub clonal variation among cancer cells allows greater adaptability of the tumor to therapy, promoting its evolution.

Multiple molecular mechanisms have been identified as contributing factors to MDR development. Among these, the interplay between pre-existing and drug-induced mechanisms, including defects in the apoptotic machinery, mitochondrial dysfunction, altered autophagy activity, aberrant cell signaling, reduction in drug concentration and genetic and epigenetic changes, plays a significant role (105–108).

Moreover, the major cause of primary therapy resistance is represented by unresponsive subpopulations, such as cancer stem cells (CSCs) that can increase by up to 30% following long-term drug treatment (109).

Stochastically CSCs are distributed within tumors, but preferably they reside in specific niches, characterized by hypoxia, low pH, and fewer nutrients, which in addition to conferring them stemness features, allow the generation of differentiated progenies (110, 111).

CSCs are frequently quiescent and poorly differentiated cell populations with a lower level of intracellular ROS that share with normal stem cells both properties (i.e., self-renewal, self-sufficiency, and differentiation), and stemness signaling pathways (e.g., Notch, Sonic hedgehog, WNT/β-Catenin, JAK/STAT, and NF-kB). Their origin is still debated but it has been suggested that, at the moment of tumor initiation, the acquisition of CSC phenotype from either transformed differentiated cells (stochastic model) or transformed tissue-resident stem cells (hierarchical model) is promoted by the overexpression of oncogenes and the inhibition of tumor suppressor genes (e.g., APC, TP53, TGFBR2, SMAD4, PTEN, and RAS). Instead, following chemotherapy or radiotherapy regimen, new CSCs derive from either non-CSC subpopulations or therapy-induced senescent tumor cells (112).

Standard chemotherapy is not a valid therapeutic option for CSCs because they can effectively counteract the chemotherapy-induced oxidative stress through their free radical scavenging systems, such as GSH, and overexpression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl2) (113–115). Moreover, the enhancement of ATP-binding cassette (ABC) transporters and aldehyde dehydrogenase (ALDH) expression, the increased resistance to apoptosis, and the activation of DNA damage sensor and repair machinery contribute to give to CSCs a survival advantage against anti-cancer therapy (116). Additionally, CSCs can transiently and reversibly switch between epithelial and mesenchymal states and *vice versa* (i.e., epithelial-mesenchymal plasticity) via Wnt/b-catenin signaling (117, 118). Such versatility, consequently, results in metabolic reprogramming in cellular bioenergetics, where energy supply can alternatively depend on aerobic glycolysis or mitochondrial OXPHOS.

It has been shown that metformin potentially offers therapeutic advantages by inhibiting the mitochondrial respiration, forces CSCs to a metabolic shift from OXPHOS to glycolysis (119, 120). The temporary OXPHOS suppression renders CSCs more prone to apoptosis. However, tumor relapse under metformin treatment cannot be excluded since CSCs can acquire resistance mainly due to MYC overexpression, promoting a Warburg-like glycolytic phenotype (120). Also, progressive

increase in glycolysis-derived lactate may promote the activation of proteases, leading to ECM degradation, and resistance to chemotherapy (121).

The release of IL-4 from colorectal CSCs promotes their survival and hampers the CD8+T cell-mediated antitumor immune response, while the presence of inflammatory cytokines, including IL-1, IL-4, IL-6, IL-8, IL-10, and TGF-β, fuels an inflammatory loop, via Stat3/NF-κB pathways, for stimulating the self-renewal of CSCs (118, 122). Moreover, the tumorigenic and self-renewal capacity of CSCs also depend on the hyperactivation of β-catenin, Notch, and Hedgehog signaling pathways (123, 124).

Although several CSC biomarkers have been identified for CRC, their preclinical application is still unavailable due to the intrinsic features of CSCs, i.e., phenotypic heterogeneity, and the influence of the TME or CSC behavior. In this regard, previous studies have focused the attention on both the CSC-related signature and immune cell infiltration as important prognostic factors. The correlation between infiltrating portion of immune cells, i.e., tumor immune microenvironment (TIME), and hallmark gene sets may represent a possible starting point for developing CSC-targeted therapeutic strategies (122, 125).

## 3.4 New drug delivery approaches and latest strategies implemented in the treatment of CRC

Increasing evidence suggests that the use of nanoscale nanoparticles (NPs) as drug delivery systems (DDS), including liposomes, nanoemulsions, hydrogels, multifunctional inorganic materials (e.g., carbon nanotubes, gold nanoparticles, quantum dots), and peptides, could provide a novel therapeutic approach useful in overcoming MDR and improving the pharmacokinetics and biodistribution of anticancer compounds, resulting in reduced side effects (126). These NPs refers to nanometer scale systems (10-1000 nm) capable of protecting encapsuled molecules from degradation and passively or actively delivering drugs, small molecules, proteins, peptides, DNA and RNA into specific targets. However, their bio-distribution and clearance in the body depend not only by the NP chemical, physical and biological properties (*e.g.*, size, stability, surface charge, solubility, and so on) but also by factors, such as the administration route (e.g., intravenous, oral, pulmonary and dermal administration) and host environment (e.g., pre-existing inflammation) (127).

Recently, it has been reported that NPs can accumulate in tumor tissues by passive or active delivery. Passive delivery of drug-loaded nanoparticles (i.e., the Enhanced Permeability and Retention, EPR, effect) is mainly due to fenestrated and immature new tumor vessels (128–131) while, the active delivery is due to a ligand-binding mechanism (e.g., nanoparticles targeting EpCAM, the folate receptor, EGFR and CD44) (132–134) (Figure 3). However, regardless of NP delivery, nanoparticle-protein complex, namely protein corona (PC), can permanently change the NP fate. The protein profile of the corona complex does not have a standard composition, because it varies not only among NPs of different chemical designs but even across the NPs of the same type. This latter is explained by the so-called Vroman effect, in which protein turnover depends not only by the high-affinity binding of proteins, but also on their exchange kinetics (135). In general, on the basis of the binding affinity between plasma proteins

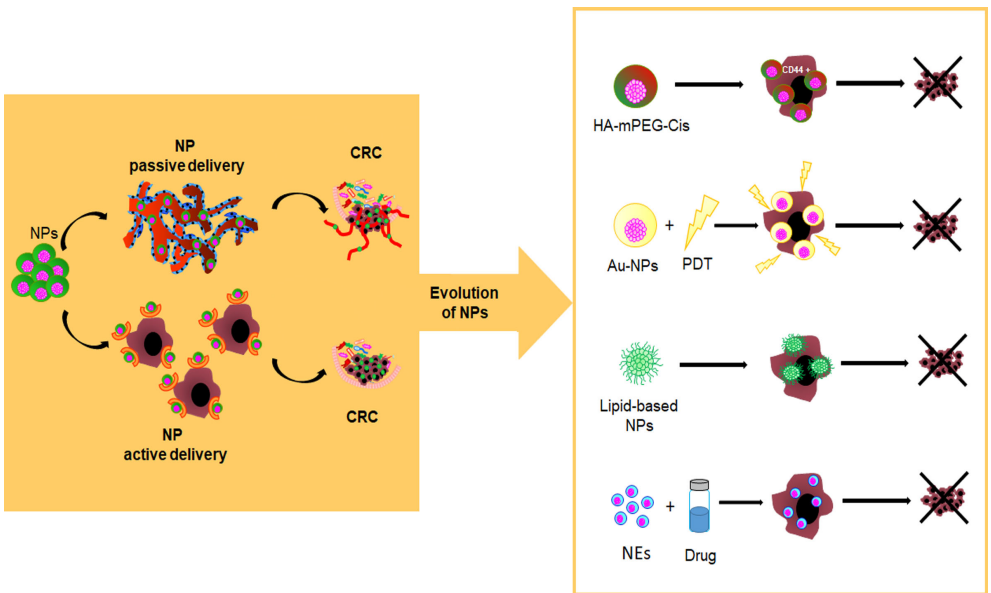


FIGURE 3
Evolution of nanoparticles as innovative drug delivery systems. pH-sensitive pegylated nano drug delivery systems (HA-mPEG-Cis NPs) are able to target CD44+ cells; Gold nanoparticles (AuNPs) find application in photodynamic therapy (PDT); Lipid-based NPs; Nanoemulsions (NEs) are a system to deliver hydrophobic drugs and hydrophilic or hydrophobic compounds.

and NP surfaces a "hard corona" and a "soft corona" are distinguished, respectively (136). Moreover, proteins participating in the complex influence the cell recognition pathway by the reticuloendothelial system (RES) and promote biological processes against NPs, including aggregation, opsonization, and phagocytosis. Therefore, either second-generation NPs or PEGylation technique enhance the effect of cancer therapy by ensuring drug delivery within the tumor and evading phagocytosis (137). In this regard, emerging self-assembly pH-sensitive pegylated nano drug delivery systems, namely HA-mPEG-Cis NPs, are able to target CD44-CRC-positive cells and dissolve the hydrated PEG in the acidic tumor environment. These drug delivery systems improve drug circulation time and tumor targeting while reducing the side effects of the loaded drug (138) (Figure 3).

Inorganic nanocarrier, of controlled size and shape, such as gold NPs (AuNPs), show a certain versatility of use, including chemical sensing, imaging, and drug delivery due to their favourable optical and physical properties coupled with a reasonable biocompatibility with regard to biological environment (139). Interestingly, AuNPs, including gold nanorods, nanocages, nanostars, nanocubes, and nanospheres, find application in photodynamic therapy (PDT) for their specific physical features (i.e., optical and Surface Plasmon Resonance properties, proton-capture cross-section) (140) (Figure 3).

Lipid-based NPs are already FDA-approved for various therapeutic purposes, including cancer treatment (e.g., Doxil®, DaunoXome®, Myocet®, DepoCyt®, Marqibo® and Onivyde®), severe infections or immunocompromised conditions (e.g. AmBisome®) and RNAi therapeutic (Onpattro®) (127, 141) (Figure 3).

Liposomes are small-size vesicles consisting of an outer lipid bilayer, synthetic or natural, and an aqueous core, widely used to encapsulate/entrap drugs or nucleic acids (i.e. gene therapy) (142). Currently, the manipulation of liposome lipid membrane components (e.g., neutral and/or negatively charged lipids plus cholesterol, sphingomyelin plus cholesterol, hydrogenated soy phosphatidylcholine plus cholesterol) as well as specific key parameters (e.g., size and shape) has improved their biological performance, in term of enhanced delivery efficiency, maximizing so-called nano-bio interactions (143).

Nanoemulsions (NEs) are another system to deliver hydrophobic drugs and hydrophilic or hydrophobic compounds through different routes of administration (e.g., aerosols, ingestion, and injections). NEs are made as single (i.e., oil-in-water [o/w], water-in-oil [w/o]) or dual (w/o/w, o/w/o) emulsions with biocompatible and FDA-approved biodegradable oils (143). Previous *in vitro* studies have shown that natural active compounds encapsulated within NEs, acting synergistically with chemotherapy, can improve the therapeutic value of treatment despite the use of a lower dosage of drug (144, 145). Also, the entrapment of active or cytotoxic drugs within nanoemulsions can be useful to sensitize CSCs to apoptosis (146) (Figure 3).

Over the past decade, nanotechnology has been widely explored to develop cytotoxic drug carriers. Although further improvements are needed, different types of NPs are already considered reliable systems for drug delivery due to their ability in targeting the tumor before releasing the drug.

## 4 Conclusion

Considering the critical nature of this review, and the variety of the included studies, it highlights that the sporadic CRC is a multi-stage and multi-step process in which the early mutational events seem to be driven by dysbiosis, chronic inflammation, and ROS. Moreover, treatments with standard cytotoxic agents, such as FOLFOX and FOLFIRI regimens, also contribute to the variation in the molecular profile of CRC in the advanced stage. Furthermore, this review also highlights that the limitation in treatment approaches for advanced CRC patients is mainly represented by both extrinsic (chemotherapy) and intrinsic mutation burden in cancer subpopulations (CSCs) developing MDR phenotype. In this regard, many strategies have been studied to overcome this issue, including the inhibition of crucial signaling involved in the self-renewal and metabolism of CSCs, as well as the redox-targeting approach. Moreover, using anti-vasculature therapies (e.g., bevacizumab and cetuximab) to modulate the tumor microenvironment represents a valid approach for enhancing cytotoxic drug uptake. Lastly, the development of novel DDS and promoter drugs can improve the delivery and the effectiveness of anti-cancer agents, opening up to personalized treatment protocols for CRC.

## Author contributions

Conceptualization: SV. Investigation: SV, ST. Writing – original draft: SV, ST, VA. Supervision: SV, AMB, CD, BM. Writing – review & editing: SV, BM, CD. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# Genetics of enzymatic dysfunctions in metabolic disorders and cancer

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Inherited metabolic disorders arise from mutations in genes involved in the biogenesis, assembly, or activity of metabolic enzymes, leading to enzymatic deficiency and severe metabolic impairments. Metabolic enzymes are essential for the normal functioning of cells and are involved in the production of amino acids, fatty acids and nucleotides, which are essential for cell growth, division and survival. When the activity of metabolic enzymes is disrupted due to mutations or changes in expression levels, it can result in various metabolic disorders that have also been linked to cancer development. However, there remains much to learn regarding the relationship between the dysregulation of metabolic enzymes and metabolic adaptations in cancer cells. In this review, we explore how dysregulated metabolism due to the alteration or change of metabolic enzymes in cancer cells plays a crucial role in tumor development, progression, metastasis and drug resistance. In addition, these changes in metabolism provide cancer cells with a number of advantages, including increased proliferation, resistance to apoptosis and the ability to evade the immune system. The tumor microenvironment, genetic context, and different signaling pathways further influence this interplay between cancer and metabolism. This review aims to explore how the dysregulation of metabolic enzymes in specific pathways, including the urea cycle, glycogen storage, lysosome storage, fatty acid oxidation, and mitochondrial respiration, contributes to the development of metabolic disorders and cancer. Additionally, the review seeks to shed light on why these enzymes represent crucial potential therapeutic targets and biomarkers in various cancer types.

KEYWORDS

**inherited metabolic disorders, enzymatic dysregulation, cancer, urea cycle, glycogen storage, lysosome storage, fatty acid oxidation, mitochondrial respiration**

## 1 Introduction

Inherited metabolic disorders can be caused by mutations of genes involved in the biogenesis, assembly or activity of metabolic enzymes, which can lead to enzymatic deficiency and severe life-threatening metabolic impairments (1). Metabolism is the process by which macromolecules (lipids, carbohydrates, nucleic acids, and proteins) are

broken down to produce energy (catabolism) or used for energy storage (anabolism). In normal cells, macromolecules go through a series of biochemical reactions catabolized by metabolic enzymes in the presence of oxygen to produce ATP through mitochondrial respiration. By-products resulting from this metabolic activity are then recycled or eliminated. Dysregulation of the activity of these enzymes due to mutation or changes in levels of expression (upregulation and downregulation) can lead to several metabolic disorders and also have been associated with cancer development (Figure 1) (2).

The "Warburg effect" or aerobic glycolysis, one of the altered metabolisms in cancer, was first described in 1927 by Otto Warburg who observed that cancer cells have altered glucose metabolism due to increased glucose uptake in the cytoplasm where glucose is converted into lactate, even in the presence of oxygen (3). This discovery showed for the first time how cancer cells can benefit from metabolic adaptation to ensure their survival and proliferation. It has been proposed that cancer cells, which require high energy consumption, use aerobic glycolysis to facilitate the uptake and incorporation of nutrients into their biomass (4). However, the reasons why some cancer cells switch from oxidative phosphorylation to aerobic glycolysis remain unclear (5), and recent research has shown that oxidative phosphorylation can also drive cancer growth (6). Since the "Warburg effect" discovery, considerable research has been conducted on the importance of metabolism for cancer development, making metabolism reprogramming one of the hallmarks of cancer and cancer itself a metabolic disease (7, 8). Cancer cell rewiring of metabolism plays a key role in tumorigenesis, tumor progression, and drug resistance, which can be influenced by the tumor microenvironment (TME) and the genetic context in which tumors arise and progress. Enzymatic deficiency can notably lead to an accumulation of metabolites, known as oncometabolites, which can act as signaling molecules for regulating gene expression and promoting tumor growth (9–11).

The upregulation or downregulation of metabolic enzymes can promote and sustain the activation of metabolic pathways that play a key role in cancer cell proliferation and survival, notably by preventing nutrient depletion (2). However, whether the expression of metabolic enzymes is a cause or a consequence of metabolic adaptations often remains unclear. As the interplay between cancer and metabolism reprogramming is becoming established, more research is needed to fully understand how cancer cells take advantage of metabolic enzyme dysregulation. In this context, here we review enzymatic dysregulation of the metabolic pathways for the urea cycle, glycogen storage, lysosome storage, fatty acid oxidation and mitochondrial respiration, with regard to their role in the development of metabolic disorders and cancer, and why these enzymes represent important potential therapeutic targets and biomarkers in most cancer types.

## 2 Urea cycle disorders

Urea cycle defects or disorders (UCDs) arise from an inherited deficiency in one of the five catalytic enzymes that play a crucial role in the urea cycle pathway. This leads to an accumulation of ammonia (hyperammonemia), which in turn results in neurocognitive deficits and/or chronic liver dysfunction. The urea cycle is the primary pathway for the elimination of nitrogenous waste, mainly in the liver, such as ammonia and glutamine, into urea (12). The five catalytic enzymes in the urea cycle are Carbamoyl phosphate synthetase I (CPS1), Ornithine transcarbamylase (OTC), Argininosuccinate synthetase (ASS1),

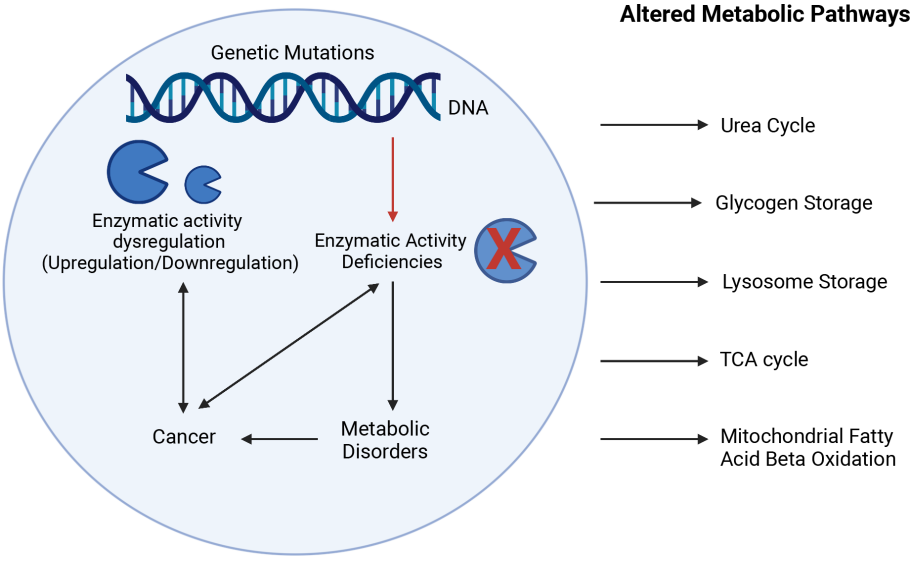


FIGURE 1
Interplay between enzymatic deficiencies, metabolic disorders, and cancer. Enzymatic activity deficiencies due to genetic mutations or changes in levels of expression (upregulation and downregulation) can lead to several metabolic disorders and have been associated with cancer development affecting metabolic pathways such as urea cycle, glycogen storage, lysosome storage, fatty acid oxidation, and mitochondria respiration. The Figure was partly generated using Biorender under the agreement number: WM25LGLSAK (www.Biorender.com).

Argininosuccinate lyase (ASL), and Arginase (ARG1) (Figure 2A) (13). The manifestation of deficiency in any of these enzymes has been linked with the progression of cancer due to the generation of nucleotide imbalances that instigate the occurrence of mutation patterns. This highlights the importance of these enzymes in maintaining normal cellular function and preventing the development of cancer.

The first step in the urea cycle is catalyzed by CPS1, converting ammonia into carbamoyl phosphate (CP). CPS1 deficiency is characterized by complete or partial absence of the CPS enzyme, leading to patients experiencing vomiting, seizures, progressive lethargy, coma, and even death (14). CPS1 overexpression has been linked to poor prognosis in various types of cancer, including colorectal (15), cholangiocarcinoma (16), glioblastoma (17), lung adenocarcinoma (18), and non-small cell lung cancer (NSCLC) (19, 20). Upregulated CPS1 expression in tumor cells produces significant amounts of CP, which is then translocated to the cytoplasm and incorporated into the reaction catalyzed by a trifunctional enzyme, the CAD protein (21, 22). CAD is composed of carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase, necessary to maintain cellular fundamental function (i.e., DNA and RNA biosynthesis) by initiating pyrimidine synthesis (23). However, in other types of cancer, such as small intestine adenocarcinoma (24) and hepatocellular carcinoma (HCC) (25), the levels of CPS1 are downregulated, which associates with decreased survival and an increase of CAD expression, resulting in the reuse of ammonia for the synthesis of glutamine as a means to initiate *de novo* pyrimidine synthesis (21).

The second step in the urea cycle is catalyzed by OTC, converting ornithine and carbamoyl phosphate into citrulline, which detoxifies the ammonia produced from amino acid catabolism. OTC deficiency is a rare X-linked genetic disorder identified by complete or partial lack of the enzyme OTC, leading to impairment of the central nervous system, which has the potential to result in permanent brain damage and is fatal in newborn infants (26). The downregulated OTC expression level results in accumulated ammonia and has been associated with larger tumor size, advanced grade, and poor prognosis for patients with hepatocellular carcinoma (HCC) (27). The downregulation of mitochondrial NAD-dependent protein deacetylase sirtuin-3 (SIRT3) in HCC cells may contribute to the protection of these cells from apoptosis. SIRT3 is a regulator of OTC deacetylation, and the acetylation of lysine 88 inhibits the enzyme activity of OTC, highlighting the important role of a deacetylase in regulating the function of OTC (26, 28, 29).

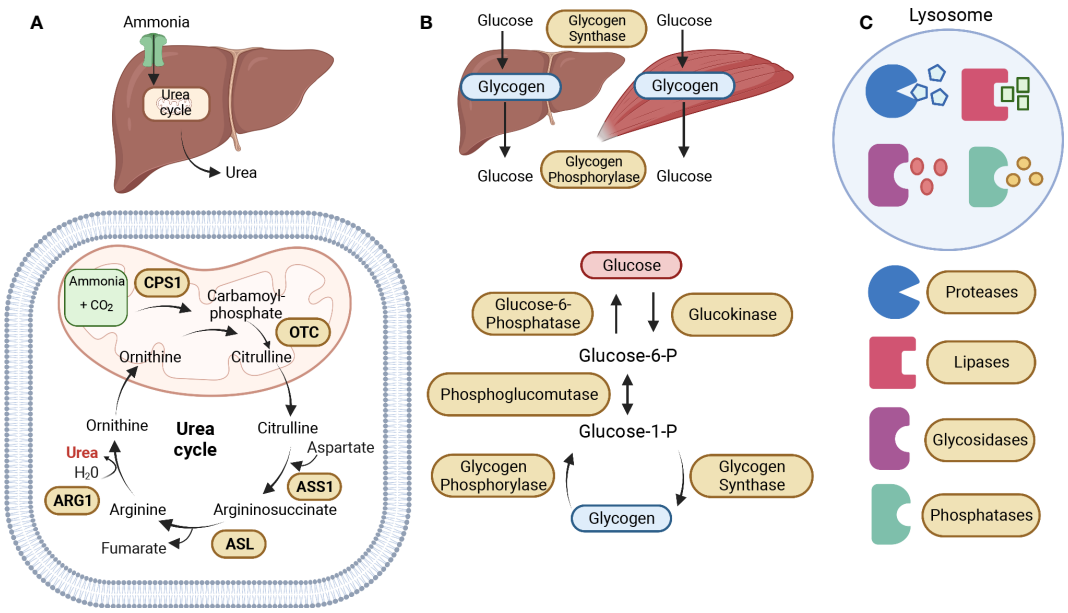


FIGURE 2
Metabolic Enzymatic Pathways. **(A)** The urea cycle is the primary pathway for the elimination of nitrogenous waste, mainly in the liver, such as ammonia and glutamine, into urea. The five catalytic enzymes in the urea cycle are Carbamoyl phosphate synthetase I (CPS1), Ornithine transcarbamylase (OTC), which are both located in the mitochondrial matrix, Argininosuccinate synthetase (ASS1), Argininosuccinate lyase (ASL), and Arginase (ARG1), located in the cytoplasm. The urea cycle starts in the mitochondrial matrix with the conversion of ammonia into carbamoyl-phosphate, which is then converted into citrulline by OTC. Citrulline is exported to the cytoplasm where it is converted into argininosuccinate by ASS1. The ASL enzyme then converts argininosuccinate into arginine, which is then converted into ornithine by ARG1, leading to the production of urea. Ornithine enters the mitochondria to participate in the conversion of carbamoyl phosphate into citrulline. **(B)** Glycogen serves as the main storage form of glucose in humans, mostly in the liver and muscles. The primary enzymes involved in glycogen synthesis (glycogenesis) and breakdown (glycogenolysis) are glycogen synthase and glycogen phosphorylase. During glycogenesis, glucose is converted into glucose-6-phosphate (Glucose-6-P) by glucokinase. Glucose-6-P is converted into glucose-1-phosphate (Glucose-1-P) by phosphoglucomutase. Then, Glucose-1-P is converted to glycogen by the enzyme glycogen synthase. During glycogenolysis, glycogen is converted to Glucose-1-P by glycogen phosphorylase, which is then converted back into Glucose-6-P by phosphoglucomutase. And finally, Glucose-6-P is converted to glucose by Glucose-6-phosphatase. **(C)** The lysosome is an essential catabolic organelle that provides an acidic environment, where macromolecules are metabolized by hydrolytic enzymes, such as proteases, lipases, glycosidases, and phosphatases. The Figure was partly generated using Biorender under the agreement number: TR25LH0BWF (www.Biorender.com).

The third step in the urea cycle is catalyzed by ASS1, in which citrulline is condensed with aspartate to form argininosuccinic acid and functions as an enzyme for arginine metabolism (30). Citrullinemia type I (CTLN1) is caused by a deficiency or absence of the enzyme ASS1, resulting in increased intracranial pressure (ICP), increased neuromuscular tone, seizures, loss of consciousness, and death (31). The incidence of ASS1 deficiency changes significantly with the tumor type and tissue of origin (32). Increased levels of ASS1 have been observed in human non-small cell lung cancer (NSCLC) and colon carcinomas, which may be supporting arginine synthesis and facilitating cellular survival under low-nutrient stress conditions (33). In contrast, decreased ASS1 levels have been shown in breast cancer, primary hepatocellular carcinoma (HCC), melanoma, sarcomas, renal cell carcinoma, and prostate cancer (32). ASS1 loss in tumors hinders arginine biosynthesis, leading to dependence on extracellular arginine for survival. Thus, arginine depletion therapy is a promising strategy for ASS1-negative tumors, which constitute nearly 70% of tumors (30). Rabinovich S et al. were also able to demonstrate that ASS1 deficiency in cancer increases cytosolic aspartate levels leading to increased activation of the enzymatic complex CAD (carbamoyl-phosphate synthase 2, aspartate transcarbamylase, dihydroorotase complex) by upregulating its substrate availability and by increasing its phosphorylation by S6K1 through the mTOR pathway. They were able to show that decreased activity of ASS1 in cancers supports proliferation by activating CAD and facilitating pyrimidines synthesis (34). Furthermore, ASS1 plays a crucial role as a biomarker for the response to glutamine deprivation. Impairment of ASS1 activity elevates sensitivity towards arginine and glutamine deprivation, whereas upregulation of ASS1 activity augments resistance towards arginine and glutamine deprivation (35).

The fourth reaction in the urea cycle is catalyzed by ASL, leading to the breakdown of argininosuccinic acid to arginine and fumarate. Argininosuccinic aciduria is an inherited disorder described by deficiency or lack of the enzyme ASL, leading to an accumulation of citrulline and argininosuccinic acid, causing vomiting, drowsiness, seizures, and/or coma (36). ASL is highly expressed in melanoma, HCC, and breast tumor tissues (37, 38). ASL and nitric oxide synthase (NOS) form the citrulline-argininosuccinate-arginine cycle, facilitating nitric oxide (NO) production. Overproduction of NO has been associated with the progression of cancer (38, 39).

The fifth reaction in the urea cycle is catalyzed by ARG1, involved in the hydrolysis of arginine to ornithine and urea, which regulate the proliferation, differentiation, and function of different cell types. Arginase-1 deficiency is identified by either a complete or partial absence of the arginase enzyme in the liver and red blood cells, with symptoms that can include vomiting, poor growth, seizures, and stiff muscles with increased reflexes (spasticity) (40, 41). Increased expression of arginases (either Arg1 or Arg2) is considered a poor prognostic factor in several types of cancer, including lung cancer (42, 43), head and neck cancer (44), neuroblastoma (45), acute myeloid leukemia (46), pancreatic ductal carcinoma (47), ovarian carcinoma (48), and colorectal cancer (49). Arginine metabolism plays a crucial role in T-cell activity and survival. Increased enzymatic activity of arginase depletes arginine levels in the tumor microenvironment, leading to immunosuppression and impaired T-cell function, which is critical for effective immune surveillance and anti-tumor response (50).

The urea cycle and its five catalytic enzymes play a crucial role in maintaining normal cellular function, and their deficiencies have been associated with cancer progression. Table 1 provides a comprehensive overview of enzyme mutations in the urea cycle,

TABLE 1 Urea Cycle Disorders (UCDs).

| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
|---|---|---|---|---|
| Carbamoyl phosphate synthetase I (CPS1) | Synthesizes carbamoyl phosphate (CP) from ammonia, bicarbonate, and 2 molecules of ATP. | Carbamoyl phosphate synthetase I deficiency (CPS1 deficiency) | Colorectal (15), cholangiocarcinoma (16), glioblastoma (17), lung adenocarcinoma (18), and non-small cell lung cancer (NSCLC) (19, 20). | Small intestine adenocarcinoma (24) and hepatocellular carcinoma (HCC) (25). |
| Ornithine transcarbamylase (OTC) | Catalyzes the reaction between CP and ornithine to form citrulline and phosphate. | Ornithine transcarbamylase deficiency (OTC deficiency) | | HCC (27). |
| Argininosuccinate synthetase (ASS1) | Catalyzes the synthesis of argininosuccinic acid from citrulline and aspartate | Arginosuccinate synthetase deficiency (ASS deficiency), also known as Citrullinemia type I (CTLN1) | NSCLC and colon carcinomas (33). | Breast, HCC, melanoma, sarcomas, renal cell carcinoma, and prostate (32). |
| Argininosuccinate lyase (ASL) | Catalyzes the reversible hydrolytic cleavage of argininosuccinic acid into arginine and fumarate | Argininosuccinate lyase deficiency (ASL deficiency), also known as Argininosuccinic aciduria | Melanoma, HCC, and breast (37, 38). | |
| Arginase (ARG1) | Catalyzes the breakdown of arginine into urea and ornithine | Arginase deficiency (ARG1 deficiency), also known as Argininemia or Hyperargininemia | Lung (42, 43), head and neck cancer (44), neuroblastoma (45), acute myeloid leukemia (46), pancreatic ductal carcinoma (47), ovarian carcinoma (48), and colorectal (49). | |

their respective enzymatic roles, associated diseases, and the regulation status (up or down) of both the enzyme and genes in different types of cancer. Further research is needed to explore the interplay between the urea cycle and cancer progression. Understanding the molecular mechanisms underlying these defects may provide potential targets for therapeutic interventions to prevent cancer development and improve patient outcomes.

## 3 Glycogen storage disorders

Glycogen Storage Disorders (GSDs) are a set of hereditary metabolic disorders that affect glycogen metabolism, which is responsible for regulating glycogen synthesis or degradation (51). Glycogen serves as the main storage form of glucose in humans, mostly in the liver and muscles (52). The primary enzymes involved in glycogen synthesis (glycogenesis) and breakdown (glycogenolysis) are glycogen synthase and glycogen phosphorylase (Figure 2B) (51). GSDs are classified based on the specific enzyme deficiency and the primary affected tissues with an increasing number of GSD types being identified. We will be focusing on Type 0 due to its two distinct forms of glycogen synthase, and Types I, II, III, and IV, which are the four most common types of GSD (53). Table 2 describes the mutated names of enzymes involved in glycogen storage, alongside their enzymatic roles and the corresponding diseases they are associated with, and information about whether the enzymes or genes are upregulated or downregulated in various types of cancer.

GSD type 0 is caused by mutations in the *GYS1* gene, leading to muscle glycogen synthase deficiency, and mutations in the *GYS2* gene, leading to liver glycogen synthase deficiency. The two isoforms of glycogen synthase share a common role of forming glycogen by linking glucose molecules (66). A study by Favaro et al. showed that *GYS1* is rapidly induced in glioblastoma, breast, and colon cancer cell lines under hypoxic conditions, followed by a decrease of glycogen phosphorylase (PYGL), an enzyme that degrades glycogen. This results in glycogen accumulation, decreased nucleotide synthesis, and increased reactive oxygen species (ROS) levels that contribute to p53-dependent growth arrest and impaired tumorigenesis *in vivo* (54). Meanwhile, the knockdown of *GYS2* in HCC promotes cell proliferation *in vitro* and tumor growth *in vivo* by regulating p53 expression. Interestingly, p53 is capable of transcriptionally regulating *GYS2*, *PYGL*, and other genes involved in glycogen synthesis (55). In addition, p53 has been identified as a key regulator of glucose metabolism through its ability to suppress glucose uptake and glycolysis in tumor cells (67).

GSD type I, also known as Von Gierke disease, has three subtypes: GSD 1a is caused by *G6PC* gene mutations involving glucose-6-phosphatase (G6Pase) deficiency. G6Pase is a membrane-bound protein associated with the endoplasmic reticulum (ER) involved in providing glucose during starvation by catalyzing the hydrolysis of glucose-6-phosphate (G6P) (68). While GSD 1b and 1c are caused by *SLC37A4* gene mutations resulting in glucose-6-phosphate translocase (G6PT) deficiency. G6PT is a transmembrane protein involved in translocating G6P from the cytosol into the lumen of the ER for glucose hydrolysis (69). Abnormal expression of *G6PC* is observed in different cancers, with low expression in HCC (59) and clear renal cell carcinoma (60), likely resulting in the accumulation of G6P. This accumulation of G6P may lead to increased glucose metabolism by producing ribose-5-phosphate through the hexose monophosphate (HMP) shunt pathway (an alternative pathway to glycolysis) in tumor cells, resulting in cell division, cell survival, and tumor growth

TABLE 2 Glycogen Storage Disorders (GSDs).

| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
|---|---|---|---|---|
| Glycogen synthase | Catalyzes the rate-limiting step in glycogenesis by transferring glucose monomers to growing glycogen chains | GSD Type 0, also known as Glycogen synthase deficiency (Muscle glycogen synthase deficiency (encoded by *GYS1*) and liver glycogen synthase deficiency (encoded by *GYS2*)). | *GYS1* in glioblastoma, breast, and colon (54). | *GYS2* in HCC (55). |
| Glucose-6-Phosphatase (G6Pase) | Provides glucose during starvation by catalyzing the hydrolysis of glucose-6-phosphate (G6P). | GSD Type I or Von Gierke disease, also known as Glucose-6-phosphate deficiency. GSD Type 1a (GSD1a) glucose-6-phosphatase (G6Pase) deficiency (encoded by *G6PC*). Type 1b and 1c (GSD1b or 1c) glucose-6-phosphate translocase (G6PT) deficiency (encoded by *SLC37A4*). | *G6PC* in ovarian (56), glioblastoma (57), and cervical (58). | *G6PC* in HCC (59) and clear renal cell carcinoma (60). |
| alpha-1-4-glucosidase (acid maltase) | Breaks down glycogen into glucose in the lysosome. | GSD Type II or Pompe disease, also known as alpha-1,4-glucosidase deficiency. | | Pancreatic cells (61). |
| Glycogen debranching enzyme | Breaks down glycogen and mobilizes glucose reserves from glycogen deposits in the muscles and liver. | GSD Type III, Cori disease or Forbes disease, also known as Glycogen debrancher deficiency. | | Bladder (62). |
| Glycogen branching enzyme | Adds branches to the growing glycogen molecule during glycogenesis | GSD Type IV or Andersen disease, also known as Glycogen branching enzyme deficiency. | Lung adenocarcinoma (63–65). | |

(59). In contrast, overexpression of *G6PC* affects glucose metabolism in ovarian (56), glioblastoma (57), and cervical cancer (58), contributing to tumor proliferation, metastasis, and poor prognosis in patients. The overexpression of *G6PC* increases the amount of blood glucose, leading to an increase in the rate of glycolysis. This may be occurring by inducing alteration in other pathways, such as cell cycle regulation via the Forkhead box protein O1 (FOXO1) pathway in ovarian cancer (56), intracellular glycogen degradation by hypoxia-inducible factor 1-alpha (HIF1α) and signal transducer and activator of transcription 3 (STAT3) in glioblastoma (57), and by regulating the activation of PI3K/AKT/mTOR pathway in cervical cancer (58). In turn, *G6PT* regulates glucose homeostasis in glioblastoma leading to inhibition of cancer cell proliferation, extracellular matrix (ECM) degradation, or inducing cell death. *G6PT* may be functioning as a "bioswitch" allowing cells to switch between migration or cell death in response to external stimuli, such as hypoxia or intracellular metabolic changes (i.e., $Ca^{2+}$ flux and cytosolic ATP) controlled by the PTEN/Akt/PI3K/mTOR pathway (70). Furthermore, overexpression of *G6PT* in glioblastoma cells induced cell migration by regulating calcium-mediated signaling (71) and *G6PT* expression regulates bone marrow-derived stromal cells (BMSC) survival, ECM degradation, and mobilization by inhibiting the activation of pro-matrix metalloproteinase-2 (proMMP-2) mediated by membrane type 1 matrix metalloproteinase (MT1-MMP) (72).

GSD type II (known as Pompe disease), also classified as lysosomal storage disease, is caused by mutations in the *GAA* gene resulting in a deficiency of alpha-1-4-glucosidase (acid maltase) causing marked accumulation of glycogen in lysosomes (73). Hamura et al. showed that knockdown of *GAA* decreased cell proliferation and increased apoptotic signals in pancreatic cells, accompanied by accumulation of dysfunctional mitochondria, caused by the suppression of the transcription factor EB (TFEB), which plays a critical role in lysosomal biogenesis (61).

GSD type III, also known as Cori or Forbes disease, is caused by mutations in the *AGL* gene, which results in glycogen debranching enzyme deficiency, an enzyme that helps facilitate the breakdown of glycogen and mobilize glucose reserves from glycogen deposits in the muscles and liver. A recent study by Guin et al. showed that *AGL* serves as a prognostic marker for bladder cancer survival, and decreased *AGL* enhances tumor growth by increasing glycine synthesis through increased expression of serine hydroxymethyltransferase 2 (SHMT2), an enzyme that allows cells to process glycogen into glycine (62).

GSD type IV, also known as Andersen disease, results from mutations in the *GBE1* gene, causing a deficiency in the glycogen branching enzyme, which adds branches to the growing glycogen molecule during the synthesis of glycogen, allowing for easy and quick glycogen utilization when it is broken down (74). Studies have revealed that *GBE1* expression is upregulated in hypoxia-conditioned primary lung adenocarcinoma cells mediated by HIF1α, while decreased *GBE1* expression inhibits lung cancer cell growth by directly affecting glycogen production and glucose metabolic signaling pathways. These findings suggest that *GBE1* expression protects cells from hypoxia and allows them to survive, thereby further promoting proliferation and metastasis (63–65).

Glycogen accumulation has been shown to play a crucial role in promoting cell survival under hypoxic conditions in both normal and cancer cells, as demonstrated by various studies including cancer cell lines such as breast, kidney, uterus, bladder, ovary, skin, and brain cancer cell lines (54, 64, 75–79). Furthermore, a recent study found that glycogen accumulation is essential for tumor initiation in human and mouse liver tumors, which commonly exhibit hypoxic stress in the early stages (80). The elimination of glycogen accumulation has been shown to abrogate liver cancer incidence, while increasing glycogen storage accelerates tumorigenesis. These findings suggest that glycogen metabolism plays a crucial role in tumor initiation and growth and could be a potential target for cancer treatment.

# 4 Lysosomal storage disorders

Lysosome Storage Disorders (LSDs) are caused by heritable mutations in genes encoding lysosomal enzymes (known as hydrolytic enzymes), resulting in the buildup of various unmetabolized macromolecules (i.e., proteins, lipids, carbohydrates, and nucleic acids) impairing lysosomal homeostasis and activity (81). The lysosome is an essential catabolic organelle found in eukaryotic cells and provides an acidic environment, where macromolecules are metabolized by hydrolytic enzymes, such as glycosidases, lipases, proteases, sulfatases, nucleases, and phosphatases (Figure 2C) (82, 83). Hydrolytic enzymes facilitate the breakdown of chemical bonds within different types of compounds including proteins, nucleic acids, starch, fats, phosphate esters, and other macromolecules (84). LSDs represent over 70 disorders, characterized by lysosomal dysfunction, in which 50 of these disorders are caused by enzyme deficiencies. Table 3 offers insights into enzyme mutations and their respective enzymatic functions in lysosome storage, along with associated diseases. Additionally, it highlights whether the enzymes or their corresponding genes are upregulated or downregulated in different types of cancer. Depending on the accumulated material in the lysosomes, these enzyme deficiencies can be classified into three categories: sphingolipidoses, glycoproteinosis, and mucopolysaccharidoses (118).

## 4.1 Sphingolipidoses

Sphingolipidoses are a group of heterogeneous inherited metabolic disorders characterized by an accumulation of glycolipids or phospholipids, which have ceramide as a common structure (119). Sphingolipidoses can lead to several diseases, the most common are Gaucher's disease (GD), Fabry disease, Farber disease, and Niemann-Pick disease.

The most common LSDs is Gaucher Disease (GD), an autosomal recessive disorder caused by mutations in the *GBA* gene, resulting in β-Glucocerebrosidase (β-glucosidase) deficiency.

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TABLE 3 Lysosome Storage Disorders (LSDs).

| Sphingolipidoses | | | | |
|---|---|---|---|---|
| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
| β-Glucocerebrosidase (β-glucosidase) encoded by *GBA* | Breaks down glucocerebroside into glucose and ceramide. | Gaucher Disease (GD), also known as Glucocerebrosidase deficiency | | *GBA* in liver (85). |
| α-Galactosidase A | Breaks down globotriaosylceramide (known as Gb3 or CD77) | Fabry disease, also known as Alpha-galactosidase A deficiency | Gb3 in breast (86, 87), ovarian (88), and colon cancer (89). | |
| Acid ceramidase | Metabolizes ceramides into sphingosine and a fatty acid | Farber disease, also known as Farber lipogranulomatosis or Acid ceramidase deficiency | Prostate cancer (90, 91), head and neck squamous cell carcinoma (92), liver (93), and breast (94). | |
| Acid sphingomyelinase (ASM) | Metabolizes the hydrolysis of sphingomyelin into phosphorylcholine and ceramide. | Niemann-Pick Disease Types A and B (NPD-A and B), also known as Sphingomyelinase deficiency | | Breast, lung, thyroid, and bladder (95). |
| **Glycoproteinoses** | | | | |
| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
| Lysosomal α-mannosidase, encoded by *MAN2B1* | Breaks down oligosaccharides containing a mannose. | α-mannosidosis, also known as Alpha-mannosidase deficiency or Mannosidosis | *MAN2B1* in bladder urothelial carcinoma, breast invasive carcinoma, colon adenocarcinoma, glioblastoma multiforme, low-grade gliomas, and laryngeal cancer (96, 97). | |
| α-L-fucosidase, encoded by *FUCA1* | Cleaves fucose-rich oligosaccharides, glycoproteins, and glycolipids. | Fucosidosis, also known as Alpha-fucosidase deficiency | *FUCA1* in glioblastoma multiforme (98), papillary thyroid cancer (PTCs) samples (99), and breast cancer (100). | *FUCA1* in colorectal cancer (101), HCC (102), and anaplastic thyroid cancer (ATCs) samples (99). |
| Lysosomal neuraminidase-1 (NEU1; also known as sialidase) | Removes terminal sialic acid residues from sialo-rich oligosaccharides, glycoproteins and glycolipids. | Sialidosis, also known as Mucolipidosis Type I | HCC (103, 104), ovarian (105), and colon (106). | |
| **Mucopolysaccharidoses** | | | | |
| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
| Alpha-L-iduronidase (IDUA) | Breaks down glycosaminoglycans, such as dermatan sulfate and heparan sulfate. | MPS I, also known as IDUA deficiency, Hurler syndrome, Scheie syndrome or Hurler-Scheie syndrome | | Breast (107) and ovarian (108). |
| Iduronate-2-sulfatase (IDS) | Breaks down glycosaminoglycans, such as dermatan sulfate and heparan sulfate. | MPS II, also known as Hunter Syndrome or Iduronate 2-sulfatase deficiency | | Breast (109). |
| Arylsulfatase B (ARSB; also known as N −acetylgalactosamine −4−sulfatase) | Breaks down glycosaminoglycans, such as dermatan sulfate and chondroitin sulfate. | MPS VI, also known as Maroteaux-Lamy syndrome or Arylsulfatase B deficiency | | Melanoma (110), colorectal (111), prostate, and breast (112, 113). |
| β−glucuronidase, encoded by *GUSB* | Breaks down glycosaminoglycans, such as dermatan sulfate and keratan sulfate. | MPS VII, also known as Sly syndrome or Beta-glucuronidase deficiency | Colorectal (114), gastric (115), and pancreas (116). *GUSB* in HCC (117). | |

β-glucosidase is an enzyme that helps break down glucocerebroside into glucose and ceramide. Deficiency of β-glucosidase leads to an accumulation of glucocerebroside (also called glucosylceramide) and glucosylsphingosine in macrophages through the body, called Gaucher cells, mainly affecting the liver, spleen, and bone marrow (120, 121). GD has been classified by type and severity of neurological involvement: Type 1 GD (GD1) is defined as the non-neuronopathic subclass, acute neuronopathic GD (GD2) is characterized by acute neurological decline, and chronic neuronopathic GD (GD3) is identified by a highly variable spectrum of associated neurological and non-neurological manifestations (122). According to the Gaucher Registry, GD1 is the most common accounting for 90% - 95% of all documented cases of GD in Europe and North America (123). Notably, several case reports have shown a link between patients with GD1 and different cancers, including bone (124), breast (125), colon (126, 127), hematologic (125, 128), kidney (125, 129), liver (125, 130, 131), melanoma (125), multiple myeloma (125, 132), and non-Hodgkin lymphoma (125, 133). The development of cancer in GD patients could be explained by the accumulation of glucocerebroside in macrophages, leading to lipid-engorged macrophage activation, which affects immune system regulation in several different ways. The levels of pro-inflammatory, as well as, anti-inflammatory cytokines, chemokines, and growth factors, mostly those involved in inflammation and B-cell function are altered in the serum of GD patients compared to normal controls (134–138). The thymus shows the most prominent dysregulation, causing severe impairment of T-cell differentiation and maturation, abnormal B-cell recruitment, upregulation of CD1d and major histocompatibility complex (MHC) class II expression, which are mostly expressed on antigen-presenting cells (APCs), such as dendritic cells, thymic epithelial cells, and B cells. Suggesting impaired immune surveillance, which can support the development of malignancy (139–144). Furthermore, the downregulation of *GBA* expression in liver cancer tissues increased cellular glucosylceramide levels, promoting the metastasis ability by supporting the epithelial-mesenchymal transition (EMT) through activation of the Wnt/β-catenin signaling pathway (85).

Fabry disease is caused by mutations in the *GLA* gene leading to a deficiency of the α-Galactosidase An enzyme, causing the accumulation of globotriaosylceramide (known as Gb3 or CD77), a glycosphingolipid functioning as a receptor for pathogens and pathogenic products (145). This receptor particularly binds to the Shiga-like toxin 1 (SLT-1), a high-affinity harmless natural ligand that, upon binding to the receptor, the toxin is internalized and travels retrograde (against the flow) through the Golgi network and the ER, preventing the endo-lysosomal vesicular pathway, therefore, avoiding the degradation of the toxin (146, 147). Glycosphingolipids have been associated with oncogenesis (148). Several studies have shown that upregulation of Gb3 expression is necessary for cell invasiveness and correlates with metastasis in several cancer types, such as breast (86, 87), ovarian (88), and colon cancer (89). Suggesting that elevated Gb3 expression could act as an indicator of the transformation of tumor cells from their primary cancer state to the metastatic state, proposing that various invasive tumor types could share common mechanisms for metastasis (89). Moreover, a recent study showed that using the toxin internalization mechanism, they were able to deliver Shiga toxin-coated nanoparticles directly into the cytoplasm of Gb3-expressing head and neck cancer cells, demonstrating a novel way to deliver peptides or therapeutic nanomaterials inside cells (147).

Farber disease is a rare autosomal recessive disorder, also known as Farber lipogranulomatosis, caused by a mutation in the *ASAH1* gene, which leads to ceramide accumulation in several organs and tissues due to lysosomal acid ceramidase deficiency (149). Acid ceramidase is the enzyme that metabolizes ceramides into sphingosine and a fatty acid, products that are then recycled to create new ceramides. Ceramides are pro-apoptotic lipids and are part of the outer membrane surrounding cells, where they sense stress and other external factors and can mediate growth arrest, differentiation, and apoptotic cell death (150). In addition, acid ceramidase enzyme activity and sphingosine kinase can promote the formation of sphingosine-1-phosphate (S1P), a potent anti-apoptotic lipid mediating cell proliferation and survival (90). Acid ceramidase is overexpressed in prostate cancer (90, 91), head and neck squamous cell carcinoma (92), liver (93), and breast (94). The increase of acid ceramidase causes decreased ceramide accumulation and increased levels of sphingosine and S1P, indicating its involvement in metabolizing a significant portion of ceramides in tumor cells, resulting in tumor growth, survival, and resistance to therapy (90, 91, 93, 94). Suggesting that targeting the enzymes, acid ceramidase and sphingosine kinase, will block the tumor cell's ability to metabolize ceramide, leading to an increase of pro-apoptotic ceramide levels, which will result in apoptosis (151, 152), growth inhibition (93, 152, 153), and increased sensitivity to radiation (154), and chemotherapeutics (155, 156).

Niemann-Pick Disease Types A and B (NPD-A and B) are rare autosomal recessive LSDs, categorized as sphingolipidoses due to sphingomyelin accumulation. These diseases arise from the deficiency of the acid sphingomyelinase (ASM) enzyme, caused by mutations in the sphingomyelin phosphodiesterase 1 (*SMPD1*) gene (157). ASM metabolizes the hydrolysis of sphingomyelin into phosphorylcholine and ceramide. A recent study demonstrated the incidence of cancer in patients diagnosed with ASM deficiency was abnormally elevated with four types of cancers being observed: breast, lung, thyroid, and bladder (95). Moreover, dysfunction of the ASM enzyme can alter sphingolipid metabolism leading to the downregulation of ceramide (a pro-apoptotic lipid) and the upregulation of S1P (a proliferative lipid) in cancer, possibly resulting in tumorigenicity and/or the potential to metastasize (158).

## 4.2 Glycoproteinoses

Glycoproteinoses are characterized as LSDs affecting glycoprotein degradation, causing an increased accumulation of undegraded oligosaccharides and/or glycoconjugates in lysosomes (159). Glycoproteinoses are rare and can lead to several diseases with high prevalence, such as α-mannosidosis, fucosidosis, and sialidoses (160).

α-mannosidosis is an autosomal recessive disorder caused by mutations in the *MAN2B1* gene, which encodes lysosomal α-mannosidase and results in α-mannosidase deficiency, leading to accumulation of mannose-rich oligosaccharides (161). Elevated expression of *MAN2B1* has been found in several cancers, including bladder urothelial carcinoma, breast invasive carcinoma, colon adenocarcinoma, glioblastoma multiforme, low-grade gliomas, and laryngeal cancer (96, 97). Specifically, the overexpression of *MAN2B1* in glioma tissues is associated with immune response and anti-inflammatory functions by correlating with the expression of tumor-associated macrophages and M2 macrophages, and correlates with malignant clinical features and poor outcome for glioma patients (96). In addition, expression of α-mannosidases has been shown in human papillomavirus (HPV)-associated cervical tumors (162) and nasopharyngeal carcinoma (163), resulting in tumor growth and metastasis. Furthermore, an inhibitor of α-mannosidases, known as swainsonine, was shown to block metastasis of melanoma and lymphoid tumor cells in mice and reduce the growth rate *in vitro* and *in vivo* of human melanoma cells. These data suggest that the expression of oligosaccharides associated with a malignant phenotype may be involved in tumor growth (164). However, other *in vivo* studies with HPV-associated cervical tumors, showed that swainsonine led to tumor growth, by inducing the accumulation of myeloid cells in the spleen of tumor-bearing mice, thereby inhibiting T-cell activation and aggravating the tumors system effects on the immune system, thus enabling tumor growth (162).

Fucosidosis is caused by mutations in the *FUCA1* gene, resulting in defective lysosomal α-L-fucosidase, which leads to the accumulation of fucose-rich oligosaccharides, glycoproteins, and glycolipids in tissues and urine (165). Several studies have shown that *FUCA1* is a p53 target gene, involved in tumorigenesis, and is capable of hydrolyzing various fucosylation sites on the epidermal growth factor receptor (EGFR), which ultimately determines the activation of EGFR (98, 99, 166, 167). According to various studies, it appears that the expression of *FUCA1* in human cancers is complex. Decreased *FUCA1* expression has been observed in colorectal cancer (101), hepatocellular carcinoma (102), and anaplastic thyroid cancer (ATCs) samples (99). While increased *FUCA1* expression has been observed in glioblastoma multiforme (98), papillary thyroid cancer (PTCs) samples (99), and breast cancer (100). Tsuchida et al., observed a potential relationship between *FUCA1* expression and p53 status, with a decreased expression of *FUCA1* and the presence of mutated p53 in ATCs, and an increased expression of *FUCA1* in PTCs, which predominantly harbor wild-type p53 (99). In addition, Ezawa et al. were able to demonstrate that tumor suppressor protein p53 is involved in protein glycosylation and targets *FUCA1* gene expression, resulting in its upregulation. This upregulation leads to the removal of fucose from the EGFR protein, ultimately suppressing cancer cell growth and inducing cell death. Furthermore, the study suggests that the upregulation of *FUCA1* expression contributes to the repression of the EGFR signaling pathway and has tumor-suppressing activity in various human cancers (166). Moreover, Xu et al. showed that *FUCA1* is highly expressed in glioma tissues, leading to poor prognosis in glioma patients. The inhibition of *FUCA1* suppressed glioma growth *in vitro* and *in vivo*, promoting autophagy through the formation of large acidic vacuoles and by lowering levels of tumor-infiltrating macrophages (98).

Sialidosis, also known as Mucolipidosis Type I, is caused by autosomal recessive mutations in the *NEU1* gene, encoding the lysosomal enzyme neuraminidase-1 (NEU1; also known as sialidase), a glycosidase that removes terminal sialic acid residues from sialo-rich oligosaccharides, glycoproteins and glycolipids (168). Sialidase deficiency leads to the accumulation of sialyloligosaccharides and glycopeptides (169). *NEU1* is also involved in other cellular processes, such as cell proliferation/migration/differentiation, macrophage-associated immune and pro-inflammatory responses, and lysosomal exocytosis (103, 170–173). NEU1 is upregulated in HCC tumor tissues, which correlates with advanced stage, grade, and worse survival of HCC patients. Higher expression of NEU1 is associated with increased proliferation, migration, and lower levels of B cells, T-cells, and natural killer (NK) cells, regulating several tumor-related proteins and pathways, such as lysosome, spliceosome, and mTOR signaling pathways (103, 104). In pancreatic cancer cells, NEU1 forms a complex with MMP-9 and G protein-coupled receptors (GPCRs) to regulate EGFR activation and cellular signaling, playing a crucial role in the activation of receptor tyrosine kinases and downstream signaling pathways, making it a potential therapeutic target (174, 175). Oseltamivir phosphate (Tamiflu), anti-NEU1 antibodies, and broad-range MMP inhibitor galardin (GM6001) were found to inhibit NEU1 activity associated with EGF-stimulated cells (174). In addition, aspirin and celecoxib were also shown to inhibit NEU-1 activity in pancreatic cells, suggesting a novel multimodality mechanism of action for these drugs as anti-cancer agents (175). The inhibition of NEU1 activity in breast cancer cells, using oseltamivir phosphate or siRNA, also suppressed cell growth and induced apoptosis (176). Furthermore, NEU1 is overexpressed in ovarian cancer tissues compared with adjacent normal tissues. The siRNA of NEU1 in human ovarian cancer effectively inhibited proliferation, apoptosis, and invasion of cells by targeting lysosome and oxidative phosphorylation signaling (105). In contrast, NEU1 overexpression in colon cancer suppresses metastasis *in vivo*, and *in vitro* decreases cell migration, invasion, and adhesion, which involves downregulation of MMP-7, through integrin beta4-mediated signaling (106).

## 4.3 Mucopolysaccharidoses

Mucopolysaccharidosis (MPS) is an inherited disorder caused by a deficiency or malfunction in lysosomal enzymes responsible for breaking down glycosaminoglycans (GAGs), such as dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate. The ECM contains significant amounts of GAGs, which play a crucial role in promoting cell-to-cell and cell-to-ECM adhesion (177). The deficiency of the enzymes responsible for the proper degradation of GAGs can lead to systemic accumulation of GAGs in cells, blood, brain, spinal cord, and connective tissues. MPS is categorized as seven types of diseases, some of which are further

categorized into subtypes. Six MPS types are autosomal-recessive inherited, and one type is inherited by the X-linked gene, known as MPS II or Hunter Syndrome (178). A retrospective study showed that the highest incidence rate at birth and prevalence rate was found for MPS I, II, and III in the US (179). Since MPS I and II have the relatively highest incidences at birth, compared to the other types of MPS, we will mainly focus on these two types of diseases. In addition, we will present types of MPS VI and VII, which have shown a link with cancer development, even though they have very low incidences at birth.

MPS I is an autosomal recessive disorder characterized by alpha-L-iduronidase (IDUA) enzyme deficiency, which is caused by a mutation in the *IDUA* gene, leading to the accumulation of dermatan sulfate and heparan sulfate in several organs and tissues. MPS I can show various degrees of clinical manifestations, and therefore is categorized according to its severity: the most severe form of MSP I is Hurler syndrome, the moderate form is Hurler-Scheie, and the least severe is Scheie syndrome (177). Currently, there is limited knowledge about the involvement of IDUA in cancer. One study found that tumors from breast cancer patients with visceral metastasis had significantly decreased IDUA expression levels compared to those without visceral metastasis. Suggesting an association between IDUA gene expression with the development of visceral organ metastasis and survival of breast cancer patients (107). Another study by Liu et al. performed a bioinformatics analysis with data from the Gene Expression Omnibus (GEO) database and obtained a glycometabolism-related gene set associated with the overall survival of patients with ovarian cancer. They were able to identify IDUA as a prognostic gene of ovarian cancer. In addition, they analyzed the expression of IDUA in ovarian cancer cells. Results showed that IDUA expression was significantly downregulated compared to human ovarian epithelial cells (108). However, additional studies are needed to elucidate the role and understand the mechanistic relationship between IDUA and cancer.

MPS II, also known as Hunter Syndrome, is caused by an inherited mutation in the *IDS* gene encoding for the iduronate-2-sulfatase (IDS) enzyme, resulting in dermatan sulfate and heparan sulfate accumulation. Presently, there is one research study exploring the potential relationship of IDS with cancer. Singh et al. found depleted IDS levels in invasive malignant epithelia of breast cancer sections compared to non-invasive or untransformed breast tissues. Simultaneously, there was a rise in levels of dermatan sulfate in the extracellular environment. Following a reduction in IDS levels, non-invasive breast cancer (MCF-7) cells displayed an increase in invasion and a shift towards a mesenchymal morphology with cytoplasmic protrusions on collagen matrices, whereas control cells retained their polygonal shape. These findings suggest that transformed cells may secrete dermatan sulfate, which can modify the mechanical characteristics and polymeric organization of nearby collagen fibers. This, in turn, may promote improved interaction between cells and the ECM, and facilitate mesenchymal migration of breast cancer cells (109).

Furthermore, it should be noted that other types of MPS, such as Type VI (Maroteaux-Lamy syndrome) caused by mutations in the *ARSB* gene, leading to deficiency of arylsulfatase B (ARSB; also known as N–acetylgalactosamine–4–sulfatase), has also been found to be associated with cancer development. The main role of ARSB is to break down GAGs into dermatan sulfate and chondroitin sulfate (180). In melanoma cells, ARSB activity was decreased compared to normal melanocytes. The decrease of ARSB activity resulted in the overexpression of melanoma progression factors, such as chondroitin sulfate proteoglycan 4 (CSPG4) and pro-matrix metalloproteinase 2 (pro-MMP2), causing increased invasiveness of melanoma cells (110). A decrease of ARSB activity in colorectal cancer cells compared to colonic epithelial cells, demonstrated an increase in cell adhesion, migration, and invasion, through upregulation of MMP9 expression and RhoA activation, which are mediators of cellular motility, implicating a key role of ARSB activity in the metastatic potential of epithelial cells (111). In addition, a decline in ARSB activity has been shown in prostate and breast carcinoma cells, which is associated with an increase in total sulfated GAGs and chondroitin sulfate content in malignant cells, suggesting a role in cell-to-cell and cell-to-matrix interactions (112, 113).

MPS Type VII (Sly syndrome) caused by β–glucuronidase deficiency involving the *GUSB* gene, has also been linked to cancer development. Several studies have reported that β–glucuronidase activity was higher in different cancers, such as highly invasive colorectal carcinoma cells compared to poorly invasive cells (114), gastric cancer compared to non-cancerous tissues (115), and pancreatic cancer due to an increased steady-state level of the enzyme compared to healthy pancreas (116). These results suggest that increased β-glucuronidase is closely related to tumor progression and metastasis. Moreover, a recent article investigating the resistance mechanism of anti-PD1 (programmed cell death 1 protein) found that *GUSB* expression was higher in HCC tumors that do not respond to anti-PD1 treatment compared to responding tumors. Anti-PD1 therapy has been shown to play a major role in inhibiting effector immune cell depletion, resulting in successful treatment advances (181, 182). However, HCC tumors can develop resistance against anti-PD1 (183). It was found that increased *GUSB* expression in HCC cells promotes cancer cell growth, reduced PD-L1 expression, and immunosuppression. In contrast, silencing *GUSB* prevents proliferation, invasion, and migration of HCC human cells, upregulation of PD-L1 expression, increased NK and T-cells in the tumor microenvironment, and decreases immunosuppressive cells such as regulatory T-cells (Tregs) and M2 macrophages. Therefore, inhibiting *GUSB* expression offers a novel strategy to reduce HCC cell progression and improve the sensitivity to anti-PD1 therapy (117).

## 5 Fatty acid oxidation disorders

Mitochondrial Fatty Acid Beta Oxidation (FAO) is a major multi-step process by which cells break down fatty acids and catabolize them into acetyl-coA, which subsequently enters the tricarboxylic acid (TCA) cycle resulting in the production of more ATP than the oxidation of carbohydrates (Figure 3) (184). Beta-oxidation is an important source of energy, especially during

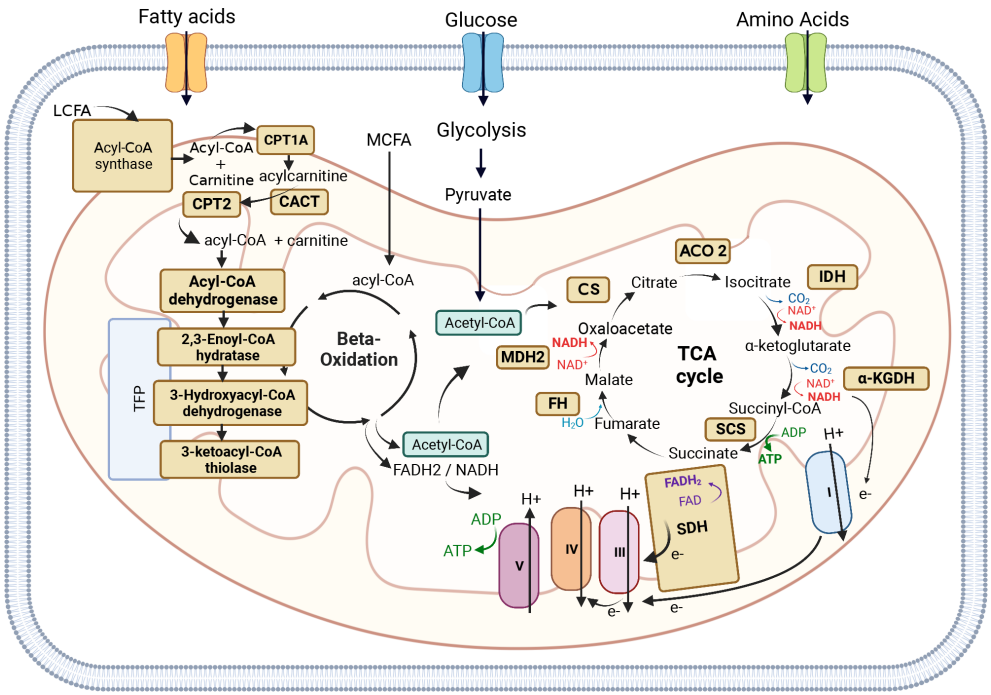


FIGURE 3
Mitochondrial Fatty Acid Beta Oxidation and TCA cycle. Mitochondrial Fatty Acid Beta Oxidation (FAO) is a major multi-step process by which cells break down fatty acids and catabolize them into acetyl-coA, which subsequently enters the tricarboxylic acid (TCA). The FADH2 and NADH produced by FAO are used by the Electron Transport Chain (ETC) to produce ATP. Long-chain fatty acids (LCFA) cannot enter mitochondria through passive diffusion, like Medium-chain fatty acids (MCFAD), and need to be activated into fatty acyl-coenzyme A in the cytosol by acetyl-CoA synthetase, and then conjugated to carnitine to be imported into the mitochondrial matrix. The shuttle of LCFA into mitochondria is carried out by three enzymes: the outer mitochondrial membrane enzyme Carnitine palmitoyltransferase 1A (CPT1A), the mitochondrial intermembrane space enzyme Carnitine-acylcarnitine translocase (CACT), and the inner mitochondrial membrane enzyme Carnitine palmitoyltransferase 2 (CPT2). Once fatty acyl-CoA is inside the mitochondrial matrix, it can enter the beta-oxidation cycle to produce acetyl-CoA, which can subsequently enter the TCA cycle. Four main enzymes are involved in the beta-oxidation cycle: acyl-CoA dehydrogenase, 2,3-Enoyl-CoA hydratase, 3-Hydroxyacyl-CoA dehydrogenase, and 3-Ketoacyl-CoA thiolase. The other beta-oxidation steps are catalyzed by the mitochondrial trifunctional protein (TFP or MTP), a protein complex attached to the inner mitochondrial membrane composed of two types of subunits: the alpha subunit (TFPα) and the beta subunit (TFPβ). The TFPα subunit comprises the 2,3-enoyl-CoA hydratase and 3-hydroxyacyl- CoA dehydrogenase activities, whereas the TFPβ subunit comprises the 3-Ketoacyl-CoA thiolase activity. The TCA cycle is a key metabolic node whose main function is to generate electrons to fuel the mitochondrial ETC (mETC) for ATP production. The breakdown of fatty acids (beta-oxidation), glucose (glycolysis), and some amino acids leads to the production of Acetyl-CoA, which can then enter the TCA cycle. Acetyl-CoA is a key substrate that participates in the first reaction of the TCA cycle, ensured by Citrate Synthase (CS) enzyme which converts oxaloacetate into citrate. The second reaction of the TCA cycle leads to the conversion of citrate into isocitrate by Aconitase (ACO2), which converts citrate into isocitrate. Isocitrate is then converted by Isocitrate Dehydrogenase (IDH), during the third reaction of the TCA cycle into α-ketoglutarate. α-ketoglutarate is converted into Succinyl-CoA by α-Ketoglutarate Dehydrogenase (α-KGDH). Succinyl-CoA is then converted by Succinyl-CoA synthetase (SCS) into succinate, which is then converted by Succinate dehydrogenase (SDH or mETC Complex II) into fumarate. Fumarate is converted into malate by Fumarate Hydratase (FH). The last reaction of the cycle is the conversion of malate into oxaloacetate by Malate Dehydrogenase (MDH2). The mETC is composed of 5 enzymatic complexes: Complex I-V. Electrons generated by the TCA cycle funnel through the mETC allowing the complexes I, III, and IV to pump protons generating a membrane potential used by the complex V to generate ATP. The Figure was partly generated using Biorender under the agreement number: VS25LLE9OH (www.Biorender.com).

periods of high-energy demand such as fasting or exercise, but also for high-energy dependent tissues, such as the heart, muscle, liver, and brain. This is why mutations in the genes coding for the enzymes involved in either the beta-oxidation cycle or the transport of long-chain fatty acids (LCFA) into mitochondria can lead to severe inherited metabolic FAO disorders (FAODs) (185, 186). Unlike Medium or Short-chain fatty acids, LCFA cannot enter mitochondria through passive diffusion and need to be activated into fatty acyl-coenzyme A in the cytosol by acetyl-CoA synthetase, and then conjugated to carnitine to be imported into the mitochondrial matrix (187).

The shuttle of LCFA into mitochondria is carried out by three enzymes: the outer mitochondrial membrane enzyme Carnitine palmitoyltransferase 1A (CPT1A), the mitochondrial intermembrane space enzyme Carnitine-acylcarnitine translocase (CACT), and the inner mitochondrial membrane enzyme Carnitine palmitoyltransferase 2 (CPT2). CPT1 catalyzes the rate-limiting step of FAO by converting fatty acyl-CoA into acyl-carnitine, which is then transported into the mitochondrial matrix via CACT. CPT2 carries out the last reaction by converting carnitine into Acyl-CoA, which can then enter the beta-oxidation cycle (Figure 3) (185, 186, 188, 189). Alterations in either of the three enzymes (CPT1A, CACT, and CPT2) can prevent the body from using certain types of fatty acids leading to hypoketotic hypoglycemia (decreased glucose in the blood) under fasting conditions or during exercise. Moreover, CPT2 deficiency has more severe clinical presentations

than CPT1 deficiency (189). It is interesting to note that while CPT1A and CPT2 are involved in the same metabolic pathway, their levels of expression, such as CPT1A upregulation and CPT2 downregulation, can have opposite effects in different types of cancer. CPT1A upregulation has been found to promote the proliferation, survival, and invasion of several cancer types, including colorectal cancer (190–192), nasopharyngeal cancer (193), ovarian cancer (194, 195), glioblastoma (196), gastric cancer (197), and HCC (198), and in many cases is associated with poor prognosis and metastasis. In contrast, downregulation of CPT2 was found to be associated with poor prognosis and tumorigenesis in colorectal cancer (199–201) and HCC (198, 202).

The elevated expression of CPT1A has been observed in metastatic tumors compared to primary tumors of colorectal cancer patients (191). Wang et al. showed that CPT1A upregulation promotes metastasis of detached colorectal cancer cells by inhibiting anoikis, a programmed cell death that occurs when cells detach from the ECM, while a decrease in metastasis was observed in CPT1A-depleted colorectal cancer cells (190). An *in vitro* study showed that adipocytes co-cultured with colon cancer cells release fatty acids which are taken up by cancer cells, allowing them to survive in nutrient-deprived conditions by upregulating mitochondrial FAO. Whereas *in vivo* studies showed that co-injection of adipocytes with colon cancer cells promotes tumor growth (191), silencing CPT1A in colon cancer cells eliminated the protective effect of fatty acids against nutrient deprivation and decreased the expression of genes associated with cancer stem cells downstream of the Wnt/β-catenin pathway (192). This suggests that the presence of adipocytes in the TME are a source of energy and metabolic regulators, facilitating the survival and proliferation of colon cancer cells. Additionally, CPT1A upregulation has been observed in radiation-resistant nasopharyngeal cancer cells associated with Rab14 (a GTPase), which facilitates fatty acid trafficking from lipid droplets to the mitochondria where FAO takes place, resulting in decreased radiation-induced lipid accumulation, demonstrating a role for CPT1A in radiation resistance (193). Moreover, CPT1A is overexpressed in most ovarian cancer cell lines, primary ovarian serous carcinomas, and a subset of high-grade serous ovarian cancers (HGSOCs) (194, 195). Studies *in vitro* showed CPT1A deficiency in ovarian cancer cell lines results in decreased cellular ATP levels, cell cycle arrest, suppression of anchorage-independent growth, and reduced xenograft formation through the induction of p21 (cyclin-dependent kinase inhibitor) by activation of the transcription factor FoxO by AMPK, JNK, and p38 (194).

On the other hand, the downregulation of CPT2 in colorectal cancer promotes cell proliferation capacity (199, 200) and inhibits apoptosis by decreasing p53 expression (200). In addition, the downregulation of CPT2 in colorectal cancer can promote cancer stemness and oxaliplatin (chemotherapy drug) resistance through the activation of the Wnt/β-catenin pathway by inducing glycolytic metabolism (201). In HCC tissues and serum of HCC patients, the accumulation of acylcarnitines, which serve as carriers to transport activated LCFA into the mitochondria for beta-oxidation, could be attributed to CPT2 downregulation, leading to the suppression of beta-oxidation and metabolic reprogramming to escape lipotoxicity and promote hepatocarcinogenesis (198). Moreover, the downregulation of CPT2 has been shown to have a link to human nonalcoholic fatty liver disease (NAFLD)-related hepatocarcinogenesis. Elevated levels of transcription factors E2F1 and E2F2 were observed in NAFLD, suggesting that these transcription factors may be metabolic drivers of HCC by promoting a lipid-rich environment (203). In glioblastoma multiforme, enhanced fatty acid metabolism by co-enhancement of CPT1A and CPT2 and immune checkpoint CD47, which functions as an anti-phagocytic signal, promotes the growth of radioresistant glioblastoma multiforme cells. By blocking FAO there is a reduction of CD47 anti-phagocytosis and tumor growth. Targeting the FAO-CD47 axis could therefore be an efficient way to block the growth of radioresistant glioblastoma multiforme cells (196).

Once fatty acyl-CoA is inside the mitochondrial matrix, it can enter the beta-oxidation cycle to produce acetyl-CoA, which can subsequently enter the TCA cycle. Four main enzymes are involved in the beta-oxidation cycle: acyl-CoA dehydrogenase, 2,3-Enoyl-CoA hydratase, 3-Hydroxyacyl-CoA dehydrogenase, and 3-Ketoacyl-CoA thiolase (also known as acetyl-CoA transferase) (Figure 3). The beta-oxidation cycle can be described in four steps: (i) Fatty acyl-CoA is dehydrogenated by acetyl-CoA dehydrogenase resulting in the formation of 2,3-enoyl-CoA, (ii) 2,3-enoyl-CoA is hydrated to form 3-hydroxyacyl-CoA by 2-enoyl-CoA hydratase, (iii) 3-hydroxyacyl-CoA is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase to form the 3-ketoacyl-CoA compound, and (iv) 3-ketoacyl-CoA is cleaved by thiolase yielding acetyl-CoA and an acyl-CoA two carbons shorter than the original, which can re-enter at the first step in the beta-oxidation pathway.

The first beta-oxidation step is catalyzed by various acyl-CoA dehydrogenases, each with a specific affinity towards different fatty acyl chain lengths. Acyl-CoA Dehydrogenase Very-Long Chain (ACADVL or VLCAD) and Acyl-CoA Dehydrogenase Medium-chain (ACADM or MCAD), are two types of Acyl-CoA dehydrogenases that initiate beta-oxidation of Very-Long Chain Acyl-CoA esters and Medium-Chain Acyl-CoA esters, respectively. Deficiencies in these enzymes are common in FAOD and result in hypoketotic hypoglycemia, liver dysfunction, and liver failure. VLCAD deficiency is clinically distinct, causing rhabdomyolysis (muscle tissue breakdown releasing myoglobin) and cardiomyopathy, which are not observed in MCAD deficiency (204). Recent studies have shown that the downregulation of VLCAD in human HCC tissues and cells promotes cell proliferation and metastasis (205). On the other hand, in glioblastoma, MCAD plays a crucial role in protecting cancer cell integrity against the accumulation of toxic by-products that would otherwise affect mitochondrial activity, demonstrating the non-energetic role of FAO enzymes in the dependence on fatty acid metabolism in cancer (205, 206).

The other three beta-oxidation steps are catalyzed by the mitochondrial trifunctional protein (TFP or MTP), a protein complex attached to the inner mitochondrial membrane (207, 208). TFP is composed of two types of subunits: the alpha subunit (TFPα), encoded by the *HADHA* gene, and the beta

subunit (TFPβ), encoded by the *HADHB* gene. The TFPα subunit comprises the 2,3-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, whereas the TFPβ subunit comprises the 3-Ketoacyl-CoA thiolase activity (Figure 3) (209). Mutations of *HADHA* or *HADHB* genes leads to TFP deficiency, an autosomal recessive disorder affecting LCFA oxidation characterized by hypoglycemia, hypotonia (decreased muscle tone), and liver dysfunction (210, 211). The TFP is a promising target to restrain tumor growth in lung carcinomas by targeting the activity of the *HADHA* enzyme (212, 213). Ameodo et al. observed a metabolic heterogeneity between human biopsies of lung adenocarcinomas and divided them into two subgroups: (i) tumors with a low mitochondrial respiration and (ii) tumors with a high mitochondrial respiration. This second group was poorly relying on glucose and was presenting an increased expression of the TFP enzyme *HADHA* compare to the adjacent tissue. Inhibition of the TPF activity *in vivo* leads to a reduction of tumor growth (212). Moreover, both *HADHA* and *HADHB* enzymes have been found overexpressed in malignant lymphoma progression (214, 215), relying on fatty acid metabolism and notably FAO as a key metabolic pathway for tumor progression and survival (216, 217). Additionally, in colorectal cancer and stomach adenocarcinoma, *HADHB* has been proposed as a tumor suppressor, its expression being significantly lower in tumors compared to normal tissue (218, 219).

Glucose and amino acids have been well-studied in cancer metabolism and are considered important sources of energy to fuel tumor growth and survival. It is also well known that cancer cells can rely on fatty acid metabolism, and notably *de novo* lipid synthesis, an anabolic pathway, for their proliferation and survival. Over the last decade, research has highlighted how cancer cells can also rely on FAO (catabolic pathway) reshaping our view on how tumors can use lipid metabolism to their advantage (220, 221). Table 4 encompasses details about mutated enzyme names, enzymatic roles, diseases linked to the enzymes, and the regulation (up or down) of these enzymes in different cancer types involved in Fatty Acid Oxidation. Further research is needed to fully characterize the energetic and non-energetic roles that FAO enzymes can play to promote cancer progression.

## 6 Mitochondrial disorders

The most common inherited metabolic disorders are mitochondrial disorders caused by dysfunction of mitochondrial activity (222). The mitochondria is a key cellular organelle, known as the powerhouse of the cell, which ensures energy production in the form of ATP. The mitochondrial machinery relies on genes from both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). The mtDNA codes for 2 rRNAs, 22 tRNAs, and 13 proteins, which are part of the multi-subunit enzymatic complexes of the electron respiratory chain (ETC) (223, 224). The TCA cycle, also known as the Krebs cycle, is a key metabolic node whose main function is to generate electrons to fuel the ETC for ATP production (225). The TCA cycle comprises 8 enzymes, all encoded by genes located in the nDNA. Electrons generated by the TCA cycle allow the ETC to generate a membrane potential, which is used to convert ADP into ATP, a process called oxidative phosphorylation (OXPHOS) (Figure 3). Mutations in genes encoding the enzymes involved in the TCA cycle and OXPHOS can lead to mitochondrial disorders and cancer, due to the inability of mitochondria to produce energy. Table 5 encompasses details about enzyme

TABLE 4 Fatty Acid Oxidation Disorders (FAODs).

| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
|---|---|---|---|---|
| Carnitine palmitoyltransferase 1A (CPT1A) | Catalyzes the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to carnitine. | Carnitine palmitoyltransferase I (CPT I) deficiency or CPT 1A deficiency | Colorectal cancer (190–192), nasopharyngeal cancer (193), ovarian cancer (194, 195), glioblastoma (196), gastric cancer (197), and HCC (198). | |
| Carnitine palmitoyltransferase 2 (CPT2) | Catalyzes the re-conjugation of long and very-long-chain acyl-carnitines to acyl-CoA | Carnitine palmitoyltransferase II (CPT II) deficiency or CPT2 deficiency | | Colorectal cancer (199–201) and HCC (198, 202). |
| Acyl-CoA Dehydrogenase Very-Long Chain (ACADVL or VLCAD) | Breaks down a group of very long-chain fatty acids | Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency | | HCC (205). |
| Acyl-CoA Dehydrogenase Medium-chain (ACADM or MCAD) | Breaks down a group of medium-chain fatty acids. | Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency | Glioblastoma (205, 206). | |
| Mitochondrial trifunctional protein (TFP or MTP), composed of two types of subunits: the alpha subunit (TFPα; *HADHA* gene), and the beta subunit (TFPβ; *HADHB* gene). | Catalyzes the last three reactions in the fatty acid β-oxidation process. Breaks down long-chain fatty acids. | Mitochondrial trifunctional protein deficiency or MTP deficiency | *HADHA* in lung carcinomas (212, 213) and both *HADHA* and *HADHB* in malignant lymphoma (214, 215). | *HADHB* in colorectal cancer and stomach adenocarcinoma (218, 219). |

TABLE 5 Mitochondria Disorders – TCA Cycle.

| Enzymes | Role | Disease Name | Upregulated Cancers | Downregulated Cancers |
|---|---|---|---|---|
| Citrate synthase (CS) | Binds with the oxaloacetate and reacts with acetyl-CoA, leading to the production of citrate. | | Ovarian (226), pancreas (227), and colon (228). | Cervical (229). |
| Aconitase (ACO2) | Catalyzes the conversion of citrate into isocitrate | Cerebellar-retinal degeneration (230, 231) and with severe optic atrophy and spastic paraplegia (232). | HCC (233). | Gastric cancer (234) and colorectal cancer (235). |
| Isocitrate dehydrogenase (IDH). Three IDH isoforms exist IDH1, IDH2, and IDH3. | Converts of isocitrate into α-ketoglutarate (α-KG) | | *IDH1* and *IDH2* in glioblastoma (236) *IDH2* in colorectal (237) and lung (238). IDH3-a in glioblastoma (239) and in HCC (240). | |
| α-ketoglutarate dehydrogenase (α-KGDH), also called 2-oxoglutarate dehydrogenase (OGDH). | Converts α-KG into succinyl-CoA | alpha-ketoglutarate dehydrogenase complex (KGDHC) deficiency | Gastric (241). | |
| Succinyl-CoA synthetase (SCS), also known as Succinyl-CoA ligase.<br>SCS is composed of two subunits, an α-subunit which is encoded by the gene *SUCLG1*, and the β-subunit which is encoded by the gene *SUCLA2* (specificity for ADP), or by the gene *SUCLG2* (specificity for GDP). | Breaks down succinyl-CoA into succinate and free CoA, and converts ADP or GDP into ATP or GTP, respectively. | Succinyl-CoA ligase deficiency | *SUCLG1* in acute myeloid leukemia (242). | *SUCLA2* in prostate (243). |
| Succinate dehydrogenase (SDH), also known as Succinate-coenzyme Q reductase (SQR). SDH is composed of four subunits, *SDHA*, *SDHB*, *SDHC* and *SDHD*. | Catalyzes the oxidation of succinate to fumarate and transfers electrons from succinate to ubiquinone (coenzyme Q). | Succinate dehydrogenase (SDH) deficiency | | *SDHB* in ovarian (244). |
| Fumarase, also known as fumarate hydratase | Catalyzes the hydration of fumarate into L-malate. | Fumarase deficiency, also known as Fumarate hydratase deficiency or Fumaric aciduria. | | Clear cell renal carcinomas (245). |
| Malate dehydrogenase (MDH2) | Catalyzes the reversible conversion of malate into oxaloacetate | Mitochondrial malate dehydrogenase (MDH2) deficiency | Prostate (246). | |

mutations involved in the TCA cycle, their respective enzymatic roles, associated diseases, and the regulation status (up or down) of the enzymes and genes across diverse cancer types.

## 6.1 Tricarboxylic acid cycle

The first reaction of the TCA cycle is catalyzed by citrate synthase (CS), which binds with the oxaloacetate and reacts with acetyl-CoA, leading to the production of citrate. Chen et al. found that CS was upregulated in human ovarian tumors and human ovarian tumor cell lines. Knockdown of CS in ovarian cancer cells leads to decreased cell proliferation accompanied by downregulation of ERK phosphorylation, inhibition of cell migration and invasion with decreased expression of p-FAK, MMP2, and Vimentin, and decreased drug resistance by downregulation of *ATG12* (226). Additionally, the activity of CS is significantly higher in human pancreatic ductal carcinoma compared with adjacent nonneoplastic tissue, contributing to the conversion of glucose to lipids, which provides the substrate for membrane lipid synthesis in pancreatic cancer (227). In colon cancer cells, CS has been shown to interact with SIRT5, a nicotinamide adenine dinucleotide $(NAD)^+$-dependent deacetylase. SIRT5 dessucinylates CS, regulating its enzymatic activity, whereas hypersuccinylation of CS reduces its enzymatic activity and inhibits the proliferation and migration of colon cancer cells (228). Furthermore, Lin et al. found that reduced expression of CS in human cervical cancer cells leads to a change in cellular energy production, from mitochondrial aerobic respiration to cytosolic glycolysis. This change is accompanied by the induction of EMT, which results in accelerated tumor malignancy due to the deregulation of p53 functions and abnormal cell growth signaling (229).

The second reaction of the TCA cycle is ensured by an aconitase (ACO2) which catalyzes the conversion of citrate into isocitrate. ACO2 is a key enzyme of the TCA cycle and is also involved in lipid

metabolism. Citrate can be exported from the mitochondrial matrix to the cytosol to be converted back into oxaloacetate and acetyl-CoA, which can be used for fatty acid synthesis. The reduced ACO2 enzyme activity in cells can lead to a deficiency in cellular respiration, mitochondrial DNA depletion, and altered expression of some TCA components and electron transport chain subunits (247). ACO2 mutations have been associated with cerebellar-retinal degeneration (230, 231) and with severe optic atrophy and spastic paraplegia (232). ACO2 expression has been found dysregulated in different types of cancers and linked to tumor progression. Decreased expression of ACO2 is associated with poor prognosis in gastric cancer (234) and colorectal cancer (235) by promoting a switch from mitochondrial oxidative phosphorylation to glycolysis in the cytosol. The knockdown of ACO2 in colorectal cancer promotes cell proliferation and colorectal cancer growth (235). However, compared with normal hepatocytes, ACO2 was overexpressed in HCC cells, promoting cell proliferation and migration by affecting molecular pathways involved in cellular energy metabolism, metabolite changes, and fatty acid metabolic pathway (233).

The third step of the TCA cycle is the conversion of isocitrate into α-ketoglutarate (α-KG) by the enzyme isocitrate dehydrogenase (IDH). Three IDH isoforms exist IDH1, IDH2, and IDH3. IDH1 is present in the cytosol and the peroxisome, while IDH2 and IDH3 are present in the mitochondrial matrix. IDH1 and IDH2 are both $NADP^+$-dependent homodimers and catalyze the reversible conversion of isocitrate into α-KG. By contrast, IDH3 is an $NAD^+$-dependent heterotetrameric protein composed of two α subunits (*IDH3A*), one β subunit (*IDH3B*), and one γ subunit (*IDH3G*), that catalyzes the irreversible conversion of isocitrate into α-KG (248). The α subunit ensures the catalytic activity of the holoenzyme, requiring the function of the β and γ subunits (249). IDH2 and IDH3 are both involved in the TCA cycle. The IDH2 catalytic activity results in the reduction of $NAPD^+$ into NAPDH, and the IDH3 catalytic activity results in the production of the electron donor NADH. *IDH1* and *IDH2* are the most frequently mutated metabolic genes in human cancer, and their mutations have been identified in different types of cancer, notably in gliomas, secondary glioblastomas, cartilaginous and bone tumors, and acute myeloid leukemia (236, 250–252). Mutated *IDH1* and *IDH2* acquire a new ability by converting α-KG into the oncometabolite 2-HG (2-hydroxyglutarate), accumulation of which can lead to the modification of the epigenome, notably by inhibiting α-KG-dependent dioxygenases (248, 252–254). While the role of the mutant IDH2 in cancer has been well characterized, recent studies have shown there is a pro-tumorigenic role for wild-type IDH2 as well. In colorectal cancer, wild-type IDH2 protects cancer cells against ROS-mediated DNA damage (237). Additionally, in lung cancer cells, the overexpression of IDH2 decreases α-KG concentrations, enhances the production of 2-HG, and decreases ROS levels, protecting cancer cells against DNA damage. The downregulation of α-KG promotes the transcription of HIF1α-targeted glycolytic genes (238). While mutated IDH1 and IDH2 are cancer-driver genes through the production of 2-HG and its impact on the epigenome, IDH3 has not been characterized as such in cancer. A study showed that IDH3-a is elevated in glioblastoma, and loss of function decreases TCA cycle turnover and inhibits oxidative phosphorylation (239). Moreover, IDH3-a is upregulated in HCC tissues and is associated with increased tumor size and greater clinicopathologic stage of HCC. *In vitro* studies showed that IDH3-a promotes EMT by increasing metastasis associated 1 (MTA1), an oncogene involved in the progression of cancer cells to metastasis, thereby enabling migration and invasion of HCC cells (240).

The fourth reaction of the TCA cycle is the conversion of α-KG into succinyl-CoA, leading to the reduction of $NAD^+$ into NADH, an electron donor which directly transfers electrons to complex I of the ETC. This reaction is catalyzed by α-ketoglutarate dehydrogenase (α-KGDH), also called 2-oxoglutarate dehydrogenase (OGDH), a highly regulated enzyme, whose role in carcinogenesis has been unclear until recently (255). The levels of OGDH in gastric cancer tissues are highly upregulated compared to normal tissues, which correlates with poor clinicopathological parameters for gastric cancer patients. The overexpression of OGDH results in decreased EMT epithelial markers, mitochondrial membrane potential, oxygen consumption rate, intracellular ATP product, and upregulation of EMT mesenchymal markers, ROS levels, and $NADP^+$/NAPDH ratio, and facilitated the activation of Wnt/β-catenin signal pathway. In addition, the overexpression of OGDH promoted tumorigenesis of gastric cancer cells in nude mice (241).

The fifth reaction of the TCA cycle is catalyzed by Succinyl-CoA synthetase (SCS; also known as succinate-CoA ligase), which breaks down succinyl-CoA into succinate plus free CoA, and converts ADP or GDP into ATP or GTP, respectively. SCS is composed of two subunits, an α-subunit which is encoded by the gene *SUCLG1*, and the β-subunit which is encoded by the gene *SUCLA2* (specificity for ADP), or by the gene *SUCLG2* (specificity for GDP) (256). Mutations in both *SUCLG1* and *SUCLA2* have been associated with encephalomyopathic mtDNA depletion syndrome with methylmalonic aciduria (257). *SUCLG1* mutations can lead to severe lactic acidosis and elevated levels of methylmalonic acid and pyruvic acid in the blood and urine. While, *SUCLA2* mutations can lead to hypotonia (decreased muscle tone), muscle weakness, Leigh syndrome (a severe neurological disorder), dystonia (movement disorder), and sensorineural hearing loss (256). Increased *SUCLG1* expression in acute myeloid leukemia patients is associated with a decreased percent survival and identifies as a risky prognostic gene (242). *SUCLA2* has been previously shown to be significantly downregulated in prostate cancer (243). A model presented by Wang et al. predicts that in malignant prostate cancer cells, the GTP-specific beta subunit of succinyl-CoA synthetase (*SUCLG2*) is selectively lethal because the alternative route via ATP-specific succinyl-CoA synthetase (*SUCLA2*) is not present in these cells, creating a selective vulnerability to *SUCLG2* knockdown in malignant cells (258). Additionally, a recent study found that the overexpression of the epidermal growth factor receptor (EGFR) in prostate cancer cells leads to the upregulation of the ligand for the LIF receptor (LIFR). The upregulation of LIFR in turn leads to the overexpression of *SUCGL2*, an enzyme involved in the production of succinate. The increased production of succinate promotes the neuroendocrine differentiation of prostate cancer cells, which

makes them more resistant to androgen deprivation therapy (ADT) (259).

The sixth reaction of the TCA cycle is ensured by Succinate dehydrogenase (SDH), also called Succinate-coenzyme Q reductase (SQR), a mitochondrial metabolic enzyme complex (respiratory complex II) involved in both the TCA cycle and OXPHOS. SDH catalyzes the oxidation of succinate to fumarate and then transfers electrons from succinate to the ubiquinone pool of the ETC via the electron donor FADH2 (260–262). SDH is composed of four subunits, *SDHA* and *SDHB* subunits that ensure the catalytic activity of the SDH complex, and *SDHC* and *SDHD* subunits that anchor the complex to the inner mitochondrial membrane (263, 264). The subunits of this complex are exclusively encoded by genes located in the nDNA (260, 265). Mutations have been identified in the genes *SDHA*, *SDHB*, and *SDHD* and in one assembly gene factor (*SDHAF1*) in patients presenting Complex II deficiency (266, 267). Moreover, germline mutations of *SDHB*, *SDHC*, or *SDHD*, are associated with an increased risk of aggressive variants of renal cell carcinoma (264, 268–270). In addition, *SDHB* was found to be decreased in ovarian tumors. The knockdown of *SDHB* in mouse ovarian cancer cells increases proliferation, promotes EMT, and leads to histone hypermethylation. In *SDHB*-depleted cells, the amount of glucose fueling the TCA cycle is decreased and is compensated by an increase of glutamine, a contribution to sustaining TCA cycle activity. This suggests that the glucose entering the pentose phosphate pathway is increased in *SDHB*-deficient cells to sustain nucleotide biosynthesis and rapid proliferation (244).

The seventh step of the TCA cycle is ensured by fumarase or fumarate hydratase (encoded by the gene *FH*), which catalyzes the conversion of fumarate into malate. *FH* deficiency results in neonatal and infantile encephalopathy (271–273). Germline mutations of *FH* are associated with Multiple Cutaneous Leiomyomas with Uterine Leiomyomas (MCUL) syndrome, also known as Reed syndrome, and share features with hereditary leiomyomatosis and renal cancer cell (HLRCC) (274–276). HLRCC is a hereditary condition that causes the development of multiple leiomyomas (fibroids) in the skin and uterus, and an increased risk of developing renal cell carcinoma (277). Individuals with hemizygous germline *FH* mutations have an increased risk of renal cancer. The remaining wild-type allele in these tumors is often functionally inactivated, suggesting that FH inactivation promotes tumor development. The study shows that *FH* inhibition and the resulting elevation of intracellular fumarate leads to the upregulation of hypoxia-inducible factors (HIFs), which are involved in many cancers including clear cell renal carcinomas (245). In addition, an aggressive subtype of renal cell carcinoma caused by mutations in the *FH* gene is Fumarate hydratase (FH)-deficient renal cell carcinoma (FHdRCC), which can lead to fumarate accumulation, resulting in the activation of HIF through the inhibition of prolyl hydroxylases. HIF activation promotes tumorigenesis by inducing a metabolic shift to glycolysis, promoting the transcription of genes such as vascular endothelial growth factor (VEGF), and a tumor-promoting mechanism between HIF and EGFR (278).

The eighth and last reaction of the TCA cycle is ensured by Malate Dehydrogenase (MDH2), which catalyzes the reversible conversion of malate into oxaloacetate. MDH2 deficiency has been shown recently to lead to early-onset severe encephalopathy, a cause of Leigh syndrome, and has been identified as a pheochromocytoma and paraganglioma susceptibility gene (279, 280). Moreover, the overexpression of the gene MDH2 was associated with shorter relapse-free survival in prostate cancer patients who underwent chemotherapy. The knockdown of MDH2 in prostate cancer cell lines decreased cell proliferation, increased sensitivity to the chemotherapy drug docetaxel, and affected signaling pathways and metabolic efficiency by influencing JNK signaling and oxidative metabolism (246).

## 6.2 Oxidative phosphorylation

Glycolysis and FAO fuel the TCA cycle which transfers electrons to the ETC to generate ATP through OXPHOS. The ETC is composed of 5 enzymatic multi-subunit complexes (CI-CV) (Figure 3). Complex I, also known as NADH dehydrogenase, plays a crucial role by facilitating the oxidation of NADH to NAD+. Complex II, also known as succinate dehydrogenase, facilitates the conversion of succinate to fumarate through oxidation. Complex III, commonly known as Cytochrome c reductase, has the pivotal function of reducing cytochrome c. Complex IV, known as Cytochrome c oxidase, has a crucial function in the oxidation of cytochrome c. Finally, Complex V, commonly referred to as ATP synthase, earns its name from its essential role in the synthesis of ATP utilizing the proton motive force (281). Electrons go through a series of redox reactions when passing through the ETC complexes CI, CIII, and CIV releasing energy used by the complexes CI, CIII, and CIV to pump protons ($H^+$) from the mitochondrial matrix resulting in the generation of a membrane potential. This membrane potential is then used by Complex V to catalyze the conversion of ADP and inorganic phosphate into ATP (282). Mutations in genes involved in the respiratory chain complex biogenesis or activity leads to mitochondrial diseases, notably Leigh syndrome, MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) syndrome, MERRF (myoclonic epilepsy with ragged red fibers (MERRF) syndrome, and mitochondrial myopathies (283).

As cancer cells rewire their metabolism to use glucose through aerobic glycolysis, one of the causes could be mitochondrial defects (284). However, it has been shown that dysfunctional OXPHOS can also promote the dependence of cancer cells for aerobic glycolysis (285–287). Recent studies have highlighted that cancer cells can be highly reliant on OXPHOS for their proliferation and survival, and that the mitochondrial ETC can play an essential role in tumor growth (288–292). Birsoy et al. showed that cancer cells sensitive to low glucose levels harbor glucose use deficiencies or Complex I mutations that lead to mitochondrial dysfunction, and that these two phenomena constitute two distinct mechanisms (293). The OXPHOS pathway has also been found to be part of tumor metabolic heterogeneity. In a murine model of pancreatic ductal

adenocarcinoma (PDAC), mutations of the oncogene KRAS, known to play a critical role in PDAC, lead to the death of most cancer cells but induce the survival of a subpopulation of dormant tumor cells relying on OXPHOS (294). Moreover, in PDAC (295), Acute Myeloid Leukemia (AML) (292), and triple-negative breast cancer (TNBC) (296), chemotherapy-resistant cells have been found to rely on a high OXPHOS status, while in high-grade serous ovarian cancer (HGSOC), high OXPHOS cells are chemo-sensitive (297). Metabolic heterogeneity observed in some cancers highlights the importance of combining drugs targeting different metabolic pathways to synergistically impair cancer cell proliferation and survival. Suggesting OXPHOS as a cancer vulnerability and a new potential therapeutic target (298).

Several studies have deciphered the role played by the OXPHOS complexes, specifically Complex I, for cancer cell proliferation, and the impact of their inhibition (299, 300). Mutations of genes located in both nDNA and mDNA genes coding for Complex I subunits have been found associated with Complex I deficiencies (301, 302). Complex I activity can be inhibited in cancer cells with different compounds, such as Metformin, an anti-diabetic drug, which has been investigated as a potential treatment for cancer (303, 304). Diabetic patients present increased cancer mortality compared to those without diabetes., While cancer mortality is increased when diabetic patients are treated with insulin or sulfonylureas, it is decreased when they are treated with Metformin, which slows down tumor growth (305). In human cancer cells, Metformin decreases cell proliferation in the presence of glucose and reduces hypoxic activation of HIF-1, but increases cell death upon glucose deprivation, indicating that cancer cells rely exclusively on glycolysis for survival in the presence of Metformin (306). Masoud et al. suggested that high OXPHOS cells are protected against stress induced by chemotherapy due to high mitochondrial respiration (295). Furthermore, a clinical-grade small-molecule inhibitor of Complex I, known as IACS-010759, is currently in phase I clinical trials and has been investigated in tumor growth of different types of tumors. Molina et al. has shown that IACS-010759 inhibits cell proliferation and induces apoptosis in brain cancers and AML, which are known to rely on OXPHOS, by elevating NADH levels and nucleotide monophosphates and decreasing nucleotide triphosphates (307). The inhibition of Complex I by IACS-010759 in Chronic Lymphocytic Leukemia (CLL), showed a minor effect on cell death and lead to upregulation of glucose uptake and glycolysis as a compensatory mechanism. However, the inhibition of both glycolysis and OXPHOS results in increased cell death, showing the importance of targeting multiple metabolic pathways to obtain a synergic effect (308). In addition, a study identified the therapeutic potential of targeting OXPHOS in lung tumors with SWI/SNF mutations, and demonstrated the selective anti-tumor effects of IACS-010759 in these specific tumor types (309).

Understanding the dysregulation of the TCA cycle and OXPHOS in mitochondrial disorders provides valuable insights into the pathogenesis of cancer and other related diseases. Targeting these metabolic pathways holds promise for the development of novel therapeutic strategies to fight mitochondrial disorders and improve patient outcomes.

# 7 Conclusions

The dysregulation of metabolic enzymes is intricately linked to both metabolic disorders and cancer. Metabolic reprogramming in cancer cells, characterized for a long time as the "Warburg effect," plays a crucial role in tumorigenesis, tumor progression, and drug resistance. Understanding the dysregulation of metabolic enzymes in different metabolic pathways provides insights into the mechanisms driving these diseases. Similarities among the mechanisms described for the different groups of disorders (UCDs, GSDs, LSDs, FAODs, and mitochondrial diseases) are related to their involvement in various aspects of cellular metabolism and signaling pathways, as well as their impact on tumor growth, invasion, migration, and metastasis. Disruptions in metabolic pathways, such as pyrimidine synthesis, arginine biosynthesis, glucose metabolism, fatty acid oxidation, and mitochondrial function are some of the mechanisms that can affect energy production, nucleotide synthesis, and other essential cellular processes. In addition, several mechanisms contribute to tumor growth and proliferation, by promoting cell cycle progression, DNA synthesis, and cell division. Dysfunctional enzymes or regulators may lead to increased cell proliferation or impaired growth arrest, allowing tumors to evade normal control mechanisms and immune surveillance, leading to immunosuppression and impaired T-cell function.

Moreover, dysfunctional enzymes or metabolic alterations can impact various signaling pathways involved in tumor growth and progression. Some signaling pathways are regulated in several of the metabolic disorders, which include Wnt/β-catenin, known to regulate key cellular functions such as proliferation, differentiation, migration, genetic stability, cell death, and stem cell renewal (310). The HIF-1α signaling pathway mediates the transcription of genes, allowing cells to adapt to hypoxic environments and lead to changes in glycolysis, nutrient uptake, waste handling, angiogenesis, cell death, and cell migration that may promote tumor survival and metastasis (311). The PI3K/AKT/mTOR pathway plays a vital role in controlling cell survival, metabolism, cell and tumor growth, and protein synthesis in various conditions, including normal physiological processes and pathological states, with a particular emphasis on cancer (312). And p53 signaling acts as a multifunctional transcription factor that activates and represses a growing number of target genes implicated in cell cycle control, apoptosis, programmed necrosis, autophagy, metabolism, stem cell homeostasis, angiogenesis, and senescence (313). Aberrant activation or suppression of these pathways can promote tumorigenesis, angiogenesis, and metastasis. Additionally, EMT is a crucial process in cancer progression, where epithelial cells acquire a mesenchymal phenotype, allowing increased invasion, migration, and metastasis. Furthermore, multiple studies consistently demonstrate the involvement of MMPs (notably MMP1, MMP2, MMP7, and MMP9) in the processes of migration associated with ECM degradation and EMT. The several mechanisms described involve the regulation of EMT-related genes and pathways, contributing to tumor invasiveness and metastatic potential. It's important to note that these mechanisms are not exclusive to the mentioned disorders but are commonly observed in various types of cancers.

Most of the patients with an inherited enzymatic disorder will receive a supportive, multidisciplinary treatment to alleviate their symptoms and their multisystemic conditions (314–318). However, specific treatments are available for some of these enzymatic disorders. Specifically, in UCDs and some GSDs, liver transplantation is the most effective treatment option (314, 319). Furthermore, multiple clinical trials are investigating treatment options for metabolic disorders such as the administration of recombinant protein (NCT no. 03378531), gene replacement (NCT no. 02991144), and mRNA administration (NCT no. 03767270) (314). For LSDs, a common therapy is substrate reduction, used to inhibit the synthesis of the accumulating macromolecule, or administration of chaperones, which help proteins to fold into their correct conformation. In addition, another common and effective treatment for some LSDs is enzyme replacement therapy (ERT), in which the deficient enzyme is administered intravenously to patients. The recombinant enzyme is taken up by the cells and the accumulated macromolecules are catabolized in lysosomes. ERT works specifically well for LSDs through the mannose-6-phosphate (M6P) receptor, which can bind and transport M6P-enzymes to lysosomes, therefore the intravenously M6P-tagged enzymes can be taken up by cells through the receptor and then delivered to lysosomes where they will catalyze the accumulated substrate (317). Moreover, several of the metabolic enzymes mentioned here seem to influence the efficiency of some chemotherapeutic drugs. The upregulation or downregulation of some genes in various tumors was associated with chemoresistance against some drugs, and depletion or inhibition of the enzymes can contribute to a higher sensitivity to chemotherapeutic drugs.

Nevertheless, there remain significant information gaps in the understanding of the genetics that underlie enzymatic dysfunction in metabolic diseases and cancer. While there is now a rich literature and well-established understanding of the metabolomics of metabolic diseases and of cancer, as well as gene alterations, including mutations, amplifications and loss of heterozygosity, as well as transcriptional alterations, there is only a poor understanding regarding translational regulatory alterations. Transcriptional changes often are not reflected in the proteome due to post-transcriptional regulatory events, including the selective translational regulation of many mRNAs, and targeted protein degradation. Ultimately, there will need to be a concerted effort to begin integrating these many layers of gene control to build a more complete understanding of enzymatic dysfunction in metabolic diseases and cancer. The identification of metabolic enzymes as potential therapeutic targets and biomarkers holds promise for improving cancer therapy and developing new treatment options. Continued research into the interplay between cancer and metabolic enzyme dysregulation will contribute to our understanding of cancer biology and potentially lead to the development of novel therapeutic strategies.

## Author contributions

Conceptualization: TR-F. Design: TR-F and MM. Resources: TR-F, MM, and AK. Writing—original draft preparation: TR-F, MM, and AK. Writing—reviewing and editing: TR-F, MM, and RS. Figures: TR and MM. Supervision: RS. Funding acquisition: TR-F, AK, and RS. All authors have read and approved the submitted version of the manuscript. All authors contributed to the article.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Alterations in the amino acid profile in patients with papillary thyroid carcinoma with and without Hashimoto's thyroiditis

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**Purpose:** Amino acids (AAs) play important physiological roles in living cells. Some amino acid changes in blood are specific for autoimmune disorders, and some are specific for thyroid cancer. The aims of this study were to profile AA metabolites in the serum of patients with papillary thyroid carcinoma (PTC0) without Hashimoto's thyroiditis (HT) and patients with PTC with HT (PTC1) and predict whether AA metabolites are associated with thyroid disease, thyroid hormone and thyroid autoantibodies.

**Methods:** A total of 95 serum samples were collected, including 28 healthy controls (HCs), 28 PTC0 patients and 39 PTC1 patients. Serum samples were analyzed by high-performance liquid chromatography-triple stage quadrupole-mass spectrometry (HPLC-TSQ-MS), and twenty-one amino acids (AAs) were detected.

**Results:** The serum concentration of glutamic acid was significantly elevated in PTC1 patients compared with PTC0 patients. Lysine was the second amino acid that differentiated these two groups of PTC patients. In addition, the serum concentrations of glycine, alanine and tyrosine were significantly reduced in both PTC patient groups compared to the HC group. These AAs were also correlated with thyroid hormones and antibodies. Five amino acid markers, namely, glycine, tyrosine, glutamic acid, glutamine and arginine, separated/distinguished PTC0 patients from healthy subjects, and eight AA markers, the same AAs as above without arginine but with alanine, leucine, valine and histidine, separated/distinguished PTC1 patients from healthy subjects based on ROC analysis.

**Conclusion:** Compared with the HCs, changes in AAs in PTC0 and PTC1 patients showed similar patterns, suggesting the possibility of a common pathophysiological basis, which confirms preliminary research that PTC is significantly associated with pathologically confirmed HT. We found two AAs, lysine and alanine, that can perform diagnostic functions in distinguishing PTC1 from PTC0.

KEYWORDS

**amino acids, Hashimoto's thyroiditis, papillary thyroid cancer, serum, LC-MS**

# 1 Introduction

Thyroid cancer (TC) is responsible for over 1% of neoplasms diagnosed every year in the general population. In Europe, there are approximately 3,500 new cases each year (1). Females are involved 3–5 times more often than men. The incidence of TC has increased rapidly in the last several years. In terms of short-term prognosis, it may become the second most common malignant neoplasm in women. Papillary thyroid cancer (PTC) accounts for more than 90% of all thyroid neoplasms (2) with a global incidence of 586,000 cases (3). Although PTC is related to an indolent disease course and has a favorable prognosis, it is a major challenge to stratify patients by risk of mortality or recurrence. Currently, clinicopathologic features associated with an unfavorable prognosis include older age, large tumor size, extrathyroidal extension (ETE), lymph node metastasis (LNM) and distant metastasis. Individuals with those features require more aggressive treatment (4, 5). On the other hand, low-intensity treatment or even active surveillance may be sufficient for patients who do not have these risk factors. Although some recent studies have indicated that Hashimoto's thyroiditis (HT) (6) may be a tumor-promoting factor, HT-related issues have barely been mentioned in current TC treatment guidelines. The link between chronic inflammation and cancer is well described (7); however, it is generally associated with indolent potential in the GI tract, liver, and skin (8). There are several theories to explain the potential relationship; for example, misbehaved follicular epithelial regeneration following chronic inflammatory damage (9) or enhanced TSH stimulus together with additional inflammatory cytokines act as potential activators of aberrant cell proliferation (10). However, the exact molecular pathomechanism remains unclear. Autoimmune thyroid diseases, including Hashimoto's thyroiditis (HT), are T-cell-mediated organ-specific autoimmune diseases, and the annual incidence of Hashimoto thyroiditis worldwide is estimated to be 0.3-1.5 cases per 1000 persons (11). HT, affects women 7-10 times more often than men (12). Prevalence increases with age, especially in patients diagnosed with other autoimmune conditions. There are various signs and symptoms of HT mainly due to hypothyroidism, including cool and dry skin, coarse hair, loss of body hair, and hyperlipidaemia (13). Chronic HT-induced inflammation may be associated with an increased risk of thyroid cancer. A recent meta-analysis reported that the rate of HT in PTC patients ranged between 4.75 and 38.4%, whereas the rate of PTC in HT patients ranged between 0.12 and 64.3% (14). The immune responses against PTC and HT are different. In PTC, the immune system is more silent and allows tumor progression, while in HT, the reaction is aggressive, destroying the proper functioning of the gland. According to some authors, HT is associated with a better prognosis due to an enhanced immune response and better control of tumor progression (15). However, the role of HT in PTC seems ambiguous and should be elucidated. It has been proven that HT plays a role in protein metabolism (16). As the basic building blocks of peptides and proteins, amino acids have a variety of physiological functions. Serum levels of polyamine metabolites were found to differ between patients with autoimmune thyroid disease and healthy controls (17). Hypothyroid status is related to lower alanine, aspartate, and glutamate concentrations. It is mainly caused by decreased whole-body proteolysis and maldigestion (18). Amino acids are the primary units of proteins and are involved in multiple physiological and pathophysiological processes (19). According to many publications, amino acids (AAs) may play a critical role in cancer cell metabolism. In contrast to hypothyroidism status, it is known that in cancer, especially in the early stages, the amino acid turnover rate is increased because of hypermetabolism. Glutamine, as a nitrogen and carbon source, is involved in the metabolic reprogramming in cancer and plays a pivotal role in the growth and proliferation of cancer cells (18, 20–22). Most thyroid cancer studies have presented increased concentrations of glutamine and glutamate in tumor samples (23–25), as well as in serum (25–27). High glutamine uptake is related with upregulated glutaminase in several tumor models (28–30). Glutaminase initiates glutaminolysis by converting glutamine to glutamate. This pathway is involved in the maintenance of the TCA cycle and synthesis of non-essential amino acids, nucleotides and fatty acids (11) as well as in cell signaling (29, 30). Also, alteration of arginine metabolism is characteristic for cancer metabolism. It is necessary for growth of cancer cells, but paradoxically, arginine is important for immune surveillance (31). Alanine also is desired amino acid in most of the perturbed pathways (25). Glycine and serine provide crucial substrates for the synthesis of nucleic acids, proteins and lipids, which are essential for cancer cell growth (32). High levels of glycine have been observed in cancer thyroid tissues (24, 33) and in malignant nodules (34) compared to samples from healthy subjects. Also, other amino acid are necessary for maintenance of cellular redox homoeostasis (35). Some derivatives produced from AAs may support cancer growth, but tryptophan induces immunosuppression by weakening the ability of dendritic cells and T cells to target and eliminate cancer cells (36). Our aim was the examination of AA profile disorders in PTC1 and PTC0 and comparison of changes in AA concentrations in these 2 pathologies.

# 2 Materials and methods

## 2.1 Patients

The present study was approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk under number NKBBN/62/2021. The study was performed in agreement with the Declaration of Helsinki of the World Medical Association. Female patients who underwent thyroidectomy or lobectomy for PTC at the Thyroid Cancer Center of the Medical University of Gdansk from January 1, 2021, to March 31, 2022, were included in the study. The study groups consisted of 28 PTC0 (mean age 42.3 ± 13.7 years) and 39 PTC1 patients (mean age 42.0 ± 14.1 years). The controls were healthy participants (43.6 ± 8.87 years). An extensive medical history was taken from the control group regarding various ailments (hypertension, chronic kidney disease, heart failure, ischemic heart diseases, cerebrovascular,

dyslipidemia, diabetes mellitus, type 2 diabetes mellitus, thyroid diseases) and taken drugs. The control group consisted of women without the above diseases. Written informed consent was obtained from all participants. Data such as age, sex, preoperative serum autoantibody levels, tumor characteristics, and treatment modalities were obtained from the medical records. Standard pathologic diagnoses were based on World Health Organization criteria (37). Routine laboratory parameters were determined at the Central Clinical Laboratory at the Medical University of Gdansk, the results of which are collected in Table 1. Only patients with confirmed PTC by histopathology were included in the study. Coexistent HT was determined by elevated anti-thyroglobulin antibodies (TgAbs) and thyroid peroxidase antibody (TPOAb) and postoperative sectioning and examination of paraffin-embedded thyroid tissue specimens; a positive result was defined as the presence of diffuse lymphocytic and plasma cell infiltrate, oxyphilic cells, formation of lymphoid follicles, and reactive germinal centers. Only women participated in this study, and we ruled out other autoimmune thyroid diseases, such as Graves' disease, through the determination of the levels of thyrotropin receptor autoantibodies (TSHR-Abs). Blood samples were collected in the morning from all study subjects, and before thyroidectomy from PTC patients. After the blood was centrifuged, the serum samples obtained were stored in aliquots at -80 °C until assayed.

## 2.2 Amino acid analysis

Concentrations of amino acids were determined by liquid chromatography/mass spectrometry (LC/MS) according to the procedure described previously (38). Briefly, internal standards (a mixture of amino acids labelled with stable isotopomers C-13 and N-15, Sigma–Aldrich) were added to 0.025 ml of serum. The sample was then deproteinized by the addition of 0.1 ml acetonitrile, incubated for 15 minutes on ice and centrifuged at 12,000 x g for 15 minutes at 4° C. The collected supernatant was freeze-dried and then dissolved in 25 µl of water. Samples were analyzed by ion-pair reversed-phase high-performance liquid chromatography coupled with mass spectrometric detection. Chromatographic separation was performed using a 2.5 µm Synergy Hydro-RP 50 x 2.0 mm column. The mobile phase was delivered at a rate of 0.2 mL/min in a gradient from 0% to 60% acetonitrile over 12 minutes. A mass detector (TSQ Vantage, Thermo, USA) with a heated electrospray ion source (HESI-2) was operated in MS2 positive mode for amino acid detection. The electrospray cone voltage was set at 4.5 kV, and the heated capillary temperature was 275°

TABLE 1 Selected biochemical and anthropometric characteristics in the study groups.

| | HC | PTC0 | PTC1 | HC vs PTC0 | HC vs PTC1 | PTC0 vs PTC1 |
|---|---|---|---|---|---|---|
| Age (year) | 43.6 ± 8.87 | 42.3 ± 13.7 | 42.0 ± 14.1 | NS | NS | NS |
| BMI (kg/m2) | 25.4 ± 7.31 | 26.5 ± 4.47 | 25.1 ± 4.45 | NS | NS | NS |
| TG (mg/dL) | 98.5 ± 44.6 | 87.3 ± 32.6 | 81.6 ± 32.3 | NS | NS | NS |
| HDL (mg/dL) | 68.8 ± 18.0 | 62.0 ± 12.8 | 59.8 ± 12.5 | NS | NS | NS |
| LDL (mg/dL) | 101 ± 27.4 | 112 ± 35.4 | 109 ± 33.6 | NS | NS | NS |
| TC (mg/dL) | 160 ± 50.9 | 199 ± 42.2 | 188 ± 42.0 | *0.014 | NS | NS |
| CRP (mg/L) | 0.77 ± 0.54 | 0.84 ± 0.52 | 2.77 ± 1.99 | NS | <0.001 | <0.001 |
| Glucose (mg/dL) | ND | 93.9 ± 27.0 | 95.3 ± 20.3 | ND | NS | NS |
| HBA1C (%) | ND | 5.37 ± 0.46 | 5.29 ± 0.34 | ND | NS | NS |
| Insulin (uU/mL) | ND | 9.14 ± 5.86 | 8.24 ± 6.05 | ND | NS | NS |
| Albumin (g/L) | ND | 42.0 ± 2.48 | 40.7 ± 3.46 | ND | NS | NS |
| Creatinine (mg/dL) | ND | 0.68 ± 0.11 | 0.69 ± 0.13 | ND | NS | NS |
| 1,25-$(OH)_2$D (pg/mL) | ND | 54.8 ± 15.0 | 51.3 ± 17.1 | ND | NS | NS |
| TSH (uU/mL) | ND | 1.14 ± 0.67 | 1.19 ± 0.80 | ND | NS | NS |
| fT3 (pmol/L) | ND | 4.30 ± 0.48 | 4.30 ± 1.06 | ND | NS | NS |
| fT4 (pmol/L) | ND | 12.3 ± 1.83 | 12.8 ± 3.03 | ND | NS | NS |
| [a]Anty-TSHr (IU/l) | ND | <0.20 | <0.20 | ND | NS | NS |
| [b]Anty-TPO (IU/mL) | ND | <3.00 | 449 ± 532 | ND | ND | **<0.001 |
| [c]Anty-TG (IU/mL) | ND | <3.00 | 133 ± 471 | ND | ND | **<0.001 |

p from one-way analysis of variance followed by the all-pairwise comparisons Holm–Sidak method, * p from nonparametric Kruskal–Wallis one-way analysis of variance followed by the all-pairwise comparisons Dunn's method for ranks. ** <0.001 - comparison between the two PTC study groups was evaluated by the Mann–Whitney rank sum test for nonparametric data. [a] <0.2 IU/l – reference value for TSHr-Ab, [b]<34 IU/ml - reference value for TPO-Ab, [c] ≤115 IU/ml - reference value for TG-Ab. ND – not determined, NS – not significant.
Healthy control (HC), patients with PTC without Hashimoto thyroiditis (PTC0) and patients with Hashimoto thyroiditis (PTC1). Values are mean ± SD.

C. The sheath gas flow was set at 35 arbitrary units. Individual amino acids were identified and confirmed by the similarity of molecular masses, chromatographic retention time and fragmentation pattern.

## 2.3 Data analysis

The data analysis was performed in SigmaPlot 14.5 (Systat Software Inc., San Jose, CA, USA). All values are presented as the mean ± standard deviation (SD). The P value was considered significant at $< 0.05$. Comparisons among the three study groups were carried out with the one-way analysis of variance (ANOVA) followed by the all-pairwise comparison Holm–Sidak method. Nonparametric data were subjected to the Kruskal–Wallis one-way analysis of variance followed by the all-pairwise comparison Dunn's method for ranks. Comparison between the two PTC study groups was evaluated by the Mann–Whitney rank sum test for nonparametric data. Correlations between pairs of variables were determined by linear regression analysis.

ROC analysis was carried out in MetaboAnalyst 5.0v (39) to evaluate the area under the curve (AUC) to compare the predictive ability of significant metabolites between the tested groups. The linear SVM algorithm was used to build the ROC curve. To understand if it is possible to increase the predictive power, the single ROC curve was built for both comparisons, HC with PTC0 and HC with PTC1, using only the metabolites with a p-value <0.01. ROC curve analyses for combinatorial AAs, the 10-fold Coss Validation was used to generate a logistic regression model and calculate the performance. MetaboAnalyst 5.0v uses the MetaboAnalyst R package with metabolomic data analysis, visualization, and functional interpretation. The raw data were subjected to normalization to the total area and autoscaled.

MetPA software (39) was used to carry out an analysis of serum metabolic pathways for the identified metabolites. Metabolome analysis identified all matched pathways based on p values determined during pathway enrichment analysis and pathway impact values determined by pathway topology analysis. The raw data were subjected to normalization to the total area and autoscaled. The pathway-associated metabolite set was the chosen metabolite library, and all compounds in this library were used. Pathways with a p value <0.05 were significantly altered in serum samples.

# 3 Results

Common biochemical parameters obtained from whole blood are presented in Table 1. PTC1 patents had elevated C-reactive protein compared with HC and PTC0 patients (although the values were within the reference range). PTC0 patients had significantly elevated concentrations of total cholesterol compared with HCs. Among the other parameters, significant differences were not observed. PTC0 patients differed statistically from PTC1 patients in thyroid peroxidase and thyroglobulin antibody levels.

## 3.1 Differences in serum AA concentrations in PTC patients with and without Hashimoto's disease and healthy controls

One-way analysis of variance was used to compare individual amino acids between study groups, and the significantly different AAs among these three groups were defined (Table S1). Concentration of some of these AAs decreased in the serum of both PTC0 and PTC1 patients due to the increased metabolic rate, which is typical of cancer. Glycine, alanine and tyrosine were reduced in both PTC groups compared with the HC group (Figures 1A-C). However, the values for glycine and valine (Figure 1D) were comparable for PTC0 and PTC1 patients, while the concentration of alanine showed a declining trend in the PTC1 group compared to that in the PTC0 group. In the PTC1 group, glutamate and lysine were significantly elevated in patients' serum compared to the PTC0 group, and there were only two AAs that separated/distinguished these two groups of patients with PTC (Figures 1E, F and Table S1). All PTC patients had elevated levels of glutamic acid, aspartic acid, glutamine and valine compared to the healthy controls, and glutamic acid was noted to be almost two times higher in PTC1 patients than in PTC0 patients (Figures 1D, E, G, H). Significantly elevated concentrations of arginine, leucine and histidine were observed only in the PTC1 group compared with the healthy control group (Figures 1I-K). The increase in histidine was slight. In the PTC0 group, only leucine and arginine showed an upwards trend (Table S1).

## 3.2 Diagnostic potential of serum AA concentrations in PTC patients

ROC curve analysis of each box plot was used to evaluate the diagnostic ability of the discriminating metabolites as screening biomarkers in patients with PTC0 and PTC1. The ROC curve summarizes the specificity and sensitivity (the x-axis and y-axis, respectively) of a single feature to accurately classify data, which can then be used to compare the overall accuracy of different biomarkers.

The results showed that the AUCs of five metabolites in the PTC0 vs. healthy group (Figure 2A) were larger than 0.780, and the AUCs of eight metabolites in the PTC1 vs. healthy group were larger than 0.742 (Figure 3A). Specific changes for PTC0 were found in arginine with an AUC of 0.789, and specific changes for PTC1 were found in alanine with an AUC of 0.853, leucine with an AUC 0.825, valine with an AUC 0.759 and histidine with an AUC of 0.0.742. The remaining AAs had different AUC values between PTC0 and PTC1. The highest AUC values noted was glycine in PTC0 and PTC1 (0.834 and 0.849, respectively) (Figures 2, 3). As shown in Figures 2B, 3B, the ROC curve for the predictive power of combined index to distinguish PTC0 from HC and PTC1 from HC was plotted. The AUC was 0.831 and 0.828, respectively (Figures 2B, 3B).

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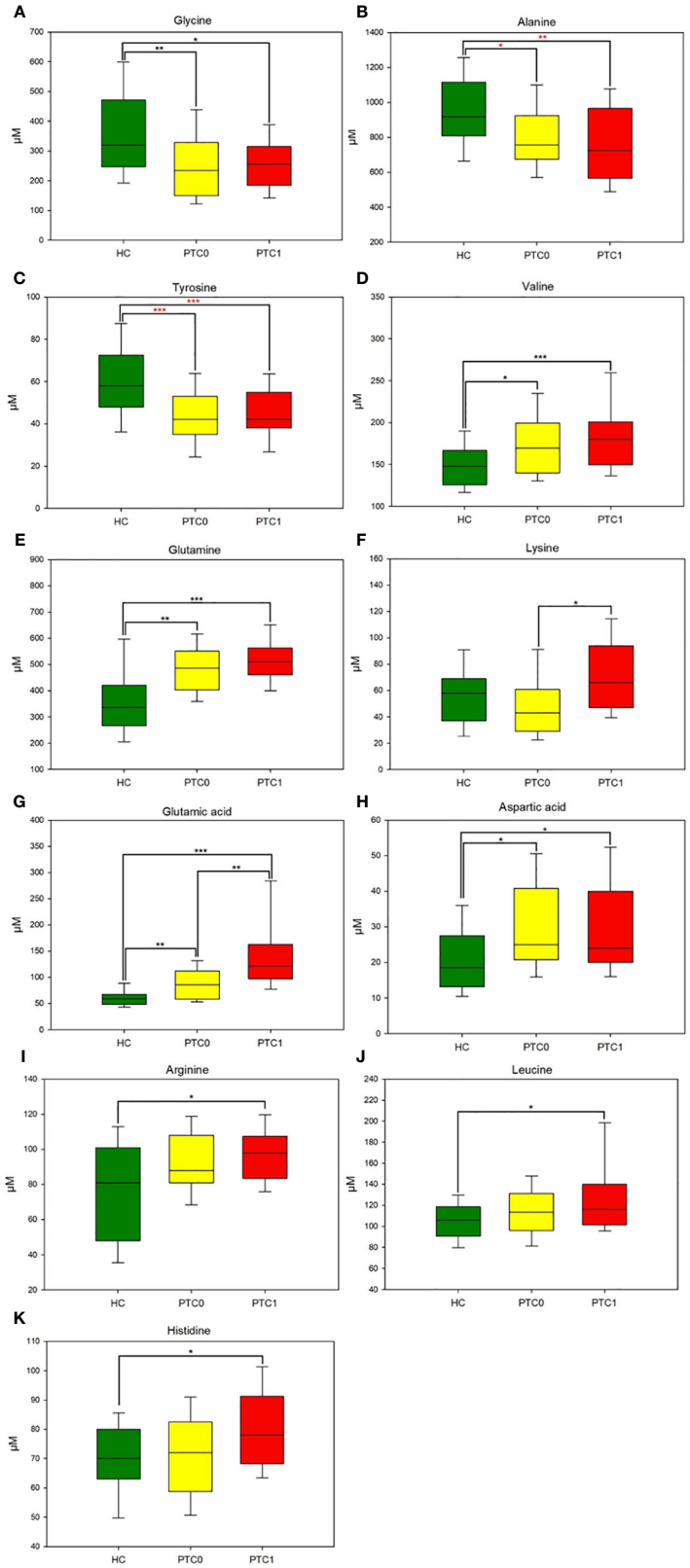


FIGURE 1
Boxplots of the 11 most significant metabolites ($p < 0.05$) in the analysis of variance results comparing the three groups (PTC0, yellow boxes; PTC1, red boxes; and healthy controls, green boxes). **(A)** glycine, **(B)** alanine, **(C)** tyrosine, **(D)** valine, **(E)** glutamine, **(F)** lysine, **(G)** glutamic acid, **(H)** aspartic acid, **(I)** arginine, **(J)** leucine, **(K)** histidine. The x-axis shows the specific metabolite, and the y-axis is the normalized peak intensity. HC, healthy control; PTC0, papillary thyroid carcinoma without Hashimoto; PTC1, papillary thyroid carcinoma with Hashimoto. Values are means ± SDs. (***$p<0.001$, **$p<0.01$, *$p<0.05$ one-way analysis of variance followed by the all-pairwise comparisons Holm–Sidak method; ***$p<0.001$, **$p<0.01$, *$p<0.05$ from nonparametric Kruskal–Wallis one-way analysis of variance followed by the all-pairwise comparisons Dunn's method for ranks).

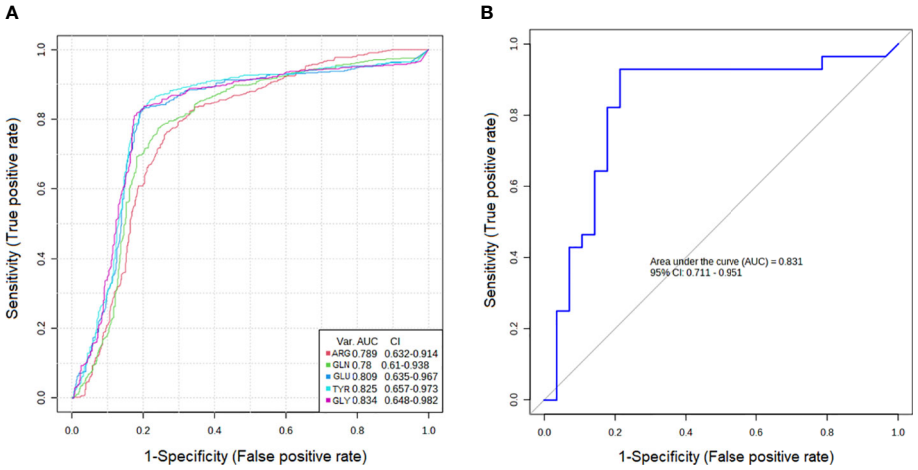


FIGURE 2
Receiver operating characteristic curve (ROC curve) analyses of the ability of 5 AAs **(A)** and a combinatorial AAs **(B)** to predict PTC0 vs. HC.

## 3.3 Analysis of correlations between serum AA and concentrations and other selected blood parameters

The next step was the analysis of correlations between patient serum parameters of thyroid function and serum AAs in the research PTC groups (Table 2). Alanine was negatively correlated with free thyroxine (fT4). Arginine was positively correlated with fT4 (0.407, $p<0.05$) and leucine with TSH (-0.428, $p<0.05$). Only proline correlated with free triiodothyronine (fT3) (-0.426, $p<0.02$). There was only one strong negative correlation of histidine with C-reactive protein (-0.626, $p<0.001$).

Significantly more relationships and stronger correlations were observed in the PTC1 group, similar to the ANOVA and ROC analysis (Table 3). Among them were AA correlations with thyroid hormones. The PTC1 entity affects a greater number of correlations. Tyrosine, which was reduced in both the PTC0 and PTC1 groups, was positively correlated with fT3 (0.469, $p<0.01$) and fT4 (0.460, $p<0.01$). Lysine positively correlated with thyroglobulin antibodies (TG-Abs) (0.434, $p<0.01$) and was one of two AAs that were different between PTC0 and PTC1 (Table S1). In turn, alanine, which was also reduced in PTC1, was strongly negatively correlated with thyroid peroxidase antibodies (TPO-Abs) (-0.567, $p<0.001$). Another strong correlation was the positive correlation of glutamic acid with thyroid-stimulating hormone (TSH) (0.530, $p<0.001$).

## 3.4 Metabolic pathway analysis of the serum AA profiles in PTC0 and PTC1 patients

Metabolic pathway analysis was performed to interpret the biological relevance of the differences in serum AA profiles in PTC0 and PTC1. The KEGG and HMDB databases were used to

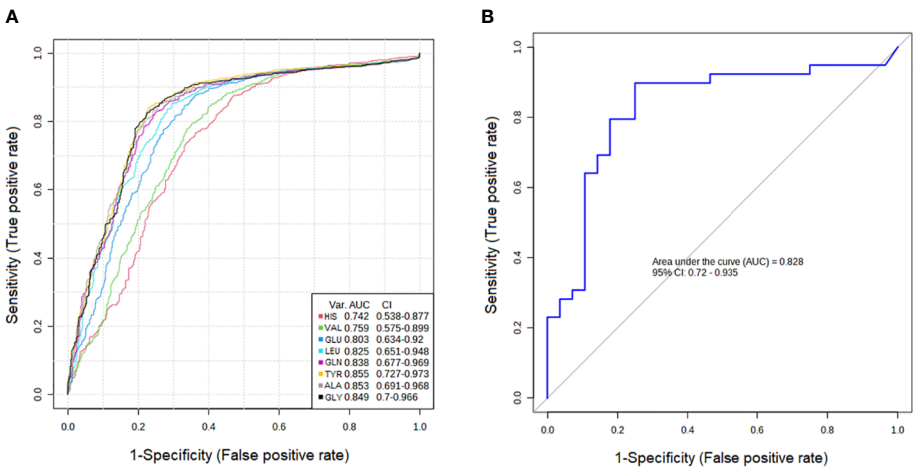


FIGURE 3
Receiver operating characteristic curve (ROC curve) analyses of the ability of 8 AAs **(A)** and a combinatorial AAs **(B)** to predict PTC1 vs. HC.

TABLE 2 Correlation coefficients between the selected blood parameters and amino acid concentrations (μM) in serum samples from patients with PTC without Hashimoto thyroiditis (PTC0) (Pearson correlation coefficient).

| | Asp | Asn | Gly | Gln | Glu | Ser | Bet | Thr | Ala | Pro | Cre | Val | Met | Tyr | His | Ile | Lys | Leu | Arg | Phe | Trp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRP | 0.220 | **-0.385** | -0.091 | -0.264 | -0.130 | -0.303 | -0.247 | -0.322 | -0.109 | -0.165 | -0.037 | -0.159 | 0.196 | 0.112 | *-0.626* | 0.006 | -0.172 | -0.004 | -0.180 | 0.177 | **-0.411** |
| TSH | -0.271 | 0.074 | -0.108 | -0.148 | -0.126 | 0.125 | -0.256 | -0.036 | -0.022 | -0.334 | **0.386** | -0.203 | -0.264 | -0.332 | 0.082 | -0.095 | -0.006 | **-0.428** | -0.297 | -0.012 | 0.020 |
| fT3 | -0.300 | 0.151 | 0.050 | -0.171 | 0.055 | -0.117 | -0.139 | -0.017 | -0.221 | **-0.426** | 0.020 | -0.270 | -0.359 | -0.104 | 0.294 | -0.154 | 0.132 | 0.081 | 0.209 | 0.138 | 0.062 |
| fT4 | -0.208 | -0.132 | 0.189 | -0.236 | 0.212 | -0.103 | -0.117 | -0.340 | **-0.397** | -0.245 | -0.111 | 0.000 | 0.100 | 0.114 | -0.290 | 0.024 | 0.235 | 0.236 | **0.407** | -0.154 | -0.087 |
| anty-TPO | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| anty-TG | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Asp – aspartic acid, Asn – asparagine, Gly – glycine, Gln – glutamine, Glu – glutamic acid, Ser – serine, Bet – betaine, Thr – threonine, Ala – alanine, Pro – proline, Cre – creatinine, Val – valine, Met – methionine, Tyr - tyrosine, His - histidine, Ile – isoleucine, Lys – lysine, Leu – leucine, Arg – arginine, Phe – phenylalanine, Trp – tryptophane.
**$p<0.05$**; *$p<0.001$*.

TABLE 3 Correlation coefficients between the selected blood parameters and amino acid concentrations (μM) in serum samples from patients with PTC with Hashimoto thyroiditis (PTC1) (Pearson correlation coefficient).

| | Asp | Asn | Gly | Gln | Glu | Ser | Bet | Thr | Ala | Pro | Cre | Val | Met | Tyr | His | Ile | Lys | Leu | Arg | Phe | Trp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRP | -0.309 | -0.212 | -0.124 | -0.294 | -0.285 | 0.149 | -0.262 | -0.006 | -0.022 | -0.067 | -0.085 | 0.137 | -0.091 | -0.137 | 0.141 | 0.174 | -0.165 | 0.266 | 0.210 | 0.041 | 0.261 |
| TSH | **0.356** | -0.095 | 0.105 | 0.109 | *0.530* | -0.051 | -0.010 | -0.228 | -0.097 | **0.362** | 0.088 | 0.046 | 0.070 | -0.005 | 0.007 | 0.126 | 0.243 | 0.198 | -0.026 | -0.002 | -0.063 |
| fT3 | -0.217 | **0.377** | -0.117 | 0.030 | -0.301 | 0.199 | 0.027 | 0.288 | 0.290 | 0.202 | 0.223 | 0.104 | -0.077 | **0.469** | 0.077 | -0.021 | -0.237 | -0.013 | -0.310 | 0.112 | 0.085 |
| fT4 | -0.308 | 0.247 | **-0.352** | -0.137 | -0.171 | 0.177 | -0.100 | 0.180 | 0.154 | 0.258 | 0.184 | -0.087 | 0.013 | **0.460** | 0.105 | -0.009 | -0.213 | 0.033 | -0.277 | 0.003 | 0.159 |
| anty-TPO | 0.103 | -0.182 | -0.278 | -0.153 | 0.268 | 0.083 | -0.244 | 0.013 | *-0.567* | -0.169 | -0.110 | -0.184 | -0.219 | -0.150 | -0.038 | -0.092 | 0.040 | -0.112 | -0.154 | -0.250 | -0.003 |
| anty-TG | 0.215 | -0.088 | -0.016 | -0.026 | 0.001 | -0.195 | -0.173 | 0.078 | -0.272 | -0.144 | -0.318 | -0.238 | -0.198 | -0.062 | 0.096 | -0.086 | **0.434** | -0.064 | -0.117 | 0.023 | 0.018 |

Asp – aspartic acid, Asn – asparagine, Gly – glycine, Gln – glutamine, Glu – glutamic acid, Ser – serine, Bet – betaine, Thr – threonine, Ala – alanine, Pro – proline, Cre – creatinine, Val – valine, Met – methionine, Tyr - tyrosine, His - histidine, Ile – isoleucine, Lys – lysine, Leu – leucine, Arg – arginine, Phe – phenylalanine, Trp – tryptophane.
**$p<0.05$**; *$p<0.001$*.



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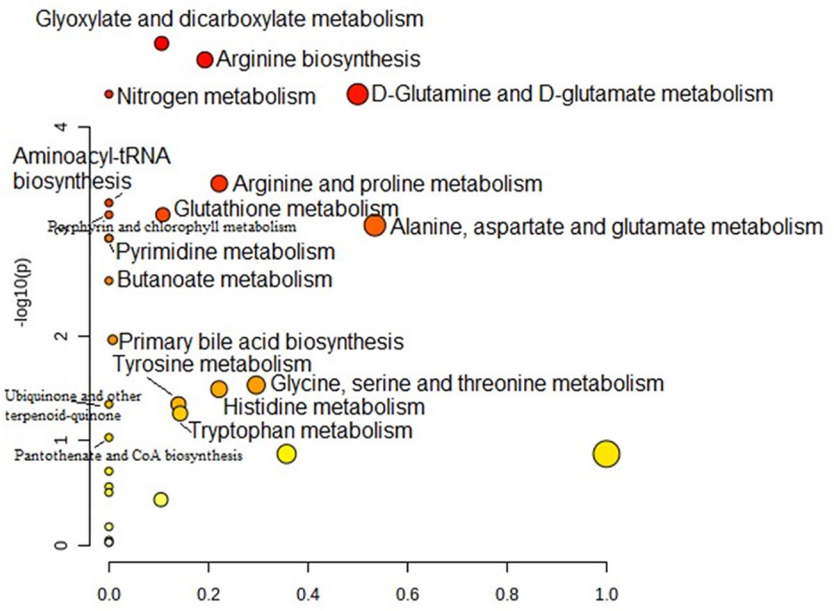


FIGURE 4
Pathway analysis of serum amino acid profiles of the papillary thyroid carcinoma without Hashimoto group compared to the control group. Pathway impact values are plotted against the X-axis, and -log (P) values are plotted against the Y-axis. For visual clarification, the pathway importance and the statistical significance are proportional to the node radius and colour, respectively. FDR p is the p value adjusted using the false discovery rate. Impact is the pathway impact value calculated from pathway topology analysis.

analyze twenty-one detected amino acids, and the results were submitted to MetaboAnalyst 5.0 to display the statistical analysis results of informatics analysis. This analysis generates a pathway impact score and the associated p value. A value >0.1 was chosen as the cut-off for less important pathways (Figures 4, 5). All the identified pathways are shown in Supplementary Tables S2 and S3.

Pathway analysis showed that "glyoxylate and dicarboxylate metabolism" was the most significant pathway characteristic of

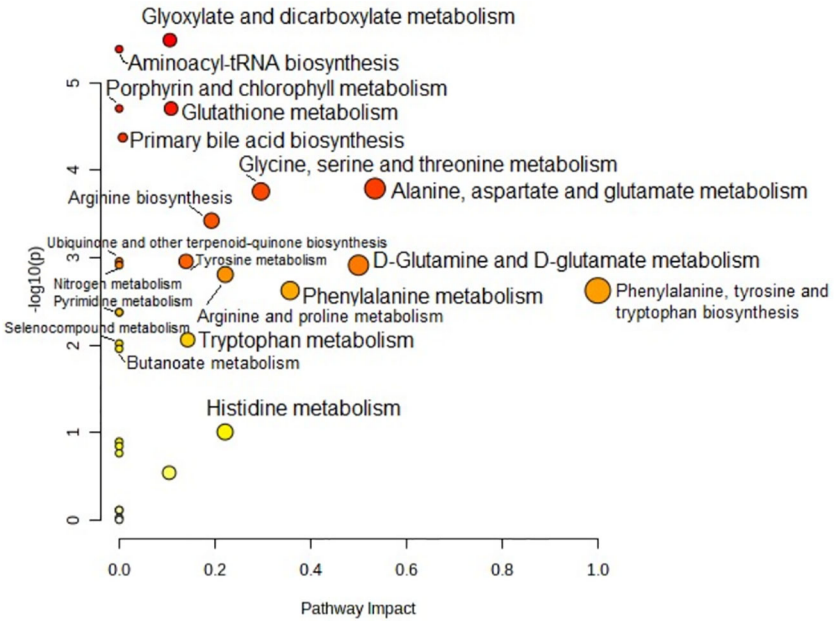


FIGURE 5
Pathway analysis of serum amino acid profiles of the papillary thyroid carcinoma with Hashimoto group and the control group. Pathway impact values are plotted against the X-axis, and -log (P) values are plotted against the Y-axis. For visual clarification, the pathway importance and the statistical significance are proportional to the node radius and colour, respectively. FDR p is the p value adjusted using the false discovery rate. Impact is the pathway impact value calculated from pathway topology analysis.

PTC0 (Figure 4), which was selected on the basis of disturbed concentrations of glycine, glutamine and glutamic acid in the serum of PTC0 patients compared with healthy controls. However, the pathway "D-Glutamine and D-glutamate metabolism" had the highest FDR value and pathway impact value calculated from pathway topology analysis (Table S3). The next most significantly changed pathways were "arginine biosynthesis" and "nitrogen metabolism" (Table S3). The most commonly changed AAs in these pathways were glutamine and glutamic acid.

A very similar set of metabolic pathways was observed in the pathway analysis based on changes in the AA profile in patients with PTC1 (Figure 5); however, there were higher FDR values and different pathway impact scores (Table S3). The two PTC groups were differentiated by the pathway "glutathione metabolism".

# 4 Discussion

The standard diagnostic tools for PTC are ultrasound and fine needle aspiration biopsies (6). In turn, HT identification is based on clinical symptoms of hypothyroidism, the presence of TPOAbs, and ultrasound features, although seronegative HT can be observed in more than 10% of cases. In such cases, diagnosis is made based on final histopathology. Additional diagnostic tools that may help to identify PTC and distinguish Hashimoto concomitant with PTC may have crucial clinical implications. Indeed, recent guidelines allow less aggressive treatment for PTC in some circumstances, which might significantly reduce postoperative complications (40). However, there is no gold standard that would allow us to distinguish between Hashimoto's and cancer, and this distinction is of great importance in further management/treatment. In the development of cancer, AA metabolism is reprogrammed. Additionally, HT affects patient catabolism, and preliminary research suggests that increased serum TSH concentration and autoimmune thyroid inflammation are involved in thyroid tumor growth (18). Therefore, is it possible to find the difference between these two diseases based on the amino acid profiles?

To the best of our knowledge, this is the first study to determine AA profiles in serum samples from PTC0 patients and PTC1 patients compared to those of healthy controls.

Lysine was one of the AAs that was elevated in PTC1 compared to PTC0. Lysine affects the production of proteins in muscles and bones, and lysine deficiency causes chronic fatigue, irritability, hair loss, anemia, susceptibility to infection, recurrent herpes and metabolic disorders. Jiang et al. (41) studied the serum of HT patients and showed that lysine degradation pathways had an impact on different clinical stages of HT (41). Additionally, lysine was increased in the serum of HT and Graves' disease patients (17). In our study, lysine was increased only in the PTC1 group. According to the referenced authors, alterations in lysine degradation affect the occurrence of HT (42).

Other AAs increased in the serum of PTC1 patients compared to HCs were leucine and arginine (Table S1). Interestingly, in PTC0 patients, we also observed a strong increase in arginine concentration (Table S1). Serum concentrations of arginine were altered in both PTC0 and PTC1 patients, which was also indicated by the MetPA analysis pathway "arginine biosynthesis" (Figures 4, 5). A positive correlation of arginine with fT4 was observed in PTC0 serum (Table 2). Interestingly, Ittermann et al. (43) found that in patients with hyperthyroidism, serum concentrations of arginine and its metabolites, including asymmetric and symmetric dimethylarginines and homoarginine, were associated with serum TSH, fT3, and fT4 concentrations. Treatment of hyperthyroidism by antithyroid drugs increased arginine levels (44). In turn, Gluvic et al. (44) described many cases of increased NO bioavailability by levothyroxine therapy. Thyroid hormones stimulate L-arginine uptake by endothelial cells by upregulating L-Arg transporters (45), and arginine is a major regulator of mitochondrial activities in cancer metabolism (31). Supplementation with arginine rewires T-cell metabolism from glycolysis to oxidative phosphorylation and promotes its survival and antitumor ability (31). Lu et al. (46), in $H^1$ NMR analysis of plasma from papillary thyroid microcarcinoma patients, reported reduced levels of valine, lysine and leucine compared with healthy groups. In a study by Jiang et al. (41), valine, leucine, and isoleucine degradation and valine, leucine, and isoleucine biosynthesis differentiated euthyroid HT patients from HT patients with subclinical hypothyroidism. However, it should be stressed that Hashimoto disease may influence the metabolism of many other tissues, and this may affect serum AA concentrations. Indeed, we observed elevated concentrations of leucine in serum from HT patients. According to Krishnamurthy et al. (47), arginine, valine, and leucine are important in immunological responses, including the synthesis of various antibodies and the activation of T cells and macrophages. It appears that the deficiency of any essential AAs, including valine, impairs T4 production and leads to primary hypothyroidism (48). Thyroid hormones have a catabolic effect on protein metabolism. In most catabolic states, uptake of branched-chain amino acids from body proteins is reduced; therefore, the increase in their concentrations does not depend on the increase in their content in the diet but results from both their reduced peripheral metabolism and increased release from fat-free tissues (49). Additionally, in our study, the concentration of valine was elevated in both PTC groups; however, much higher differences in valine levels were observed in PTC1 patients. Plasma branched-chain amino acids are decreased in short-term profound hypothyroidism and increase in response to thyroid hormone supplementation (16). Therefore, thyroid hormone supplementation can be a reason for the higher serum concentrations of BCAAs in PTC1 patients (Table S1). The next amino acid, glycine is a highly desirable compound for cancer cells (24, 33)., therefore, reduced levels of glycine in the serum of PTC patients (Table S1) could be the reason for glycine participation in cancer pathogenesis (21). We observed an inverse correlation of glycine with fT4 in PTC0 serum (Table 2). Glycine supplementation improves the conversion of fT4 to fT3, which contributes to the proper functioning of the thyroid gland. Mannisto et al. reported that intraperitoneal administration of glycine inhibited TSH secretion in rats (50).

In turn, glutamate and aspartate, which are excitatory amino acids, act by increasing the concentrations of TSH, fT3 and fT4 in rat serum (51). Indeed, in our study, glutamate and aspartate were positively correlated with TSH in the serum of PTC1 patients.

Moreover, significantly higher concentrations of glutamate and aspartic acid were detected in the serum of PTC0 and PTC1 patients in comparison to healthy controls (Table S1). Aizawa et al. (52) studied the effects of glutamic acid and glutamine on TSHβ expression in pars tuberalis (PT) slice cultures from rat brains. After 2- and 4-h treatments, glutamic acid and glutamine significantly stimulated TSHβ expression in PT slices, and the impact of glutamic acid was stronger than that of glutamine (52). TSH was also positively correlated with aspartic acid, although the correlation was weak (0.356, $p<0.05$) (Table 3). The enzymes involved in glutaminolysis were overexpressed in thyroid cancer tissue (20, 21, 53) and promoted the transformation of glutamine to glutamate to sustain the TCA cycle and anabolic processes (27). Therefore, excess products of glutaminolysis, such as aspartic and glutamic acid, can be removed into the serum of PTC patients. Furthermore, aspartic and glutamic acid are substrates for nucleotide biosynthesis, and increased amounts could replenish the levels of the metabolites of the TCA cycle that may be decreased as a result of aerobic glycolysis (Warburg effect) (25). According to Cheng et al. (54), the increased glutamate concentrations in PTC patients are a result of increased glutamine metabolism in tumour cells. Moreover, the existing association of thyroid autoimmunity with PTC may be involved in increased serum glutamine concentrations (18). One of the amino acids with a reduced concentration in serum samples in both the PTC0 and PTC1 groups compared to HCs was alanine. Additionally, Qing Huang et al. (25) found reduced concentrations of alanine in the serum of PTC patients. A similar reason is indicated by Wojtowicz et al. (26). Decreased alanine might be evidence of its fast utilization from circulating blood as an answer for energy demands (26). In our study, alanine showed a decreasing trend in the serum of PTC1 patients compared to PTC0 patients. Additionally, we observed a strong negative correlation of alanine with TPO-Abs in the serum of PTC1 patients. Thyroid hormones control a multitude of homeostatic functions, including protein proteolysis (16). In Hashimoto, the antibody titre is significantly elevated, so this may be another factor lowering the concentration of alanine in patients with HT. When comparing different studies, the differences in the abundance of these amino acids between healthy subjects and patients with benign or malignant thyroid lesions do not always match. Certainly, observed results need further investigations. One of this reason might be the use of different sampling methods, techniques and study groups. However, there is agreement that plasma/serum levels of tyrosine, a precursor of thyroid hormones, are lower in PTC patients than in controls (26, 46, 55–57). The T3 hormone, triiodothyronine, constitutes only 10% of the total thyroid hormones, although it is considered responsible for most of the thyroid's activities and is 3-4 times stronger than the T4 hormone. Tyrosine is necessary for synthesis of thyroxine, which is produced by the thyroid gland (47). Reduced values of fT4 and elevated values of fT3 accompany Hashimoto (18). Deficiency in tyrosine, as well as phenylalanine, results in altered levels of thyroid hormones (47). Tyrosine is considered a nonessential amino acid because it can be synthesized from phenylalanine; nonetheless, it has an important role in the production of proteins that are a part of signal transduction processes, acting as a receiver of phosphate groups transferred through tyrosine kinases. In turn, these enzymes have been associated with the regulation of cellular proliferation, survival, differentiation, function and motility, linking them to a cancer phenotype Tahara et al. (48) demonstrated the effects of amino acid deficiency on serum levels of T4, T3, fT4, and reverse T3; they reported that reduction of phenylalanine and tyrosine drastically affected the serum levels of thyroid hormones. We observed a positive correlation between tyrosine and fT3 and fT4 in the serum of PTC1 patients. Interestingly, in HT and other autoimmune thyroid diseases, other studies (17, 18) did not observe the phenomenon of decreased levels of tyrosine in serum patients. Jiang et al. (41) suggested that lysine degradation and tyrosine metabolism played an important role in the HTS group compared to the control group. However, this was not supported by measured tyrosine concentrations, only enrichment analysis (41). This may be due to the effects of the HT drugs.

## 4.1 Conclusion

Our study aimed to contribute to further understanding of how AAs differ between patients with papillary thyroid cancer alone and those with comorbid Hashimoto thyroiditis in relation to healthy controls. By examining the amino acid profile in the blood, we found some unique patterns that would allow us to distinguish PTC0 patients from PTC1 patients. The clinical significance of these findings remains unclear. This is due to several limitations (1), absence of serum from the HT group, (2) relatively small study groups, (3) absence of laboratory tests of thyroid function or thyroid autoantibodies from control group. Therefore, despite finding differences in several AAs depending on the analysis used, only two were actually changed in most analyses and could be used to distinguish the studied PTC groups. The AA that most strongly separated PTC0 from PTC1 was lysine. Lysine, in addition to glutamic acid, differentiated both PTC groups and was positively correlated with anti-TG. The second AA marker with high probability may be alanine. Although no statistically significant difference was found (probably due to high SD), its concentration showed a downwards trend in the PTC1 group compared to the PTC0 and HC groups. Alanine was also negatively correlated with anti-TPO and was one of the 8 markers of AAs that separated/distinguished PTC1 patients from healthy subjects based on ROC analysis. We believe that long-term studies in larger populations are needed to confirm the predictive potential of selected metabolites in diagnosing thyroid lesions.

# Data availability statement

The data presented in the study are deposited in the MetaboLights repository, accession number MTBLS8012; https://www.ebi.ac.uk/metabolights/editor/study/MTBLS8012.

## Ethics statement

The studies involving human participants were reviewed and approved by Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk under number NKBBN/62/2021. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

AH and AM, designed the study, analyzed and interpreted results, and wrote the manuscript. AH, and AT, contributed to sample and clinical data collection. JT, conducted the amino acids analysis. JK conducted the analysis of mRNA levels by real-time PCR. AZ and AM, performed the statistical analysis. All authors reviewed and edited the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1199291/full#supplementary-material

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# Do metabolic factors increase the risk of thyroid cancer? a Mendelian randomization study

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**Background:** Epidemiological studies emphasize the link between metabolic factors and thyroid cancer. Using Mendelian randomization (MR), we assessed the possible causal impact of metabolic factors on thyroid cancer for the first time.

**Methods:** Summary statistics for metabolic factors and thyroid cancer were obtained from published Genome-wide association studies. The causal relationships were assessed using the inverse-variance weighted (IVW) method as the primary method through a two-sample Mendelian Randomization (MR) analysis. To account for the potential existence of horizontal pleiotropy, four additional methods were employed, including Mendelian Randomization–Egger (MR-Egger), weighted median method (WM), simple mode, and weighted mode method. Given the presence of interactions between metabolic factors, a multivariable MR analysis was subsequently conducted.

**Results:** The results showed there was a genetic link between HDL level and protection effect of thyroid cancer using IVW (OR= 0.75, 95% confidence intervals [CIs] 0.60-0.93, p=0.01) and MR-Egger method (OR= 0.70, 95% confidence intervals [CIs] 0.50- 0.97, p=0.03). The results remained robust in multivariable MR analysis for the genetic link between HDL level and protection effect of thyroid cancer (OR= 0.74, 95% confidence intervals [CIs] 0.55-0.99, p=0.04).

**Conclusions:** This study suggests a protection role for HDL on thyroid cancer. The study findings provide evidence for the public health suggestion for thyroid cancer prevention. HDL's potential as a pharmacological target needs further validation.

KEYWORDS

metabolic factors, HDL, thyroid cancer, Mendelian randomization, public health

## Introduction

Thyroid cancer is widely regarded as the most prevalent endocrine malignancy. In numerous countries, the frequency of thyroid cancer has experienced a notable rise in recent decades (1). The treatment options for thyroid cancer encompass surgical intervention to excise the thyroid gland, radioactive iodine therapy, and hormone replacement therapy. With early detection and appropriate treatment, patients have a good chance of long-term survival and a good quality of life. However, ongoing monitoring and follow-up care is important to detect any recurrence or new cancerous growths.

The exact cause of thyroid cancer is not known. Various studies have linked metabolic factors to thyroid cancer, but the majority of the findings remain controversial. There exists empirical evidence indicating that metabolic factors are associated with an elevated risk of developing various carcinogenic mechanisms, including those affecting the liver, colon, and mammary tissue, but the association between thyroid cancer and metabolic factors is inconsistent (2, 3). Specifically, the correlation between diabetes and thyroid cancer has yielded inconsistent results across studies (2, 3). Existing research posits that metabolic hormone imbalances, including insulin and leptin, may play a role in the pathogenesis of thyroid cancer (4, 5). Elevated insulin resistance and heightened insulin levels in the bloodstream have been correlated with an augmented susceptibility to thyroid cancer (4). Furthermore, obesity, which is concomitant with insulin resistance, has been demonstrated as a risk factor for the onset of thyroid cancer (6, 7), although this was not corroborated by a Mendelian randomization study (8). Additionally, reduced levels of vitamin D have been associated with an increased likelihood of thyroid cancer (9). Nevertheless, there exists evidence that vitamin D levels are not linked to the risk of thyroid cancer (10). A retrospective cohort study has reported a positive correlation between uric acid and thyroid nodules (11), while a cohort study from China has reported an association between nonalcoholic fatty liver disease and an increased risk of thyroid cancer (12). These studies are predominantly epidemiological and clinical in nature, and the causal relationship remains unclear. Therefore, it is imperative to evaluate the causality of these associations to inform updates to thyroid cancer prevention strategies.

The Mendelian randomization (MR) technique is a statistical methodology employed to investigate the causal associations between variables in observational research (13). It is based on the principle of Mendel's laws of inheritance, which state that the distribution of genetic variations among offspring is random (14). Due to the random assignment of genotypes during the transmission from parents to offspring (14), it can be inferred that groups of individuals characterized by genetic variation related to a particular exposure at a population level are expected to have minimal association with the confounding factors commonly encountered in observational epidemiology studies. Furthermore, germline genetic variation remains unchanged after conception and is not influenced by the occurrence of any outcome or disease, thereby eliminating the possibility of reverse causation. The utilization of genetic variations as instrumental variables in MR enables the inference of the causal effect of a risk factor on a specific outcome of interest. Notably, MR offers an advantage over conventional observational studies by facilitating the establishment of a causal relationship between a risk factor and an outcome, despite the presence of confounding factors (15). Genetic variants that are correlated with the risk factor of interest are detected in MR studies and employed as surrogates for the exposure (16). These variants are then used to estimate the causal effect of the risk factor on the outcome, while controlling for the influence of other confounding variables. Because genetic variants are randomly assigned at conception, they are not subject to the biases and confounding factors that can impact the results of observational studies (17). Thus, Mendelian randomization can be conceptualized as akin to a randomized controlled trial conducted by nature. The MR method has become a popular tool in epidemiology and public health research, particularly for investigating the causal relationships between lifestyle factors and health outcomes. The results of MR studies have provided valuable insights into the causal relationships between risk factors and health outcomes and have helped to inform public health policies and interventions aimed at improving population health (18).

In this article, we applied Mendelian randomization methodology to explore the causal association between metabolic factors and thyroid cancer.

## Methods

Mendelian randomization (MR) employs genetic variation as a means to investigate causal inquiries pertaining to the potential impact of modifiable exposures on health, developmental, or social outcomes. Methods for MR are usually based on instrumental variables (IVs). Genetic variants serve as a potential exogenous source of variation in the exposure, thereby functioning as an IV. Figure 1 showed our study workflow.

We tried to cover metabolic factors as much as we can to provide evidence for the public health suggestion for thyroid cancer prevention. Metabolic factors reported to be associated with thyroid cancer but that remained controversial were included in the study (2–12). Metabolic factors reported in other solid tumors but lack of evidence in thyroid cancer were also included in the study (19–21). Finally, our study included 17 metabolic factors according to present epidemic study reporting the relevance to thyroid cancer. Firstly, we estimated the associations of metabolic factors and thyroid cancer using univariable MR analysis. Considering the metabolic factors may have interaction, multivariable MR analysis

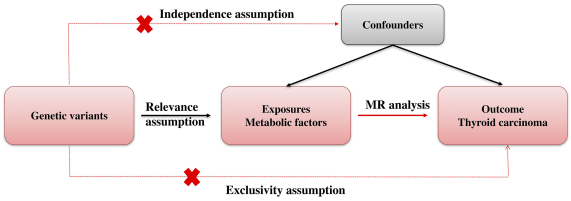


FIGURE 1
Experimental workflow. MR, Mendelian randomization.

were conducted to increase the analysis power. The study was based on publicly available, summary-level data of genome- wide association studies (GWAS), the FinnGen study (22), the UK Biobank study (23), and other large consortia. Informed consent was obtained from participants in included studies, which were approved by an appropriate ethical review board.

## Exposures chosen

Significant SNPs for 17 metabolic factors were extracted from corresponding GWAS studies (Table 1). The SNP used as the exposure instrumental variables (IVs) were selected with a p-value less than 5E-8. Then we performed linkage disequilibria based clumping to return only independent significant associations. SNPs without linkage disequilibrium r2 < 0.001 and a clump distance >10,000kb window were obtained.

## Outcomes chosen

Based on reported GWAS data, we obtained summary statistics on SNP associations with thyroid cancer. GWAS data from the largest publicly available thyroid cancer case–control study involving 218792 Europeans (989 cases, 217,803 controls) was obtained from FinnGen. 16,380,466 SNPs in finn-b-C3_THYROID_GLAND was downloaded for further analysis.

## Statistical analysis

IVs and outcome data were firstly harmonized to be relative to the same allele. MR analysis was then conducted. Various methods were employed to assess the resilience of the outcomes and identify pleiotropy, such as the inverse-variance weighted (IVW), Mendelian Randomization–Egger (MR-Egger), weighted median method (WM), simple mode, and weighted mode method, in order to compute the causal effect. Analyzing causal relationships was primarily conducted using IVW methods. Results were mostly derived from IVW (random effects) and sensitivity analysis. The meta-analysis approach employed by IVW amalgamates the Wald ratios of individual SNPs to yield precise estimates. A significance level of $P < 0.05$ was deemed indicative of a potential association. The MR-Egger method is a proficient strategy for identifying deviations from the assumptions underlying instrumental variables (24). Weighted median method can provide sensitivity analyses with multiple genetic variants. If the weight of valid instruments exceeds 50%, consistent causal estimates may be obtained (25). Although less powerful than IVW, simple mode offers robustness against pleiotropy (26). As a supplementary analysis method, weighted mode is sensitive to challenging bandwidth selections for mode estimation (27). The MR-Egger regression intercept term tests were utilized to identify horizontal pleiotropy. Heterogeneity in IVW and MR-Egger regression analyses was quantified using Cochran's test.

TABLE 1 Metabolic factors included in the Mendelian randomization study.

| Exposure | Participants Included in Analysis | Dataset |
|---|---|---|
| Body mass index | 339,224 | ieu-a-2 |
| Height | 6,974 | ieu-a-1032 |
| Waist-to-hip ratio | 224,459 | ieu-a-72 |
| Body fat | 100,716 | ieu-a-999 |
| LDL cholesterol | 440,546 | ieu-b-110 |
| HDL cholesterol | 403,943 | ieu-b-109 |
| triglycerides | 441,016 | ieu-b-111 |
| Total cholesterol | 187,365 | ieu-a-301 |
| apolipoprotein A-I | 393,193 | ieu-b-107 |
| Adiponectin | 39,883 | ieu-a-1 |
| Nonalcoholic fatty liver disease | 218792 | finn-b-NAFLD |
| Type 2 diabetes | 655,666 | ebi-a-GCST006867 |
| Hemoglobin A1c | 42,790 | bbj-a-26 |
| Serum 25-Hydroxyvitamin D levels | 496,946 | ebi-a-GCST90000618 |
| Uric acid | 109,029 | bbj-a-57 |
| hypertension | 463,010 | ukb-b-12493 |
| Systolic blood pressure | 757,601 | ieu-b-38 |
| diastolic blood pressure | 757,601 | ieu-b-39 |

For significant associations identified in the analyses, the multivariable MR was further used as a sensitivity analysis to explore whether this causal effect was robust to the adjustment.

All statistical analyses were conducted in R (version 4.2.2) using the TwoSampleMR (28), MRInstruments packages. Plots were generated using ggplot2 R package. Our code is publicly available on GitHub: https://github.com/heleliangww/MR-for-thyroid-cancer-.

## Result

IVW analysis showed there was a genetic link between HDL level and protection effect of thyroid cancer (Figure 2). Results revealed an increase in HDL level was strongly associated with a decrease in the risk of thyroid cancer (OR= 0.75, 95% confidence intervals [CIs] 0.60-0.93, p=0.01). The scatter plots in Figure 3 illustrated the SNP- thyroid cancer associations against the SNP-HDL associations. There was a consistent association in sensitivity analyses using MR-Egger method (OR= 0.70, 95% confidence intervals [CIs] 0.50- 0.97, p=0.03). Based on MR-Egger regression intercept analysis, no significant horizontal pleiotropy was detected (intercept= 0.002, SE= 0.005, p= 0.58). Using Cochran's Q test, no heterogeneity was observed among SNPs in IVW analysis and MR-Egger analysis, suggesting no strong unbalanced horizontal pleiotropy (Q_pval = 0.09 in IVW method, Q_pval= 0.09 in MR Egger method). There was a balanced pleiotropy in SNP effects around the effect estimate, as evidenced by the funnel plot (Figure 4).

IVW analysis showed there was a genetic link between diastolic blood pressure and increased risk of thyroid cancer (Figure 2). Results revealed an increase in diastolic blood pressure level may associated with an increase in the risk of thyroid cancer (OR=1.03, 95% confidence intervals [CIs] 1.00-1.06, p=0.046). While, the result was not consistent in MR-Egger method analysis (OR= 0.99, 95% confidence intervals [CIs] 0.92- 1.06, p=0.71).

Considering there were interactions between different lipid components, multivariable MR was conducted. Diastolic blood pressure was also included in the multivariable MR analysis for its positive IVW analysis (Figure 5). As with the univariate MR analysis, the results remained robust in multivariable MR analysis for the genetic link between HDL level and protection effect of thyroid cancer (OR= 0.74, 95% confidence intervals [CIs] 0.55-0.99, p=0.04).

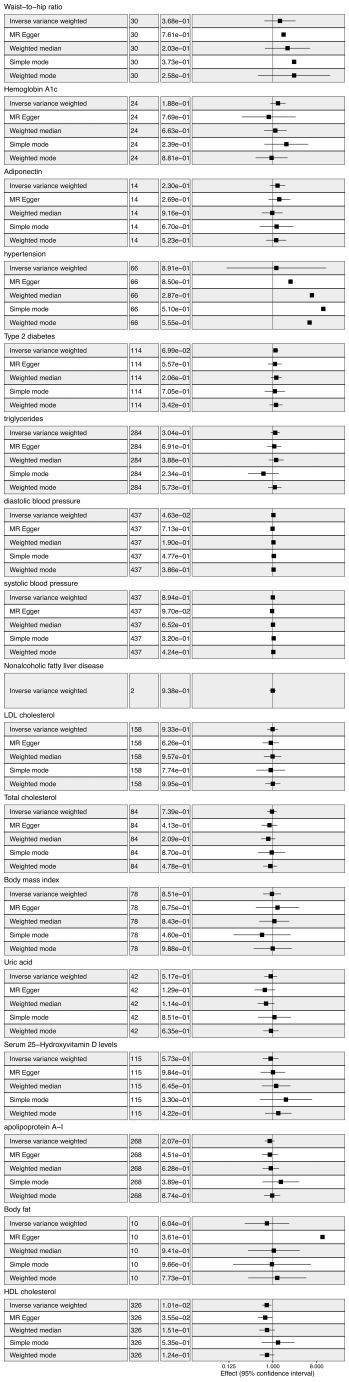


FIGURE 2
Metabolic factors and thyroid cancer in Mendelian randomization (MR) analyses. The first column from left showed the corresponding methods. The second column from left showed the number of SNPs involved in the analyses. The third column from left showed the corresponding *p* value. The forth column from left showed odds ratio and 95% confidence interval.

## Discussion

This study used GWAS summary statistics to perform MR analysis to investigate the causal association between thyroid cancer and metabolic factors. We believe this is the first MR study to identify a large number of modifiable causal risk factors for thyroid cancer. We found serum HDL-cholesterol level was associated with a reduced risk of thyroid cancer. We did not find a causal relationship between obesity, diabetes, blood pressure, NAFLD, uric acid, and serum 25-hydroxyvitamin D levels and thyroid cancer.

HDL, also known as high-density lipoprotein, is commonly acknowledged as "good" cholesterol due to its ability to eliminate excess cholesterol from the bloodstream and transport it to the liver for processing and excretion from the body. Numerous published

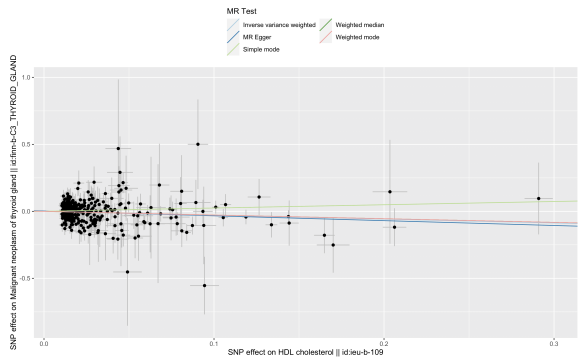


FIGURE 3
The scatter plot of five Mendelian randomization methods on HDL and thyroid cancer.

observational studies have established a consistent correlation between HDL and thyroid cancer. For instance, a Korean epidemiological study discovered that obese women with low HDL cholesterol levels were at a heightened risk of developing thyroid cancer (29, 30). Similarly, the Swedish Apolipoprotein-Related Mortality Risk (AMORIS) Cohort study demonstrated that thyroid cancer risk was associated with blood levels of total cholesterol (TC) and HDL-C (31). HDL-C level was found to be a statistically significant independent predictor of thyroid cancer in a model developed by Zhang et al. (32). Some retrospective observational studies have reported an association between total cholesterol (31) and apolipoprotein A1 (33) with thyroid cancer, which is somewhat inconsistent with the results of our study. In the observational study, HDL may be a confounding factor for other lipid profiles.

Few studies have investigated the mechanism of HDL in thyroid cancer *in vivo* and *in vitro*. HDL has been reported to play a role in the invasion, metastasis, and development of other solid tumors. When HDL levels are within a certain range, tumor development

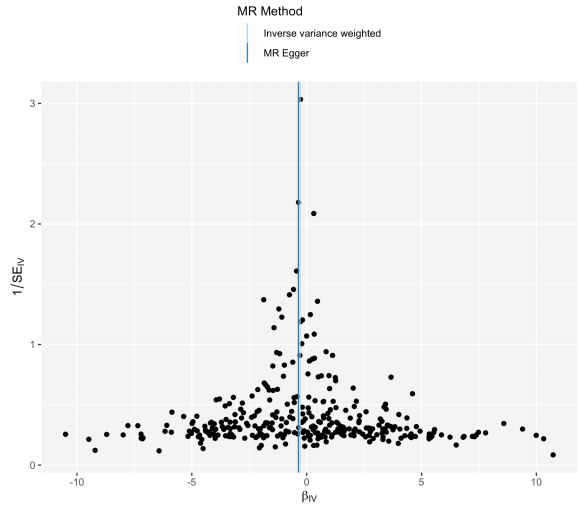


FIGURE 4
Funnel plot for HDL and thyroid cancer.

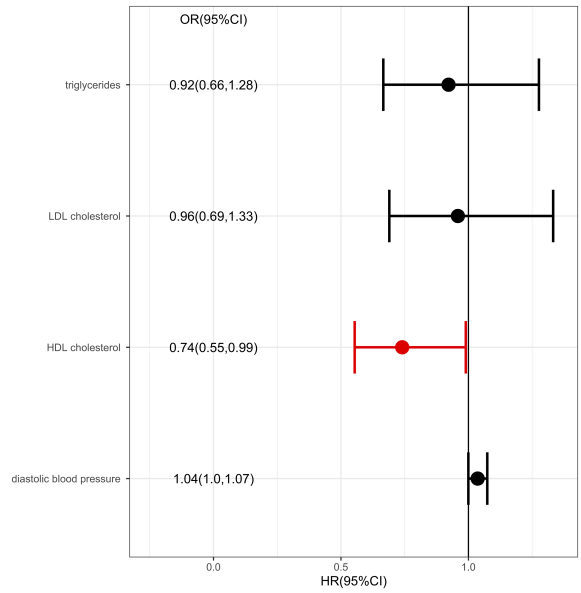


FIGURE 5
Multivariable MR result for metabolic factors and thyroid cancer.

can be inhibited *in vivo* (34). *In vitro* studies have shown that HDL inhibits tumor cell growth or promotes apoptosis by inhibiting components of tumor microenvironments (34). The HDL reduce oxidative stress and proinflammatory molecules in cancer cells (35). Additionally, HDL can inhibit angiogenesis and reverse tumor immune escape (35). In pancreatic ductal adenocarcinoma, research showed cancer cell growth is reduced by HDL-mediated cholesterol removal (36). Relevant functional studies are lacking, further research is needed to fully understand the relationship between HDL and thyroid cancer.

IVW analysis showed a genetic link between diastolic blood pressure and thyroid cancer, which was inconsistent in MR-Egger method analysis. The result might be biased by pleiotropy or other confounding factors.

Unlike observational studies, our results do not confirm a causal role for other metabolic factors in thyroid cancer. Confounding factors such as HDL levels may lead to false associations in clinical observations. By using genetic variants, we can limit those confounding factors in by using Mendelian randomization.

In this study, we address metabolic factors and related traits and the effect on thyroid cancer for the first time using Mendelian randomization. We acknowledge, however, that there are some limitations to our study. Our MR analysis power was limited by the fact that we had only 989 thyroid cancer cases. Our analysis was not stratified by gender. There is a need for further GWAS studies with a larger number of cases and detailed information on disease characteristics.

In conclusion, our study found serum HDL-cholesterol level was associated with a reduced risk of thyroid cancer. Our study provided genetic evidence that HDL might protect thyroid cancer patients. The study findings provide evidence for the public health suggestion for thyroid cancer prevention. Further validation of our findings in other cohorts and ethnicities will require independent

GWAS and large prospective studies. HDL's potential as a pharmacological target needs further validation.

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The study was based on publicly available, summary-level data of genome- wide association studies (GWAS), the FinnGen study, the UK Biobank study.

## Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

## Author contributions

WL made significant contributions to the literature search and study design, as well as the analysis and interpretation of the data, ultimately resulting in the composition of the manuscript. FS, on the other hand, played a crucial role in formatting the figures and tables, as well as revising the manuscript. Additionally, FS provided valuable insights and constructive discussions during the analysis process. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Is MG53 a potential therapeutic target for cancer?

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Cancer treatment still encounters challenges, such as side effects and drug resistance. The tripartite-motif (TRIM) protein family is widely involved in regulation of the occurrence, development, and drug resistance of tumors. MG53, a member of the TRIM protein family, shows strong potential in cancer therapy, primarily due to its E3 ubiquitin ligase properties. The classic membrane repair function and anti-inflammatory capacity of MG53 may also be beneficial for cancer prevention and treatment. However, MG53 appears to be a key regulatory factor in impaired glucose metabolism and a negative regulatory mechanism in muscle regeneration that may have a negative effect on cancer treatment. Developing MG53 mutants that balance the pros and cons may be the key to solving the problem. This article aims to summarize the role and mechanism of MG53 in the occurrence, progression, and invasion of cancer, focusing on the potential impact of the biological function of MG53 on cancer therapy.

KEYWORDS

**MG53, cancer, glucose metabolism, membrane repair, insulin resistance**

## 1 Introduction

The tripartite-motif (TRIM) family is characterized by a really interesting new gene (RING) finger domain, one or two B-box domains, and a coiled coil domain (1). Tripartite domains are highly conserved among TRIM proteins and hence perform similar functions in cellular processes (2). The vast majority of TRIM proteins contain RING finger domains in their N-terminal regions and seem to participate mostly in ubiquitination (3). B-box domains may exist solely in TRIM proteins and may mediate protein–protein interactions (1, 4). The coiled coil domain has been proven to mediate homo-oligomeric and hetero-oligomeric interactions given that self-association via this domain is believed to play a critical role in catalytic activity of TRIM proteins (5). The variation in the C-terminal domain contributes to the diverse functions of TRIM proteins.

About 80 TRIM protein genes have been identified in humans (6). Many diseases have been shown to be associated with TRIM proteins. These diseases include metabolic and neurodegenerative diseases, viral infections, and cancers (7–10). The role of TRIM proteins in cancer has received more attention. As a result of structural differences, TRIM proteins act as oncogenes and tumor suppressors in different cancers (11). However, the

relationship between some members of TRIM proteins and cancer remains unexplored (10).

TRIM72, also known as Mitsugumin 53 (MG53), is secreted by muscle tissues and is a TRIM family protein derived from an immunoproteomics pool (12). The C-terminal of MG53 contains PRY and SPRY domains, which are the most common domains in TRIM proteins (4, 13). These domains can recognize specific partner proteins, thus acting as protein-interacting modules (14). As a typical E3 ubiquitin ligase, MG53 was initially found to participate in damage repair in skeletal muscle cells, and its key feature of membrane repair in a variety of organ injuries was later confirmed (12, 13). MG53 overexpression can inhibit systemic insulin response and subsequently cause metabolic issues (15). However, other researchers take a completely opposing position (16). Evidence suggesting that MG53 may perform an anticancer role in cancers, such as hepatocellular carcinoma, colorectal carcinoma, tongue cancer, and nonsmall cell lung cancer (NSCLC), has recently emerged (17–20). In this review, we summarize the roles and mechanisms of MG53 in a variety of cancers and discuss the possible contribution of the diverse biological functions of MG53 in cancer.

# 2 Beneficial effects of MG53 on cancer therapy

## 2.1 MG53 in colorectal carcinoma

Colorectal cancer is the second most common cause of cancer deaths worldwide and is expected to cause 1.2 million deaths by 2030 (21, 22). Considering that most patients with colorectal cancer progress slowly over many years, colorectal cancer is usually curable if diagnosed at an early stage (23). Screening for colorectal cancer requires the development of sensitive biomarkers in peripheral blood. Many members of the TRIM protein family have been reported to act as oncogenic and tumor-suppressive factors in gastrointestinal cancers via different signaling pathways (24). In addition, TRIM47 may be an effective diagnostic marker for predicting colorectal cancer (25).

The gene and protein levels of MG53 were considerably lower in colon cancer tissues than in healthy colon tissues, and the same results were found in the serum of patients with colon cancer (26). In colon cancer and normal colon tissues, MG53 may be expressed and secreted by stromal cells instead of normal colon or colon cancer cells, and serum MG53 levels are negatively correlated with colon cancer stage and metastasis, suggesting that the low MG53 levels in the serum of patients with colon cancer may be due to local tissue lesions (26). Low levels of MG53 in focal tissues have also been suggested to account for the poor prognosis of stage II colon carcinoma (27). Under colorectal carcinogen induction, MG53 knockout mice present more severe tumor progression than wild-type mice, whereas mice with MG53 overexpression have relatively good colorectal structure and function (19). MG53 has also been shown to inhibit the proliferation of colorectal cancer cells in an *in vitro* study. And this study found that MG53, as an E3 ubiquitin ligase capable of targeting cyclin D1, induces its ubiquitination-dependent degradation to inhibit the proliferation of gastrointestinal cancer cells by arresting the cell cycle at the G1 phase (28). In addition, MG53 acts differently on different anticancer drugs. MG53 and pabocinib inhibit the proliferation of colon cancer cells synergistically, and MG53 could partially ameliorate drug resistance (19). The safety of recombinant human MG53 (rhMG53) has been validated in a mouse model of colorectal cancer (28). Although rhMG53 do not affect the doxorubicin sensitivity of resistant colorectal cancer cells (SW620/AD300), it inhibits the proliferation of colorectal cancer cells. Moreover, in mouse tumor xenograft models of colorectal adenocarcinoma with multidrug resistance, the combination of doxorubicin and rhMG53 appeared to be more effective than doxorubicin or rhMG53 alone (28).

## 2.2 MG53 in hepatocellular carcinoma

Although vaccination and antiviral therapy have reduced the incidence of hepatocellular carcinoma, the incidence and mortality rates of this malignancy continue to increase in many regions of the world (29). In hepatocellular carcinoma, the expression of numerous TRIM proteins tends to be altered and has been shown to be correlated with diagnosis, treatment, and prognosis (30). TRIM proteins appear to be involved in the survival, growth, aerobic glycolysis, immune infiltration, and invasion of hepatocellular carcinoma cells (31–34).

The mRNA expression of MG53 was detected in human hepatocellular carcinoma and normal human hepatocyte cell lines. In patients with hepatocellular carcinoma, the high expression of MG53 may be associated with poor overall survival (35). However, one study has shown that the gene and protein expression levels of MG53 have been suggested to be drastically lower in hepatocellular carcinoma tissue than in matched noncancerous liver tissue (17). MG53 regulates the ubiquitination and degradation of RAC1, a small GTPase with oncogenic function, this effect, in turn, inhibits the malignant progression of hepatocellular carcinoma and improves the resistance of hepatocellular carcinoma to sorafenib treatment by blocking the RAC1/MAPK signaling pathway (17).

## 2.3 MG53 in NSCLC

Although the application of precision medicine in NSCLC treatment has advanced considerably over the past decade, the 5-year survival rate of patients with metastatic NSCLC remains less than 5% due to multiple drug resistance mechanisms (36, 37). Some TRIM proteins may contribute to NSCLC or resistance to targeted drugs (38–44), whereas others have completely opposite functions (45–47).

MG53 is downregulated in metastatic tumors from patients with NSCLC relative to in nonmetastatic tumors, and MG53 knockout promotes the growth and metastasis of lung tumors in mice (48, 49). G3BP2, a protein associated with the formation of multiple tumors, was upregulated in the cytosol of tumor cells from patients with NSCLC relative to in nontumor cells. Circulating

levels of MG53 appear to influence the proliferation and migration of NSCLC cells directly via G3BP2. Instead of performing classical ubiquitination-dependent degradation functions, the amino terminus of MG53 physically interacts with G3BP2 and enhances its nuclear translocation, which may be a key mechanism by which MG53 inhibits the G3BP2-mediated formation of lung cancer tumors and stress granules (20, 50). Furthermore, an *in vitro* study showed that rhMG53 inhibited the formation of stress granules and potentiated the cytotoxic effect of cisplatin on human NSCLC cells (20).

## 2.4 MG53 in other cancers

MG53 appears to have an ameliorative effect on multiple types of cancer. However, many TRIM proteins have inconsistent effects on different cancers. A three-dimensional growth system study reported that MG53 dramatically suppressed the proliferation, invasion, and colony formation of tongue cancer cells (18). Knocking down MG53 in tongue cancer cells resulted in a remarkable increase in the phosphorylation of $AKT^{Ser308}$ and $AKT^{Thr473}$. Animal studies showed that in mice, knocking out MG53 also accelerated the progression of tongue cancer (18). O6-methylguanine DNA methyl transferase (MGMT) is an important target in cancer therapy because it blocks the beneficial effects of chemotherapy on tumor cells (51). The RING structural domain of MG53 interacts with the N-terminal region of MGMT and regulates the ubiquitination-dependent degradation of MGMT. Human uveal melanoma cells have higher MGMT levels and lower MG53 levels than normal human pigment epithelium cells. MG53 overexpression in uveal melanoma cells contributes to improved chemoresistance to dacarbazine treatment (52). MG53 is downregulated in the tumor tissue of patients with breast cancer relative to in paired adjacent nontumor tissue and is also downregulated in many breast cancer cell lines relative to in normal human mammary cell lines. *In vivo* and *in vitro*, MG53 inhibits breast cancer progression likely because it can inhibit the activation of the PI3K/Akt/mTOR pathway and reduce lactate levels through protein phosphatase 3 catalytic subunit α (53). One study analyzed ubiquitin-related genes in The Cancer Genome Atlas cohort and found that MG53 was correlated strongly with the grade, stage, and T stage of clear cell renal cell carcinoma. However, the expression of MG53 in patients with clear cell renal cell carcinoma remains to be confirmed (54).

In accordance with the current evidence, MG53 appears to be beneficial for delaying the progression of various cancers and improving resistance to some anticancer drugs in *in vitro* and animal models (Figure 1). Available studies suggest that the antitumor effect of MG53 may be mainly derived from its role as an E3 ubiquitin ligase. However, the current evidence for specific cancer types remains insufficient and lacks mechanism research. Further safety verification is required for the application of rhMG53.

# 3 Other biological functions of MG53 may contribute to cancer therapy

## 3.1 Potential role of MG53 as a plasma membrane repair protein in cancer treatment

During cancer progression and treatment, many organs suffer varying degrees of tissue damage from the tumor, cancer

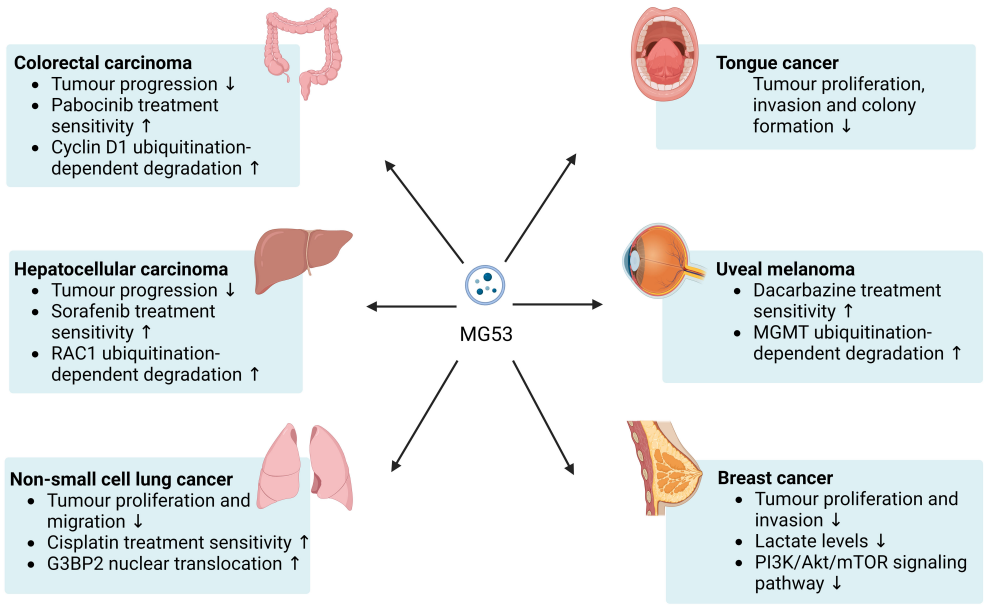


FIGURE 1
Beneficial effects of MG53 on cancer. MG53 can inhibits the progression of a broad range of cancers and helps improve the therapeutic sensitivity of numerous anticancer drugs. The antitumor capacity of MG53 may be mainly attributed to its E3 ubiquitin ligase properties.

complications, and treatment side effects, all of which are related to plasma membrane damage and may accelerate cancer progression (55–57). MG53 was initially well known for its function in the repair of muscle cell membranes. Evidence showing that MG53 can participate in the repair of various cell membranes and promote tissue regeneration has emerged with the deepening of research (13, 58). MG53 secreted by skeletal muscles is transported in the circulatory system in the form of vesicles and participates in muscle cell membrane repair. The failure of MG53-mediated membrane damage repair may cause certain skeletal muscle diseases (12, 59–61). In addition, the pathological processes of myocardial injury and cancer are intertwined, and heart failure induced by anticancer therapy has become a key focus in cardiac oncology research (62, 63). Evidence also suggests that cancer and ischemia–reperfusion injury share common pathways also exists (64).

### 3.1.1 MG53 in kidney injury

The occurrence of acute and chronic kidney injury is strongly associated with the development of kidney cancer, and early intervention for kidney injury is an effective means of kidney cancer prevention (65). Moreover, the presence of acute kidney injury is fairly prevalent in patients with cancer. The management strategies for acute kidney injury differ in accordance with predisposing factors. For example, immunotherapy-induced acute kidney injury is influenced by tumor type and treatment modality (66, 67). When renal proximal tubular epithelium cells experience acute injury, such as mechanical or chemical damage, MG53 rapidly translocates to the injured site to form a repair patch. By contrast, in injured renal proximal tubular epithelium cells with MG53 knockout, the defect in membrane repair function leads to rapid death of cells. MG53 knockout mice exhibit tubulointerstitial defects and show more severe renal injury than wild-type mice during ischemia–reperfusion. In animals, the preadministration of rhMG53 alleviates cisplatin or iodine contrast agent-induced acute kidney injury (68, 69). In chronic kidney disease, MG53 provides benefits by controlling inflammation and promoting mitochondrial autophagy (70, 71).

### 3.1.2 MG53 in lung injury

Chronic lung injury, such as chronic obstructive pulmonary disease, is strongly associated with the development of lung cancer, and this mechanistic overlap has attracted increasing attention (72, 73). Chemotherapy, surgery, medication treatment for lung cancer, and even treatment for other types of cancer can lead to lung injury (74–77). Chronic moderate liver injury tends to induce hepatic cell carcinogenesis rather than hepatocellular senescence, which can inhibit carcinogenesis (78). In several models of lung injury, MG53 shows reparative effects on pulmonary epithelial cells. Animals lacking MG53 exhibit increased susceptibility to injury induced by various factors, and rhMG53 can protect lung tissue from lung injury. MG53 may execute its membrane repair function by coregulating the endocytosis of alveolar epithelial cells with caveolin 1 (79–85).

### 3.1.3 MG53 in liver injury

The liver is susceptible to the effects of drugs, such as conventional chemotherapy drugs, small-molecule-targeting drugs, including multikinase inhibitors, or immune checkpoint inhibitors, all of which can induce varying degrees of liver injury (86–88). With the widespread application of immune checkpoint inhibitors in liver tumor therapy, the relationship between checkpoint inhibitors and liver safety has received increased attention (89). Although hepatocytes do not express MG53 mRNA, circulating MG53 leads to the ubiquitination-dependent degradation of RIPK3, which inhibits the phosphorylation and membrane translocation of MLKL and thus alleviates acetaminophen-induced hepatocyte injury (90, 91). MG53 can also ameliorate oxidative stress and hepatocyte death induced by hepatic ischemia–reperfusion through interaction with dysferlin (92).

Overall, the plasma membrane repair function of MG53 has considerable potential for application in cancer prevention and treatment. Current research focuses on the association between MG53 and the progression of tumor tissues, whereas only a few studies have investigated the contribution of plasma membrane repair by MG53 to cancer treatment. However, the fact that excessive membrane repair contributes to cancer cell invasion is also important to consider when using rhMG53 (93). For example, the annexin family, which participates in membrane repair together with MG53, is overexpressed in invasive cancer cells and promotes the plasma membrane repair of cancer cells. Inhibiting Annexin-mediated repair is beneficial for inducing cancer cell death (94–98).

## 3.2 Potential role of MG53 as an anti-inflammatory factor in cancer treatment

Inflammatory response is an important defense mechanism of the body, but it can also promote the formation of tumor microenvironment and tumor promotion, especially chronic inflammation (99). Anti-inflammatory therapy targeting inflammation-related factors such as nuclear factor-κB (NF-κB) plays an important role in cancer control (100). TRIM proteins are widely involved in regulating inflammatory responses and MG53 appears to have anti-inflammatory effects in multiple tissues (101).

MG53 interacts with the p65 subunit of NF-κB and thereby inhibits the nuclear translocation of NF-κB, which in turn alleviates inflammatory responses in kidney, nervous system and airway (70, 102, 103). After infection of macrophages or mice with virus, MG53 attenuates inflammatory response by decreasing type I interferon levels (104). In mice with Duchenne muscular dystrophy, MG53 appears to enhance mitochondrial autophagy, thereby reducing nucleotide oligomerization domain-like receptor protein 3 (NLRP3) inflammasomes and suppressing chronic inflammation in skeletal muscles (105). Similarly, it was emphasised that MG53 may improve neuroinflammation by decreasing NLRP3 inflammasomes in a study using human umbilical cord mesenchymal stem cells and mice (106).

MG53 can ameliorate inflammation in many disease models, but its role in carcinogenic inflammation, inflammation caused by cancer and inflammation triggered by cancer treatment remains to be investigated.

Furthermore, other biological functions of MG53 may be beneficial for cancer therapy. For example, angiogenesis is an important target for cancer treatment, and MG53 inhibits angiogenesis *in vivo* and *in vitro* by decreasing focal adhesion kinase phosphorylation and blocking the Src/Akt/ERK1/2 signaling pathway (107, 108). Peroxisome proliferator-activated receptor-α (PPARα) agonists have a role in anti-tumor therapy and MG53 attenuates inflammatory responses in cardiomyocytes by upregulating PPARα expression (109, 110). However, there is too little relevant evidence to demonstrate that these functions of MG53 are beneficial for cancer therapy.

# 4 Potential adverse effects of MG53 on cancer treatment

## 4.1 MG53 overexpression may disrupt glucose metabolism signals

Insulin resistance is a key factor in the occurrence and development of cancer, and a substantial proportion of patients with cancer have insulin resistance (111–114). Impaired glucose tolerance is also strongly associated with long-term cancer risk and is an important risk factor for cancer-related death (115–119). During cancer treatment, the blood glucose and insulin levels of patients must be monitored to learn about the insulin resistance induced by therapeutic measures and thus adjust the treatment protocol promptly (120).

The relationship between MG53 and insulin resistance has long been controversial. Some studies have suggested that MG53 induces insulin resistance through multiple pathways, including targeting insulin receptor substrate 1 (IRS-1), insulin receptors (IRs), and AMP-activated protein kinase for ubiquitin-dependent degradation, promoting the expression of peroxisome proliferator-activated receptor-α and its target genes to facilitate myocardial lipid uptake and thereby leading to lipid accumulation and toxicity, and binding to the extracellular structural domains of IRs to inhibit receptors allosterically (15, 121–124). In addition, the direct application of rhMG53 may exacerbate insulin resistance, and the protective effect of MG53 on myocardial cells may be counteracted by its adverse metabolism. Two mutants of rhMG53, rhMG53-C14A and rhMG53-S255A, can eliminate adverse effects on metabolism while retaining the membrane repair function of rhMG53 (121, 125–128).

However, MG53 expression is inconsistent in various models of metabolic disorders, and neither the ablation nor overexpression of MG53 in wild-type and db/db mice has been noted to alter insulin signaling. Additionally, in rats, the repeated intravenous administration of rhMG53 does not seem to affect glucose metabolism (16, 129–133). Indeed, the lack of IRS-1 does not immediately give rise to diabetes because strong compensatory mechanisms exist between different IR subtypes (134, 135).

The aforementioned controversy may be attributed to the overlooked role of MG53 in pancreatic β-cells. In the absence of global insulin resistance, the IRs of pancreatic β-cells can inhibit high glucose-induced insulin secretion and their knockout can promote insulin secretion and improve glucose tolerance. However, this regulatory function of the IRs to β-cells does not occur in the presence of global insulin resistance (136). High glucose and insulin levels can promote the secretion of MG53 in striated muscle, and MG53 can induce the ubiquitination-dependent degradation and inactivation of IRs (15, 121). Under the assumption that MG53 can affect the function of pancreatic β-cells through IRs, MG53 overexpression would have a complicated effect on glucose metabolism in healthy and insulin-resistant humans (Figure 2). A cohort study involving 283 subjects supports our hypothesis. This study found that although serum MG53 levels appeared to be unrelated to insulin resistance, subjects with impaired glucose metabolism had remarkably higher circulating levels of MG53 than healthy subjects. Furthermore, circulating levels of MG53 were found to be an independent risk factor for the development of type 2 diabetes rather than a simple disease marker, and elevated circulating levels of MG53 represent the diminished function of β-cells (137).

The above evidence suggests that MG53 has important implications for glucose metabolic disorders although the relationship between MG53 and insulin resistance is controversial. However, current research remains insufficient to elucidate its underlying mechanisms. Additional robust evidence is needed to explain the mechanism underlying the involvement of MG53 in glucose metabolism and validate the safety of rhMG53 in patients with metabolic disorders and cancer.

## 4.2 MG53 inhibits myogenesis and promotes myocardial fibrosis

The potential adverse effects of MG53 on cancer cachexia must also be considered when applying rhMG53 in cancer therapy. Cancer cachexia, a common syndrome among patients with cancer, is characterized primarily by the loss of muscle tissue and inadequately relieved by nutritional means (138, 139). Changes in factors related to protein metabolism during cancer progression or treatment led to an imbalance between protein synthesis and degradation, resulting in muscle tissue reduction (140, 141). Reduced muscle mass in cancer cachexia is partly attributed to suppression of the anabolic signaling pathway induced by insulin-like growth factor1 (140). Cardiac atrophy and fibrosis in cancer cachexia are associated with the activated transforming growth factor-β (TGF-β)-mediated SMAD2/3 catabolic signaling pathway (142–145). MG53 inhibits the IGF-induced IRS-1/PI (3)K/Akt pathway, which is the best-characterized mechanism in cardiac and skeletal muscle myogenesis, through the ubiquitin-dependent degradation of IRS-1 (125, 139, 146, 147). Caveolin-1 plays an antifibrotic role in multiple organs and reduces cardiac fibrosis by repressing the TGF-β/Smad2 pathway (148, 149). MG53 can inhibit the expression of caveolin-1, thereby promoting TGF-β1/SMAD2-induced myocardial fibrosis (150). The activation of signal transducers and activator of transcription 3 (STAT3) has been implicated in promoting the progression of many cancers as well as exacerbating the loss of skeletal muscle tissue in cancer cachexia (151, 152). MG53 overexpression promotes the phosphorylation of

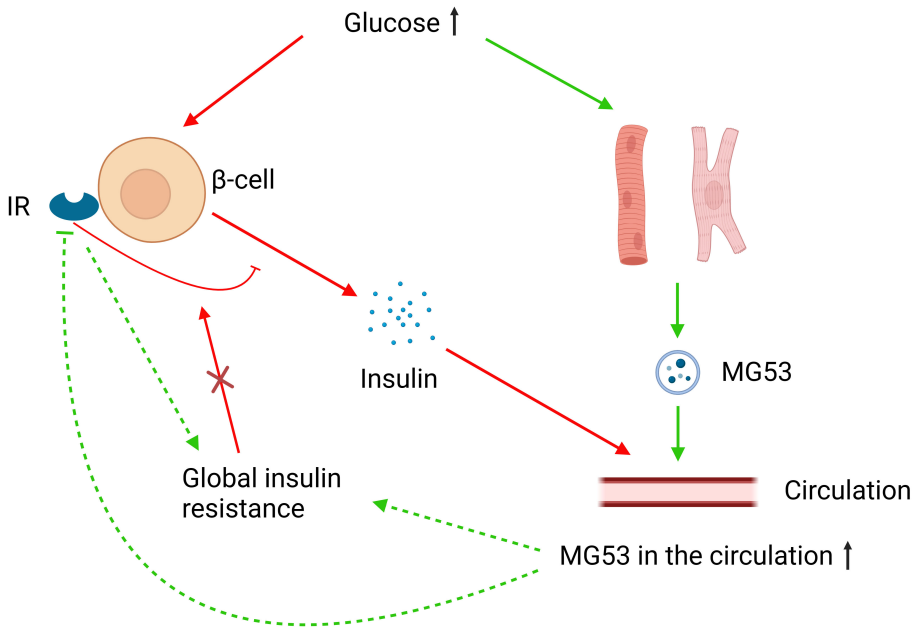


FIGURE 2
MG53 and glucose metabolism. Elevated glucose levels increase MG53 secretion from muscle tissue, and excess MG53 can lead to global insulin resistance through the inhibition of IRs or other pathways. High glucose also stimulates insulin secretion from pancreatic β-cells, where IRs play an inhibitory role. However, in the case of global insulin resistance, this inhibitory function of IRs fails. MG53 may have different effects on insulin secretion and therefore glucose metabolism in different severities of insulin resistance.

STAT3, which thereby induces cardiac fibrosis, and its effect on cardiac lesions in cancer cachexia remains to be investigated (153).

Current research strongly suggests that MG53 is an important cancer therapy target, despite its potentially negative effects. Future researches should be focused on elucidating the mechanism of MG53's role in cancer, glucose metabolism, and myogenesis, and on this basis, attempts should be made to retain the cancer therapeutic ability of MG53 while removing its side effects. MG53 mutants that retain membrane repair function without impairing glucose metabolism have been developed by eliminating the E3 ubiquitin ligase property of MG53 (128). In cancer therapy, however, the E3 ubiquitin ligase function of MG53 seems to play a crucial role. For the ubiquitination-dependent degradation of different proteins, MG53 may need to be activated at different sites, which could be the key to solving this problem (123, 128).

In summary, discussing the possible negative effects of MG53 on cancer treatment, especially in the context of varying degrees of insulin resistance and across gender, is urgently needed. If adverse effects are evident, developing safe mutants of MG53 may be a win-win approach.

## 5 Conclusions

The TRIM protein family has always been an important therapeutic target for cancer treatment, and in recent years, the role of MG53 in cancer has gradually been recognized. We found that almost all the evidence indicates that MG53 has a strong inhibitory effect on the progression of cancer and may serve as a biomarker for cancer. However, due to the lack of clinical research, the effect of MG53 on human cancer is actually undetermined. Furthermore, current research appears to overlook the contribution of the membrane repair function and anti-inflammatory properties of MG53 to cancer and does not discuss the potential adverse effects of MG53 on cancer treatment. Therefore, the safety of rhMG53 also needs further discussion. From the perspective of the biological functions of MG53, MG53 may still be a double-edged sword in cancer treatment and further research is needed to comprehensively investigate its role in cancer.

## Author contributions

YD: Writing – original draft, Writing – review & editing. TL: Writing – review & editing. MY: Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Population-enriched innate immune variants may identify candidate gene targets at the intersection of cancer and cardio-metabolic disease

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Both cancer and cardio-metabolic disease disparities exist among specific populations in the US. For example, African Americans experience the highest rates of breast and prostate cancer mortality and the highest incidence of obesity. Native and Hispanic Americans experience the highest rates of liver cancer mortality. At the same time, Pacific Islanders have the highest death rate attributed to type 2 diabetes (T2D), and Asian Americans experience the highest incidence of non-alcoholic fatty liver disease (NAFLD) and cancers induced by infectious agents. Notably, the pathologic progression of both cancer and cardio-metabolic diseases involves innate immunity and mechanisms of inflammation. Innate immunity in individuals is established through genetic inheritance and external stimuli to respond to environmental threats and stresses such as pathogen exposure. Further, individual genomes contain characteristic genetic markers associated with one or more geographic ancestries (ethnic groups), including protective innate immune genetic programming optimized for survival in their corresponding ancestral environment(s). This perspective explores evidence related to our working hypothesis that genetic variations in innate immune genes, particularly those that are commonly found but unevenly distributed between populations, are associated with disparities between populations in both cancer and cardio-metabolic diseases. Identifying conventional and unconventional innate immune genes that fit this profile may provide critical insights into the underlying mechanisms that connect these two families of complex diseases and offer novel targets for precision-based treatment of cancer and/or cardio-metabolic disease.

KEYWORDS

**innate immune variants, pleiotropic actions, cancer disparities, cardio-metabolic disparities, population-enriched variants, candidate protein targets**

# 1 Introduction

## 1.1 Double-edged swords: important factors connecting metabolic disorders and cancer development

The following perspective was written in response to an invited *Frontiers* research topic to explore methods, mechanisms, and hypotheses that may ultimately identify and exploit biological processes contributing to complex disease progression and molecular interactions enabling cross-talk between cancer and cardio-metabolic disease. Based on our hypothesis that innate immunity differences contribute to observed population disease disparities in cancer and metabolic disorders, we apply a functional genomics approach to identify specific innate immune genes as potential therapeutic targets at the intersection of these two complex disease families.

## 1.2 Framing precision drug target discovery in the context of health disparities

### 1.2.1 Defining health disparities

The US National Institute on Minority Health and Health Disparities (NIMHD) defines health disparities as "a health difference (compared with the general population), based on one or more health outcomes (such as the overall rate of disease incidence, prevalence, morbidity, mortality or survival) that adversely affect disadvantaged populations." In the US, such populations include Blacks/African Americans, Hispanics/Latinos, Asians, American Indians/Alaska Natives, and Native Hawaiians/other Pacific Islanders) (1). Diverse sources, from sponsored websites (such as 2 and associated links) to peer-reviewed articles summarizing disparities in one or more diseases between two or more populations, provide ample evidence for differences in cancer (3), cardio-metabolic disease (4) and overall health risks and outcomes (5) based on ethnic background/geographic ancestry. By way of illustration, Tables 1, 2 summarize disparities in cancer incidence and mortality among US ethnic populations (adapted from 6) and population differences in overall mortality rates of cancer and cardio-metabolic diseases (adapted from 7), respectively.

Assessing health differences between populations is complicated because results may vary depending on the size and granular composition of the populations being compared. On the one hand, evaluating larger, more heterogeneous populations improves statistical reliability, but this approach may mask disparities among subpopulations. For example, among Asians in the US (8) and Asia (9), the incidence of liver cancer varies widely based on geography and/or geographic ancestry. Further, trends in incidence and/or mortality may change due to cohort variations in age, exposure to risk, and geographic location, as is the case for liver (10) and breast cancer incidence (11) in the US and for global cancer mortality rates (e.g., 12).

Defining/distinguishing populations is a critical aspect of evaluating health disparities. Many analyses have been based on self-identified ethnicity; it stands to reason that this approach is likely to align more closely with social determinants of health. In contrast, a relatively precise biological assessment of geographic ancestry can be obtained using genetic markers to identify ethnic origins. In this approach, selected ancestry informative markers (AIMs) were initially used to evaluate genetic admixture and geographic ancestry and provide valuable background information when comparing individuals representing different populations (13). Improved methods and more extensive and complete reference datasets have further refined admixture mapping (14).

For the purposes of this perspective, we will refer to populations as they are defined by individual authors; populations in Section 3 are defined according to Karczewski (15). The interested reader is referred to a recent book chapter entitled "Using Population Descriptors in Genetics and Genomics Research: A New Framework for an Evolving Field" written by the National

TABLE 1 Ethnic Disparities in US Cancer Incidence and Mortality.

| | EA | AA | ASN/PI | NA/AN | HISP |
|---|---|---|---|---|---|
| **Cancer Incidence** | breast | prostate | *stomach* | colon<br>kidney<br>liver<br>lung<br>stomach<br>uterine | *uterine* |
| **Cancer Mortality** | lung | breast<br>colon<br>prostate<br>uterine | *stomach* | kidney<br>liver<br>stomach | *liver* |

adapted from "Table 9. Incidence and Mortality Rates for Selected Cancer by Race and Ethnicity, US" (6).
standard font indicates most frequently occuring cancer among aggregate populations; italics indicate most frequently occuring cancer for a specific ethnic group (not aggregate).
EA, European American, non-Hispanic White; AA, African American, non-Hispanic Black; ASN/PI, Asian American/Pacific Islander; NA/AN, Native American/Alaskan Native; HISP, Hispanic/Latino.

TABLE 2 Deaths from Cancer, Cardio-Metabolic, and Infectious Diseases in the US as of 2018.

| Cause of Death | Aggregate | EA | AA | NA/AN | ASN | PI | HISP |
|---|---|---|---|---|---|---|---|
| **heart disease** | 23.1% | 23.4% | **23.6%** | *18.0%* | 21.3% | 23.5% | 19.8% |
| **cancer** | 21.1% | 21.2% | 20.4% | *16.8%* | **25.1%** | 21.9% | 20.5% |
| **stroke** | 5.2% | 5.1% | 5.7% | *3.6%* | **7.6%** | 6.2% | 5.5% |
| **diabetes** | 3.0% | *2.5%* | 4.5% | 5.6% | 4.1% | **7.3%** | 4.6% |
| **infection (flu, pneumonia)** | 2.1% | 2.1% | *1.8%* | 2.3% | **3.3%** | 2.2% | 2.1% |
| **kidney disease** | 1.8% | *1.6%* | **2.8%** | 1.8% | 2.1% | 2.2% | 2.1% |
| **liver disease** | * | 1.4% | 1.0% | **6.2%** | *0.9%* | 1.3% | 3.2% |
| **hypertension** | * | *1.1%* | 1.9% | *1.1%* | **2.1%** | 1.4% | 1.4% |

adapted from Tables C & D in "Deaths: Leading Causes for 2018." Heron, M. National Vital Statistics Reports 70(4) (7).
bold indicates highest mortality rate for given cause of death.
italics indicates lowest mortality rate for given cause of death.
*aggregate data were only available for top ten causes of death.

Academies of Sciences' Committee on the Use of Race, Ethnicity, and Ancestry as Population Descriptors in Genomics Research (16) for a thorough treatment of this subject.

### 1.2.2 Past and present challenges to advancing research in the biology of health disparities

Cancer and cardio-metabolic disease disparities have multifactorial etiologies, including biological, behavioral, environmental, and social components. There is ample evidence that these disparate etiological factors are not adequately understood in isolation from one another. The interested reader is referred to reviews on the impact of physical, social, and chemical environments on the biology of health disparities (17–19) and on the biological impacts of stress (20), including racism-induced stress and increased allostatic load (21–23), all of which are beyond the scope of this perspective.

The relative contribution of biology to cancer and cardio-metabolic disparities continues to be a matter of debate among scientists in various disciplines and even among biologists themselves (24). The hesitation to consider geographic ancestral differences in biology among some mainstream biomedical scientists is just one of several obstacles that have hindered a rigorous study of the biology of health disparities.

Social forces continue to hinder the participation of minority populations in medical research and to limit their access to medical care. For example, an entrenched and well-founded mistrust of the medical establishment in the US exists among minority populations due to a long history of abuses (25). Limited access to healthcare and subpar healthcare quality further exacerbate health disparities in minority populations, leading to lower life expectancy in American Hispanic and Black populations (26).

Traditional research approaches and the most widely available resources in the biomedical sciences have also unintentionally hindered a rigorous characterization of the biological differences that underlie health disparities. *In vitro* studies employ samples and cell lines obtained most often from individuals of European descent (27, 28) and the majority of clinical trials disproportionately enroll individuals from this same population (29, 30). Thus, at multiple stages in the drug research and development cycle, biases exist towards agents optimized for those of European ancestry. Fortunately, the need to increase the diversity of human samples and cell lines and to engage diverse study populations in biomedical research and clinical trials has recently gained the attention and enthusiastic support of pre-eminent scientists (29, 31–33) and the NIH (34).

### 1.2.3 Considering geographic ancestry in the development of effective treatments

The human genome possesses a high degree of variation. According to a 2016 meta-analysis of 60,706 individuals of diverse ancestries, an average of 1 in 8 bases of the coding sequence were variants, and 72% of these had not been previously identified and/or characterized (35). Wide genetic variations within populations are at least as diverse as genetic variations between populations (36). This finding implies that not all genetic variations contribute to putative biological differences between populations.

Genetic differences associated with geographic ancestry, such as AIMs, may result in the uneven among populations distribution of gene variants. In many cases, these variants are uncommon, and/or their impact on protein expression, function, or disease is either insignificant or unknown. However, an intriguing study by Ahsan et al. (37) identified 65 "minor" drug response alleles that were present in more than 50% of individuals in at least one population; in other words, in some populations, the variant was more common than the wild type/canonical protein. Consistent with this is a body of clinical evidence that specific drug responses vary according to geographic ancestry, with outcomes that range from lack of efficacy to drug-related pathology and death in one or more minority populations (38–40). Therefore, we sought to identify population-specific potential therapeutic targets at the intersection of cancer and cardio-metabolic disease, in part by hand-curating gene variants with "minor" alleles that were common in at least one major population (as defined by 15) but that were significantly less common in at least one other major population.

## 1.3 Innate immunity as a biological driver of health disparities

Gene variants that confer protective immunity are retained in each population to optimize survival. For example, in the case of those with African ancestry, gene variants retained in the pan-African genome have been identified that provide defense against indigenous pathogens such as malaria and trypanosomiasis (African sleeping sickness/Chagas disease). The selective pressure imposed by pathogens on gene variation is impressive; in the case of malaria, variants of at least 40 different genes are thought to protect against one or more species of *Plasmodium* (41, 42).

Unfortunately, immune protection frequently involves a trade-off where protective innate immune variants may introduce new pathologies. For example, among the gene variants that protect against malaria, *HbS* also promotes sickle cell anemia, *HbE* promotes thalassemia, *G6PD* variants promote hemolytic anemia, and Duffy antigen receptor (*DARC*) variants are associated with increased breast cancer metastasis and mortality (43, 44). Similarly, the same *APOL1* variants shown to protect against severe trypanosomiasis are also associated with nephropathy (45, 46).

Several lines of evidence affirm that innate immune genes are highly adaptable and optimized to respond to local pathogens. First, within the human genome, genes associated with immunity are under the strongest selective pressure (47, 48). Second, selective pressure on immune genes is pathogen-driven (49, 50). Third, the geographic distribution of populations bearing the highest frequency of *HbS* (51) and *DARC* (52) gene variants closely resemble the geographic distribution of the malarial strains they protect against. Finally, according to their geographical ancestry, populations differ in their susceptibility to infectious disease (53), in their immune response to pathogens (54) and even in their macrophage function and circulating cytokine levels (55–57). All of these findings indicate that protective innate immune variants are distributed among individuals based on their geographic ancestry.

It is important to note that genes associated with innate immunity are structurally and functionally diverse. Some are well-characterized participants in inflammation, including but not limited to cytokines, chemokines, and pattern recognition receptors (lectins, Toll-like receptor (TLR) family members, and NLRs) and their related pathways. However, as illustrated by the variety of genes that protect against malaria (summarized in Table 3), others are pleiotropic, expressed in non-immune tissues and/or frequently better known for their "day jobs". Most of the protective variants listed in Table 3 can be tied directly to immunity. Still, a few (such as *APOE*, *G6PD*, glycophorin (*GYP*), hemoglobin (*HB*), and haptoglobulin (*HP*)) would be considered unconventional innate immune genes.

TABLE 3 Innate immune genes that provide protection against malaria (adapted from **41**, **42**).

| Gene | Name/Function | Expression | Association with Disease (based on titles available in Google Scholar) |
|---|---|---|---|
| *ABO* | ABO blood group | secreted | cancer, cardiovascular disease, diabetes, obesity, NAFLD |
| *APOE* | apolipoprotein E | secreted | cardiovascular disease, obesity, diabetes, NAFLD, cancer |
| *CD36*, thrombospondin receptor, scavenger receptor B3 | broad specificity receptor for proteins and lipids | adipose, liver, others | cardiometabolic disease, cancer |
| *CR1, CD35*, C3b/C4b complement receptor, Knops blood group antigen | | erythrocytes, leukocytes, glomerular podocytes, splenic DCs | gallbladder and liver cancer, diabetes, kidney disease |
| *DARC, FY, ACKR1, CD234, CCBP*1 | Duffy atypical chemokine receptor | erythrocytes, endothelia | breast cancer, prostate cancer, cardiometabolic disease |
| *FCGRA2, CD32* | low affinity Fc receptor | phagocytes | breast cancer, cardiovascular events |
| *G6PD* | glucose-6-phosphatase dehydrogenase rate-limiting step to pentose-phosphate, NADPH | lymphoblasts, granulocytes | cancer, diabetes, cardiovascular disease |
| *GYPA,B,C, CD235a,b,c* | glycophorin A,B,C sialoglycoproteins | A broad expression | |
| | | B,C erythrocytes | C leukemia, oral cancer |
| *HBA, HBB* | hemoglobin, O2/CO2 transport | erythrocytes | thalassemia, sickle cell anemia |
| *HLA-B* | component of MHC class I | broad expression | elimination of infected or transformed cells |

*(Continued)*

TABLE 3 Continued

| Gene | Name/Function | Expression | Association with Disease (based on titles available in Google Scholar) |
|---|---|---|---|
| *HLA-DR series (A,B1,3,4,5)* | components of MHC class II | antigen presenting cells | elimination of infected or transformed cells |
| *HP* | haptoglobulin, plasma protein that binds Hb | liver, others | diabetic nephropathy and coronary artery disease |
| *ICAM1, CD54* | intercellular adhesion molecule 1, receptor for CD11a or b/CD18 integrins and rhinovrius | immune and endothelial cells | cancer, diabetes, obesity |
| *IFNAR1,2* | interferon alpha (and beta) receptor, subunits 1 and 2 | broad expression | 1 gastric, colorectal, breast cancer; 2 lung cancer, diabetes |
| *IFNG* | interferon gamma | circulating | cancer, diabetes |
| *IFNGR1,2* | IFN gamma receptor 1 (CD119), 2 | broad expression | 1,2 cancer |
| *IL1A/IL1B* | interleukin 1A, 1B | circulating | A, B cancer, obesity; B diabetes |
| *IL1RN* | IL1 receptor antagonist | secreted | cardiovascular disease, cancer, obesity, diabetes |
| *IL10* | interleukin 10 | circulating | cancer, obesity, diabetes, atherosclerosis |
| *IL10RB* | IL10 receptor, subunit beta | broad expression | obesity |
| *IL12B* | interleukin 12, beta subunit | circulating | diabetes, cancer |
| *IL4* | interleukin 4 | circulating | cancer, diabetes |
| *IRF1* | interferon regulatory factor 1 | broad expression | cancer |
| *MBL2* | mannose binding lectin 2, collectin 1 | circulating | cancer, diabetes, atherosclerosis |
| *MST1, HGFL* | macrophage stimulating 1, hepatocyte growth factor like | secreted | cancer, diabetes, NAFLD, cardiovascular disease |
| *NCR3, CD337* | natural cytotoxicity triggering receptor 3 | NK cells | cancer |
| *NOS2A* | nitric oxide synthase 2 | liver, retina, bone, lung, cartilage, fat | cancer, diabetes |
| *PECAM1, CD31* | platelet-endothelial cell-adhesion molelcule 1 | immune and endothelial cells | cancer, cardiovascular disease, diabetes |
| *PSMB9* | proteosome 20S subunit beta 9 | MHC II expressing tissues | cancer, diabetes |
| *SCL4A1, CD233*, erythrocyte band 3 prot. | chloride/bicarbonate exchanger, Diego blood group | erythrocytes, kidney, bone, others | |
| | CO2 transport from tissues to lungs, structural protein | | cardiovascular disease, colorectal cancer |
| *SELE, CD62E* | selectin E | endothelia | cancer |
| *TCRB* | T-cell receptor, beta subunit | T-cells | diabetes, cancer |
| *TIRAP, MAL*, Myd88-2 | TIR domain containing adapter protein | broad expression | cancer, diabetes, NAFLD |
| *TL4* | Toll-like receptor 4 | broad expression | cancer, obesity, diabetes, NAFLD, cardiovascular |
| *TAP1, ABCB2* | transporter 1, ATP binding cassette, subfamily B | broad expression | cancer, diabetes |
| *TNF* | tumor necrosis factor | circulating | cancer, obesity, diabetes, NAFLD, cardiovascular disease |
| *TNFSF5* | CD40 ligand, CD154, TNF superfamily member 5 | circulating | diabetes, cancer, cardiovascular disease, obseity |

## 1.4 Inflammation is a component of cancer and cardio-metabolic diseases

### 1.4.1 Cancer and inflammation

Hanahan and Weinberg, in their seminal review, describe six hallmarks of cancer, many of which are enabled by mechanisms of immunity, including inflammation (58). Their observations are particularly relevant to this perspective since further research in the field has established that reprogrammed energy metabolism and immune evasion are additional hallmarks (58, 59).

In a previously published perspective, we presented evidence for an association between breast and prostate cancer disparities in African Americans (AAs) and classic innate immune gene variants (interleukins, Toll-like receptors, monocyte activity) more commonly found in AAs (60). Since 2019, Google Scholar (accessed 4/18/23) has listed more than 18,000 publications with titles that include "cancer" and "inflammation," "infection", "immune," "immunity," or "innate"; these publications address a wide range of topics, including immune escape by cancer cells, the contribution of chronic inflammation to tumor progression, and immune-based cancer therapies, that are beyond the scope of this perspective. Notably, less than 40 of these publications (< 0.2%) include the terms "disparity" or "disparities" in their titles. Among this small set of publications are descriptions of population differences in tumor microenvironment and immune signatures in breast (61, 62), head and neck (63–65), lung (66, 67), and colorectal (68, 69) cancers, as well as cancer generally (70). Of particular interest is a recent exploration of the link between racial differences in mitochondrial metabolism and the tumor immune microenvironment (71).

### 1.4.2 Cardio-metabolic disease and inflammation

The constellation of inter-related cardio-metabolic diseases has been collectively referred to as metabolic syndrome (MetS), and their cumulative effect on global health is massive (reviewed in 72–74). Clinical definitions of MetS vary depending on which disease(s) are of primary interest (reviewed in 75–77). The National Heart Lung and Blood Institute (NHLBI) lists the following MetS risk factors as abdominal obesity and/or insulin resistance, elevated triglycerides and LDL-cholesterol, reduced HDL-cholesterol, hypertension, elevated glucose and pro-thrombotic or pro-inflammatory states (78). Several metabolic diseases have been associated with these risk factors, including hypertension, obesity, atherosclerotic cardiovascular disease, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and stroke.

Genetic and environmental factors impact cardio-metabolic diseases, and their risk, morbidity, and mortality vary with age, gender, and race/ethnicity (4, 76). Unfortunately, the effects of MetS are not confined to cardio-metabolic co-morbidities, given that MetS is also associated with increases in the incidence and/or mortality of arthritis, chronic kidney disease, schizophrenia, depression and cancer, as noted in references (79, 80).

Inflammation is a key contributor to MetS and associated co-morbidities (81–83), just as MetS pathologies impact inflammation (c.f. 84). In general, low-grade chronic inflammation evoked during metabolic disease stimulates the production of pro-inflammatory cytokines, immuno-modulatory proteins, lipids, and other mediators of inflammation that impact systemic and/or localized tissue inflammation (82, 85). Unfortunately, the treatment of metabolic diseases is complicated by the cross-talk between pro- and anti-inflammatory mechanisms at work among MetS co-morbidities (c.f. 77, 86–88). Further, inflammation from one metabolic disease can also exacerbate other MetS co-morbidities.

As with almost all tissues, organs that regulate systemic metabolism possess innate immune response capabilities. Notably, some organs that regulate overall metabolic homeostasis also impact systemic inflammation. In the case of both adipose tissue (89–91) and liver (92–94), these organs harbor and partner with resident macrophages (ATMs and Kupffer cells, respectively) in inflammation. Further, adipose tissue and liver produce unique immunologically active biomolecules, such as adipokines (86, 95) and bile acids (96–99). Perhaps less appreciated are two additional organs associated with metabolic homeostasis that control systemic levels of immunologically active biomolecules: the gallbladder regulates bile acid levels and the pancreas controls insulin, which levels of insulin, with its known anti-inflammatory effects (100).

Just as mediators of metabolism can impact inflammation, mediators of immunity can impact metabolism. For example, innate immune receptors have demonstrated roles in metabolic disease progression (101), and pro-inflammatory cytokines produced in the adipose tissue of obese individuals contribute to the development of T2D (102). Significantly, biomolecules such as adipokines, insulin, and bile acids mediate metabolism and inflammation. Further, besides their widely recognized role in lipid transport and cellular metabolic homeostasis, serum lipids and lipoproteins also provide innate immune protection (103, 104).

# 2 A functional genomics approach to novel target discovery

Using functional genomics, we and others have observed associations between specific innate immune gene variants and cancer or metabolic disease risk or outcome that differ according to geographic ancestry (57, 60, 105). Given that immunity including inflammation contributes to the progression of both complex disease families, we have hypothesized that population differences in genetic (and epigenetic) innate immune programs contribute to complex disease disparities between populations. Based on this conceptual framework, this perspective seeks to identify innate immune gene candidates associated with both cancer and cardio-metabolic disease that differ between populations.

Genome wide association studies (GWAS) in general (106) and the Genome Aggregation Database (gnomAD) in particular (107) provide researchers with the capacity to compare thousands of complete genomes from individuals among all largely-grouped populations. These resources catalog gene variations called single nucleotide polymorphisms (SNPs) across the entire genome of each

individual. SNPs are located not only in protein coding genes (including coding exons as well as non-coding introns and remote, up-, down-, and mid-stream regulatory sites), but also across regions associated with short and long non-coding RNAs, chromosomal architecture, and other essential functions that have been previously underappreciated and mislabeled as "junk DNA" (108). The number of genes and the percentage of the human genome they occupy varies depending on their definition (109). Notably, most SNPs associated with disease states or changes in phenotype (95%) are located outside coding exons (110).

Nevertheless, in this perspective, we will focus on widely occurring gene variants that code for changes in the canonical amino acid (aa) sequence, also referred to as missense variants or nonsynonymous SNPs, as a first step towards accelerating the development of optimally safe and active drugs that target understudied protein variants widely found in patients with diverse geographical ancestries. Importantly, nonsynonymous SNPs have the potential to impact protein conformation, activity and/or protein-protein interactions, potentially altering disease states and phenotypes. For simplicity, we have also excluded synonymous SNPs (exonic point mutations that do not alter aa sequence), in spite of mounting evidence that suggests they can function in isoform selection (protein size and sequence), transcript expression levels and stability, translational folding rate, overall conformation, and posttranslational modifications, all of which possess potential functional consequences on cell behavior and disease risk (111–113).

This perspective identifies conventional and unconventional innate immune genes (summarized in Section 3) that meet the following criteria. First, there is evidence that each gene participates in, is a target of, or is associated with innate immunity including inflammation. Second, there is evidence that each gene is associated with at least one form of cancer and at least one cardio-metabolic disease. Finally, each gene occurs among the global population as at least one population-enriched variant, which we define as a widely occurring missense variant distributed unevenly among populations.

We have employed a hand-curated discovery process to identify population-specific innate immune genes at the intersection of cancer and metabolic disease. From the primary and secondary literature, gene lists associated with innate immunity (49, 114, 115), cancer (116, 117), or cardio-metabolic disease (118, 119) were vetted for the following characteristics:

1) Evidence in the primary or secondary literature (accessed through Google Scholar) indicated that the candidate gene was involved in all three disease categories: innate immunity/inflammation, cancer, and cardio-metabolic disease.
2) Indication in gnomAD that the candidate gene occurs as at least one nonsynonymous SNP/missense variant with
    a. a high minor allele frequency (MAF ≥ 0.2 in at least one of the six major populations defined by 15): African/African American (AFR/AA), East Asian (E ASN), non-Finnish European (EUR), Latino/Latina (LAT), Middle Eastern (MID E), and South Asian (S ASN),
    b. a difference in MAF among significant populations of ≥ 0.2 from the highest to lowest frequency.

Note that among genes with missense variants, we chose only those with common variants that occur widely among individuals in one or more populations, i.e., missense variants that occurred in at least 20% of individuals in one or more populations (by definition, having a minor allele frequency (MAF) ≥ 0.20 and varying widely in the frequency of their occurrence among populations. This approach was based on our rationale that variants selected and retained in the human genome provide a survival benefit for the population(s) in which they occur, even as they may also paradoxically contribute to complex disease as discussed above for *HbS* and *APOL1* variants (see Section 1.3).

# 3 Candidate innate immune genes at the intersection of cancer and cardio-metabolic disease disparities

Among the candidate innate immune genes that we identified at the intersection of cancer and cardio-metabolic disease, we found both "conventional" innate immune genes, such as cytokines and cytokine receptors, pattern recognition receptors, and other genes that have widely acknowledged roles in immune cell function, and "unconventional genes" with pleiotropic functions that include innate immunity, such apolipoproteins, biomolecule transporters, and transcription regulators. Using the approach described in Section 2, three lists of innate immune genes implicated in cancer and cardio-metabolic disease were generated. Each gene listed in the three tables below possesses at least one population-enriched variant with an amino acid replacement that differs in its distribution among populations, suggesting its potential role in both cancer and cardio-metabolic disparities. The 52 genes identified provide a representative but not exhaustive list of candidate genes, thus serving as preliminary data for further investigation.

Section 3.1 summarizes conventional innate immune genes and their corresponding population-enriched variants previously shown to impact disease or biological function. Similarly, Section 3.2 summarizes unconventional innate immune genes (better known for their non-immune functions) and their corresponding population-enriched variants that have been previously shown to impact disease or biological function. Finally, Section 3.3 summarizes genes associated with innate immunity, cancer, and cardio-metabolic diseases and their corresponding population-enriched variants whose impact on disease or biological function has not yet been established.

## 3.1 Conventional innate immune genes with previously characterized population-enriched variants

Table 4 includes 14 genes best known for their roles in immunity, including inflammation, that are present as at least one population-enriched variant shown to impact biological

function. Among these are cytokines and cytokine receptors, including macrophage inhibitory cytokine 1 (*MIC-1/GDF15*), interleukin 3 and the alpha subunit of its receptor (*IL3* and *IL3RA*), along with subunits for interleukin 4, 6 and 7 receptors (*IL4R, IL6R*, and *IL7R*), and the leptin adipokine receptor (*LEPR*). Additional immune receptors include the soluble receptor for MHC I antigens I (leukocyte Ig-like receptor A3, *LILRA3/CD85E*) and two pattern recognition receptors, the intracellular pattern recognition receptor nucleotide-binding oligomerization domain containing 2 (*NOD2*) and the five transmembrane stimulator of interferon response CGAMP interactor 1 (*STING1/TMEM173*). Also included were the catalytic enzyme in the rate-limiting step of the kynurenine pathway during inflammation indoleamine 2,3-dioxygenase 2 (*IDO2*), the temperature-sensitive cation channel *TRPM8*, and two adhesion molecules, one expressed in lymphocytes (integrin alpha L, *ITGAL/LFA-1/CD11A*) and the other expressed in leukocytes (junctional adhesion molecule-like, *JAML/AMICA*).

### 3.1.1 Interleukin 3 and interleukin 3 receptor alpha chain

IL-3 is a growth factor produced by activated T-cells (129) that regulates the growth of hematopoietic progenitor cells and activates mature neutrophils and macrophages (208). IL-3 is also implicated in priming (131) and activating (130) basophils. Intriguingly, increased serum levels of IL-3 have recently been associated with the onset of type 2 diabetes in African American women as determined by serum levels of glucose and HbA1c (133). Genetic variations in *IL3* have been noted in colon and rectal cancers (132). The Pro27Ser variant (5-132060785-C-T) has been associated with protection against malaria (134) but also with an increase in miscarriages following *in vitro* fertilization (IVF) in women of various populations (209).

The interleukin 3 receptor is a heterodimer comprised of an interleukin 3-specific alpha chain (IL-3RA, CD123) and the common cytokine beta chain CSF2RB, another candidate listed below in Section 3.3, that also forms dimers with the alpha chains of both GM-CSF and IL-5 receptors. High-affinity IL-3 binding induces hetero-dimerization of IL-3RA and CSF2RB, and subsequent disulfide linkage of these receptor chains is required for receptor activation and CSF2RB phosphorylation (210). IL-3RA expression varies among CD34+ hematopoietic cell types, with negative/low expression in primitive hematopoietic cells and little or no surface expression in early erythroid progenitors, but high expression in B-lymphoid and myeloid progenitors (135). The X-chromosome-linked *IL3RA* Val323Leu variant (X-1378751-G-C) was associated with non-complete response to neoadjuvant chemotherapy against locally advanced rectal cancer in Hong Kong patients (138).

### 3.1.2 Interleukin 4 receptor alpha chain

The IL-4R alpha chain (IL4R, CD124) forms heterodimers with at least two partners. Type 1 IL-4 receptors are composed of IL-4R complexed with the common cytokine receptor gamma chain (IL2RG, CD132), which may alternatively dimerize with IL-2, IL-7 and IL-21 cytokine receptors, so that IL-2, IL-7, and IL-21 receptors compete with IL-4R for binding to IL2RG. Type 2 IL-4 receptors are composed of IL-4R complexed with IL-13RA1 (IL13Rα1, CD213A1). Thus, IL-4 activates both Type 1 and Type 2 IL-4 receptors, while IL-13 activates Type 2 IL-4 receptors. Both IL-4 and IL-13 signaling through the IL-4R mediate type 2 (humoral, as opposed to type 1 cellular) immunity against helminths, toxins and tropical parasites such as plasmodium (malaria) and trypanosomes (African sleeping sickness/Chagas disease) (139–141, 211). Both IL-4Rα and IL13-Rα1 have also been implicated in cancer progression and were recently identified as prognostic indicators in soft-tissue sarcoma patients when present in the nucleus. IL-4 regulates lipid metabolism (143), and (142) recent findings highlight an intriguing relationship between non-hematopoietic IL-4Rα activation of a non-canonical signaling pathway that regulates a high-fat, high-carbohydrate diet-driven induction of obesity and impacts the severity of obesity-associated sequelae in mice (212). Numerous genetic epidemiological studies have also shown that *IL4* and *IL4R* and their gene polymorphisms play important roles in asthma in various populations. Notably, individuals carrying one or two copies of the *IL4R* Glu400Ala (16-27362551-A-C) minor allele were at higher risk to suffer from allergy (145) and asthma (144, 213).

### 3.1.3 Interleukin 7 receptor alpha chain

The integral membrane interleukin 7 receptor (IL-7R) transmits pro-inflammatory signals initiated by IL-7 at the cell surface. The functional IL-7 receptor is a heterodimer comprised of the IL-7 receptor alpha chain (IL7R, IL7Rα, CD127) and the same common cytokine receptor gamma chain (IL2RG, CD132) that dimerizes with the IL-4R alpha chain. The assembled IL-7R recognizes not only IL-7 but also thymic stromal lymphopoietin (TSLP), both cytokines with 4 α-helical strands (214). Multiple transcriptional and post-transcriptional mechanisms exist to regulate expression of the IL-7R protein (215). Some of these mechanisms are homeostatic, molecular and cytokine-mediated, where *IL7Rα* transcription decreases in $CD4^+$ and $CD8^+$ cells once naïve T cells become activated. Notably, IL-7 binding to IL-7R activates the Janus kinase (JAK/STAT) pathway, which plays an essential role in lipid metabolism (216). However, peripheral blood mononuclear cells (PBMCs) in breast cancer patients show defects in STAT5 phosphorylation and altered expression of IL-7Rα that ultimately impacts memory T cell development (156).

Notably, compared to the canonical gene, the *IL7R* variants 5-35874473-C-T (rs6897932), 5-35860966-T-C (rs1494558) and 5-35871088-G-A (rs1494555) alter the pathology of autoimmune and infectious diseases due to their impact on IL7R expression and alternative splicing (155). Further, all three population-enriched missense variants of *IL7R* identified in Table 4 show an association with cardio-metabolic disease: Ile66Thr (5-35860966-T-C, rs1494558) with post-transplantation diabetes (158); Val138Ile (5-35871088-G-A, rs1494555) with body mass index (BMI) in lymphoma patients (161), and Ile356Val (5-35876172-A-G, rs3194051) with severe liver disease (162). However, to date only Val138Ile has been associated with increased cancer risk, both in lung (160) and stomach (159).

TABLE 4 Candidate Conventional Innate Immune Genes at the Intersection of Cancer and Cardio-Metabolic Disease.

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***GDF15*** | | growth differentiation factor 15, macrophage inhibitory cytokine 1 | | induced by HCV | 120 | regulates hepatocellular carcinoma genes | 120 | stress, metabolic and cardiovascular disease | 121 |
| | | MIC-1 | | mediates tissue tolerance | 122 | pro- and anti-tumor activity | 121 | deficiency protects against atherosclerosis | 123 |
| 19-18386331-T-A (rs1059369) | 2 (2) | Ser48Thr | 0.38 E ASN to 0.14 MID E | systemic lupus erythematosus (SLE) risk in Chinese population | 124 | | | | |
| ***IDO2*** | | indoleamine 2,3-dioxygenase 2 | | immunomodulator | 125 | multiple cancers | 126 | NAFLD | 118 |
| 8-39982715-A-G (rs4736794) | 3 (2) | Ile140Val (2 of 4 transcripts) | E ASN 0.34 to 0.03 AFR/AA | major depressive disorder symptoms | 127 | | | | |
| 8-40005362-C-T (rs10109853) | 3 (2) | Arg248Trp (2 of 4 transcripts) | S ASN 0.54 to 0.25 E ASN | | | multiple myeloma risk in a small Japanese cohort | 128 | | |
| ***IL3*** | | interleukin 3 | | hematopoietic growth factor, mast-cell growth factor, multipotential colony stimulating factor | 129 130 131 | colon cancer risk | 132 | T2D in obese AA women | 133 |
| 5-132060785-C-T (rs40401) | 1 (1) | Pro27Ser | AFR/AA 0.53 to 0.22 EUR | protection against malaria | 134 | | | | |
| ***IL3RA*** | | interleukin 3 receptor, CD123 | | production and differentiation of hematopoietic progenitor cells | 135 | leukemia | 136, 137 | ligand IL3 implicated in T2D in obese AA women | 133 |
| X-1378751-G-C (rs17883366) | 2 (2) | Val323Leu | MID E 0.26 to 0.06 AFR/AA | | | colorectal cancer treatment response | 138 | | |
| ***IL4R*** | | interleukin 4 receptor, CD124, IL4RA, IL13 receptor | | ligand IL4 provides protection against malaria, schistosomiasis and helminths | 139–141 | IL4R overexpressed on the surface of multiple cancer types (breast, lung, etc.) | reviewed in 142 | IL-4 dysregulation caused decreased lipid metabolism, decreased lipolysis and increased adipogenesis leading to diseases such as obesity and Type 2 Diabetes | 143 |

*(Continued)*





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TABLE 4 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 16-27362551-A-C (rs1805011) | 5 (3) | Glu400Ala | 0.53 AFR/AA to 0.06 S ASN | allergy, asthma | 144, 145 | lung cancer response to radiation | 146 | type I diabetes? | yes: 147<br>no: 148, 149 |
| ***IL6R*** | | IL6 receptor, CD126, Gp80 | | receptor for pleiotropic cytokine IL6 | 150 | lung and other cancers | 151, 152 | cardiometabolic disease | 153 |
| 1-154454494-A-C (rs2228145) | 3 (2) | Asp358Ala | 0.49 LAT to 0.14 AFR/AA | | | liver cancer | 154 | | |
| ***IL7R*** | | interleukin 7 receptor, CD127, IL7RA | | variants involved in autoimmunity and infectious disease | 155 | reduced in breast cancer | 156 | type I diabetes | 157 |
| 5-35860966-T-C (rs1494558) | 5 (4) | Ile66Thr | 0.75 AFR/AA to 0.42 E ASN | | | | | post-transplantation diabetes | 158 |
| 5-35871088-G-A (rs1494555) | 3 (3) | Val138Ile | 0.87 AFR/AA to 0.48 E ASN | | | gastic cancer in EUR, increase lung cancer | 159, 160 | BMI in lymphoma patients | 161 |
| 5-35876172-A-G (rs3194051) | 3 (1) | Ile356Val | 0.34 AFR/AA to 0.07 E ASN | | | | | severe liver disease | 162 |
| ***ITGAL*** | | integrin alpha L, LFA-1, CD11A | | lymphocyte function associated antigen | | renal cancer, gastric cancer prognostic marker | 163, 164 | bioinformatic assn w aortic valve calcification in metabolic syndrome | 165 |
| 16-30506720-G-C (rs2230433) | 6 (3) | Arg791Thr | 0.66 S ASN to 0.14 E ASN | | | protection against renal cell carcinoma | 166 | | |
| | | | | | | risk of IDC breast carcinoma in Han women | 167 | | |
| ***JAML*** | | junctional adhesion molecule-like, AMICA | | regulates inflammatory cell migration | 168, 169 | lung cancer | 151, 170 | diabetic nephropathy | 171, 172 |

*(Continued)*





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TABLE 4 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 11-118198037-T-C (rs2298831) | 8 (5) | Ile322Met | 0.36 AFR/AA to 0.07 S ASN | steroid interaction with Duchenne muscular dystrophy in a multi-center European cohort (n=301 cases) | 173 | | | | |
| ***LEPR*** | | leptin receptor, CD295, OBR | | required for lymphopoiesis | | | | regulation of fat metabolism, obesity | 174, 175 |
| | | | | leptin (ligand) produced by lymphocytes, NK cells, monocytes | 176 | susceptibility to HBV induced hepatocellular carcinoma | 177 | NAFLD | 86 |
| 1-65570758-A-G (rs1137100) | 7 (6) | Lys109Arg | 0.81 E ASN to 0.10 MID E | | | colorectal cancer risk | 178 | early atherosclerosis | 179 |
| 1-65592830-A-G (rs1137101) | 7 (6) | Gln223Arg | 0.88 E ASN to 0.34 MID E | | | | | obesity in Pacific Islanders | 180 |
| ***LILRA3*** | | leukocyte Ig-like receptor A3, CD85E | | soluble receptor for MHC I antigens | 181 | benign prostatic risk hyperplasia | 182 | elevated plasma HDL | 183 |
| | | | | | 184 | lymphomagenesis risk | 185 | downregulated in obesity, metabolic syndrome | 186 |
| 19-54803504-A-C (rs6509862)* | 3 (3) | Leu107Arg | 0.79 AFR/AA to 0.12 EUR | statin intolerance | 187 | | | | |
| ***NOD2*** | | nucleotide binding oligomerization domain containing 2, CARD15, NLRC2, BLAU, IBD1 | | immune response, inflammation | 188 | triple negative breast cancer, therapeutic target | 114, 189 | deficiency promotes diabetes and NAFLD in mice | 190, 191 |
| 16-50710713-C-T (rs2066842) | 7 (4) | Pro241Ser | 0.28 MID E to 0.01 E ASN | | | assn with follicular lymphoma survival | 192 | | |
| ***TMEM173*** | | STING1, stimulator of interferon genes, MPYS | | activates IFN innate immune response genes | 193, 194 | multiple cancers | 193, 195 | cardiovascular and metabolic disease | 196 |

*(Continued)*





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TABLE 4 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 5-139477397-C-T (rs7380824) | 17 (4) | Arg293Gln | 0.41 E ASN to 0.14 EUR | LOF, decreased response to bacterial ligands, poxviruses | 197–199 | | | | |
| 5-139478340-C-G (rs78233829) | 18 (4) | Gly230Ala | 0.41 E ASN to 0.14 EUR | altered c-di-GMP lid conformation | 198 | | | | |
| 5-139481493-C-T (rs11554776) | 16 (5) | Arg71His | 0.41 E ASN to 0.03 AFR/AA | large effect on loss of function | 197, 198 | | | | |
| ***TRPM8*** | | transient receptor potential cation channel | | immune response, inflammation, temperature regulation | 200, 201 | multiple cancers | 202, 203 | obesity, blood pressure | 204, 205 |
| 2-233955144-G-A (rs7593557) | 4 (2) | Ser419Asn | 0.55 AFR/AA to 0.05 EUR | cold-induced hyperresponsiveness in bronchial asthma | 206 | | | blood lipid profile, BMI in Russian population | 207 |

Genes listed have been associated with innate immunity/inflammation, cancer, and cardio-metabolic disease and have at least one variant in the human genome that occurs in at least 20% (Minor Allele Frequency (MAF) ≥ 0.2) of one or more populations. Missense variants are described by their location in the GRCh38 reference genome (accessed from gnomAD v3.1.2), rs number (reference SNP cluster ID), and amino acid location numbers and identities of the original and coded replacement. Populations are defined by Karczewski 2020 (15): African/African American (AFR/AA), East Asian (E ASN), non-Finnish European (EUR), Latino/Latina (LAT), Middle Eastern (MID E), and South Asian (S ASN). The number of affected transcripts listed include total transcripts (first number) and transcripts with missense mutations (in parentheses) that contain the gene variant, but do not include transcripts of any overlapping genes.





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## 3.2 Unconventional innate immune genes with previously characterized population-enriched variants

Table 5 includes 18 genes representing several classes of proteins primarily associated with non-immune functions that occur as population-enriched variants shown to impact biological function. These genes include transport membrane proteins, consisting of the multidrug resistance pump (*ABCB1*), the Niemann-Pick cholesterol transporter 1 (*NPC1, SLC65A1*), and the Na+-dependent multivitamin transporter (*SLC5A6*). Among the class of regulatory metabolic enzymes are alcohol dehydrogenase (*ADH1C*), mitochondrial dihydroorotate dehydrogenase (*DHODH*), hydroxysteroid (17-beta) dehydrogenase 4 (*HSD17B4*) involved in peroxisomal fatty acid beta-oxidation, and glycogen phosphorylase B (*PYGB*) involved in regulating glycogen mobilization. Among the genes that participate in signal transduction are the membrane glycoprotein signaling co-receptor neuregulin (*NRG1*), phosphodiesterase 10A (*PDE10A*, which regulates cAMP concentrations), along with the small bioactive neuropeptide neuromedin B (*NMB*). Transcription factors and/or nucleic acid binding protein genes coded as population-enriched variants include hypoxia-inducible factor 2A (*EPAS1, HIF2A*), Iroquois homeobox 2 (*IRX2*), mismatch repair MutL homolog 3 (*MLH3*), the novel intracellular and extracellular ribonuclease T2 (*RNASET2*) and the SURP and G-Patch domain containing 1 (*SUGP1*) splicing factor. Also included are the lipid transport protein apolipoprotein B (*APOB*), the triacylglycerol lipase patatin-like phospholipase domain containing 3 (*PNPLA3*), and the adhesion cadherin family member desmoglein 2 (*DSG2*).

### 3.2.1 Multidrug resistance gene

The ATP binding cassette subfamily B member 1 (*ABCB1*) gene is commonly known as the first of two multidrug resistance (*MDR1*) genes in humans and is one of 48 ABC family members (217). ABCB1 functions at the plasma membrane as a 170 kDa monomer with 12 transmembrane domains (TMs), is glycosylated on the first extracellular loop (between TM1 and TM2), and has two intracellular ATP binding sites (one located between TMs 6 and 7, and the other in the carboxy terminus downstream of TM12). ABCB1 is expressed in a wide range of tissues (such as intestine, colon, placenta, liver, and blood-brain barrier) to protect against the intracellular build-up of xenobiotic molecules in vulnerable cells and organs by expelling toxins, including chemotherapeutics, from the cell interior. Thus, ABCB1 has become a widely-known source of and marker for chemoresistance (c.f. 219). ABCB1 also functions as a broad specificity lipid translocase (326). In a Chinese cohort, a variant in the ABCB1 promoter showed pleiotropic effects related to T2D and lipid metabolism (221). Notably, the *ABCB1* Ser893Ala variant (7-87531302-A-C, rs2032582) has been correlated with obesity in a Japanese population (220) and with increased susceptibility to lung cancer in a Spanish cohort (223). This *ABCB1* variant occurs in 91% of Africans/African Americans, but in only 35-62% of other populations (gnomAD) and was shown to impact drug (etanercept) efficacy in the treatment of Chinese Han patients with ankylosing spondylitis (222).

### 3.2.2 Mismatch repair protein MutL homolog 3

MLH3 is a homolog of the mismatch repair protein MutL. DNA mismatch repair (MMR) proteins play a vital role in maintaining genome integrity and in antibody maturation during class switch DNA recombination and somatic hypermutation (276). In cases of microsatellite instability, tumors often display somatic mutations in MLH3, while hereditary nonpolyposis colorectal cancer type 7 (HNPCC7) has been associated with germline mutations in the same gene (276, 327). Further, reduced MLH3 expression was observed in individuals diagnosed with grade II and III breast cancer, suggesting MLH3 may serve as a reliable susceptibility marker (278, 328). There was no correlation between the *MLH3* Pro844Leu variant (14-75047125-G-A, rs175080, predominantly found in the Middle East) and susceptibility to colorectal cancer in a predominantly white cohort (279). However, in Chinese patients this variant was associated with both cervical cancer (280) and hepatocellular carcinoma (281).

### 3.2.3 Apolipoprotein B

Lipoproteins enclose otherwise insoluble lipid particles (made up of a central core of cholesterol esters and triglycerides and an outer layer of phospholipids, free cholesterol, and apolipoproteins) for transport through the blood to various tissues (329). Apolipoprotein B (APOB) serves as the primary carrier for several classes of serum lipid particles, including chylomicrons, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), intermediate-density lipoprotein, and lipoprotein. In LDL particles, APOB interacts with the apoB/E (LDL) receptor, facilitating the removal of LDL cholesterol from the circulation via cellular uptake followed by intracellular LDL breakdown. In a small Japanese study correlating variants of genes related to lipid regulation (including apolipoproteins), the population-enriched missense *APOB* variant 2-21002409-C-T (rs1042034) correlated with HCV infection (235) variant has an allele frequency of 0.85 in African American populations but only 0.26 in East Asian populations (gnomAD). Another population-enriched missense *APOB* variant, 2-21008652-G-A (rs676210) (present in 73% of East Asians vs. 15% of Africans/African Americans (gnomAD)) correlated with the occurrence of initial non-cardioembolic ischemic stroke in a small European cohort (239). A third population-enriched missense *APOB* variant, 2-21028042-G-A (rs679899) (present in 85% of East Asians vs. 17% of Africans/African Americans (gnomAD)) and was protective against acute coronary syndrome in a Mexican population (238). This was associated with both hypertension and chronic kidney disease in a cohort of 3696 Japanese individuals (240).

Functional effects of additional *APOB* missense variants have also been reported. The Arg3638Gln variant (2-21005955-C-T, rs1801701), which is present in no more than 10% of any population, was associated with survival outcomes in non-small cell lung cancer (NSCLC) patients (236). Additionally, two

TABLE 5 Candidate Unconventional Innate Immune Genes at the Intersection of Cancer and Cardio-Metabolic Disease.

| Gene (SNP) | Affected Transcripts | Name/Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***ABCB1*** | | MDR1, P-glycoprotein 1, ATP binding cassette B1 | | multidrug resistance, xenobiotic protection | 217 | gallbladder carcinoma, drug resistance | 218, 219 | Japanese obesity, diabetes and serum lipids in Chinese | 220, 221 |
| 7-87531302-A-C (rs2032582) | 5 (3) | Ser893Ala | 0.91 AFR/AA to 0.35 S ASN | drug efficacy | 222 | increased lung cancer risk | 223 | | |
| ***ADH1C*** | | alcohol dehydrogenase 1C (class I), gamma | | downregulated during inflammation in ulcerative colitis, | 224 | liver cancer | 225, 226 | endogenous substrate bile acids involved in lipid, glucose and energy metabolism and impact metabolic syndrome | 227, 228 |
| | | | | increased expression reduces IL-6 and IL-8 secretion | 224 | colorectal cancer | 229 | | |
| | | | | substrates (estrogen, bile acids) impact innate immunity | 96, 230 | lung cancer | 231, 232 | | |
| 4-99339632-T-C (rs698) | 1 (1) | Ile350Val | 0.52 EUR to 0.08 E ASN | | | increased cancer risk in Africans and Asians | 233 | | |
| 4-99342808-C-T (rs1693482) | | Arg272Gln | 0.52 EUR to 0.08 E ASN | | | Japanese upper aerodigestive tract cancer | 234 | | |
| ***APOB*** | | apolipoprotein B | | HCV infection | 235 | variants associated with NSCLC survival | 236 | variants in Asian population associated with metabolic syndrome | 237 |
| 2-21002409-C-T (rs1042034) | 1 (1) | Ser4338Asn | 0.85 AFR/AA to 0.26 E ASN | HCV infection | 235 | | | protective against acute coronary syndrome in Mexican population | 238 |
| 2-21008652-G-A (rs676210) | 1 (1) | Pro2739Leu | 0.73 E ASN to 0.15 AFR/AA | | | | | stroke | 239 |
| 2-21028042-G-A (rs679899) | 3 (2) | Ala618Val | 0.85 E ASN to 0.17 AFR/AA | | | | | chronic kidney disease risk among Japanese with hyptertension | 240 |
| ***DHODH*** | | dihydroorotate dehydrogenase | | defense against bacteria, viruses and protozoa | 241–243 | multiple cancers, pro-inflammatory ferroptosis | 244, 245 | glucose metabolism, insulin resistance | 246, 247 |
| 16-72008783-A-C (rs3213422) | 4 (4) | Lys7Gln | 0.75 E ASN to 0.34 MID E | rheumatoid arthritis drug response | 248–250 | | | | |

*(Continued)*





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TABLE 5 Continued

| Gene (SNP) | Affected Transcripts | Name/Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***DSG2*** | | desmoglein 2 | | receptor for selected adenovirus serotypes | 251 | multiple cancers | 252 | pancreatic islet function, insulin resistance | 253, 254 |
| 18-31542836-G-A (rs2278792) | 1 (1) | Arg773Lys | 0.48 E ASN to 0.08 AFR/AA | | | | | cardiomyopathy in Yi population | 255 |
| ***EPAS1*** | | endothelial PAS domain protein 1, HIF2A, hypoxia-inducible factor 2A | | IL31 induction in CD4+ T cells | 256 | non-small cell lung cancer, colorectal, others | 257, 258 | dyslipidemia and NAFLD | 259 |
| 2-46382433-A-C (rs59901247) | 2 (1) | Thr766Pro | 0.41 AFR/AA to 0.01 S ASN | N-acetylaspartate levels in elite athletes | 260 | | | | |
| ***HSD17B4*** | | hydroxysteroid (17-beta) dehydrogenase 4, DBP, MFE-2, MPF-2, SDR8C1 | | peroxisomal multifunctional protein (detox) | 261 | overexpressed in prostate cancer | 262 | peroxisomal fatty acid oxidation | 263 |
| | | | | | | downregulated in non-small cell lung cancer | 264 | lipid and bile acid metabolism | 265 |
| 5-119475838-G-A (rs25640) | 18 (7) | Arg131His, Arg106Pro or His | LAT 0.56 to AFR/AA 0.17 | homozygous D-bifunctional peroxisomal protein disease | 266 | | | | |
| 5-119525243-T-C (rs11539471) | 20 (7) | Trp536Arg | AFR/AA 0.3 to 0.00 E ASN | | | protective against endometrial cancer | 267 | | |
| 5-119526018-A-G (rs11205) | 21 (7) | Ile584Val | LAT 0.53 to 0.29 S ASN | | | testicular germ cell tumor risk | 268 | | |
| ***IRX2*** | | Iroquois Homeobox 2 | | mediates expression of immune regulators MMP9 and VEGF | 269, 270 | sarcomas, breast, leukemia | 271, reviewed in 272 | VEGF altered in ischemic stroke and atherosclerosis | reviewed in 273 |
| | | | | | | nasopharyngeal cancer marker | 274 | | |
| 5-2748943-C-A (rs76906087) | 2 (1) | Glu255Asp | 0.28 S ASN to 0.03 AFR/AA | | | | | present in 5/10 Indian congenital heart defects | 275 |
| ***MLH3*** | | mutL homolog protein 3, mismatch repair, HNPCC7 | | Ig class switch | 276 | colorectal cancer microsatellite instability | 277 | mutations only found in breast cancer patients with metabolic disease | 278 |

*(Continued)*





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TABLE 5 Continued

| Gene (SNP) | Affected Transcripts | Name/Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 14-75047125-G-A (rs175080) | 4 (3) | Pro844Leu | 0.52 MID E to 0.15 E ASN | | | no assn w CRC in white population | 279 | | |
| | | | | | | susceptibility to cervical cancer in Chinese | 280 | | |
| | | | | | | hepatocellular carcinoma in Han | 281 | | |
| ***NMB*** | | neuromedin B | | innate immune response to influenza A virus | 282 | cervical and other cancers | 283 | highly expressed in adipose, variants related to obesity | 284 |
| 15-84657289-G-T (rs1051168) | 2 (2) | Pro73Ala | 0.37 MID E to 0.05 AFR/AA | | | | | obesity | 284 |
| ***NPC1*** | | Niemann Pick cholesterol transporter, SLC65A1 | | NKT cell development | 285 | breast cancer | 286 | obesity | 287, 288 |
| | | | | endosomal entry receptor for ebolavirus | 289 | | | type 2 diabetes | 290 |
| 18-23540480-T-C (rs1805082) | 3 (2) | Ile858Val | 0.63 E ASN to 0.30 MID E | | | | | obesity | 291 |
| 18-23560468-T-C (rs1805081) | 2 (1) | His215Arg | 0.41 EUR to 0.08 AFR/AA | | | | | cardiovascular disease (Iranian) | 292 |
| ***NRG1*** | | neuregulin | | macrophage response to yeast | 293 | NRG1 gene fusions drive multiple solid tumors | 294 | regulates insulin sensitivity | 295 |
| 8-32595840-G-A (rs3924999) | 21 (17) | Arg30Gln | 0.77 E ASN to 0.11 AFR/AA | susceptibility to schizophrenia in Chinese Han | 296 | | | | |
| | | | | Fin susceptilibity to reward dependence in major depression | 297 | | | | |
| ***PDE10A*** | | phosphodiesterase 10A | | mediator of lung and vascular inflammation | 298, 299 | ovarian cancer target | 300 | diabetes, diet-induced obesity, insulin sensitivity | 301 |
| 6-165654841-C-G (rs880121) | 10 (2) | Glu15Asp | 0.63 MID E to 0.04 E ASN | sporadic Parkinson's in Chinese Han | 302 | | | | |

*(Continued)*





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TABLE 5 Continued

| Gene (SNP) | Affected Transcripts | Name/Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***PNPLA3*** | | Patatin Like Phospholipase Domain Containing 3, adipnutrin | | platelet and monocyte levels | 303 | hepatic cancer (Europeans, Han Chinese) | 304, 305 | NAFLD | 118, 303 |
| 22-43928847-C-G (rs738409) | 4 (2) | Ile148Met (2 of 4 transcripts) | 0.42 LAT to 0.14 AFR/AA | | | hepatic cancer | 304, 305 | NAFLD | 303 |
| ***PYGB*** | | glycogen phosphorylase B | | TCR activation stimulates PYGB-dependent glycogenolysis | 306 | prostate, gastric, non-small cell lung cancers | 307–309 | carbohydrate metabolism | 310 |
| 20-25278370-G-T (rs2228976) | 1 (1) | Ala303Ser (1 transcript) | 0.34 E ASN to 0.08 AFR/AA | | | present in European desmoid tumors in familiall adenomatous polyposis (FAP) | 311 | | |
| ***RNASET2*** | | ribonuclease T2, RNASE6PL | | degrades microbial RNAs for recognition by TRL8 | 312, 313 | tumor suppressor in lung and ovarian cancers | 151, 313 | myocardial lipotoxicity in obesity | 314 |
| 6-166938616-C-A (rs3777722) | 14 (1) | Arg226Met | 0.4 E ASN to 0.04 AFR/AA | putative association with preterm birth | 315 | | | | |
| ***SLC5A6*** | | Na+ dependent multivitamin transporter | | anti-inflammatory in murine gut | 316 | gastric cancer | 317 | lymphocyte metabolic programming | 318 |
| | | | | B lymphocyte maturation | 318 | | | | |
| 2-27201768-G-A (rs1395) | 7 (2) | Ser481Phe | 0.86 E ASN to 0.24 AFR/AA | | | | | serum levels of glucose (during fasting) and pantothenate | 319, 320 |
| ***SUGP1*** | | SURP And G-Patch Domain Containing 1, Splicing Factor 4 | | altered splicing in innate immunity | 321 | pan-cancer | 322 | NAFLD | 118 |
| 19-19302283-C-T (rs17751061) | 8 (1) | Arg290His (1 of 8 transcripts) | 0.26 MID E to 0.00 E ASN | serum IgE levels | 323 | | | waist-hip ratio<br>fasting insulin and glucose | 324<br>325 |

Genes listed have been associated with innate immunity/inflammation, cancer, and cardio-metabolic disease and have at least one variant in the human genome that occurs in at least 20% (Minor Allele Frequency (MAF) ≥ 0.2) of one or more populations. Missense variants are described by their location in the GRCh38 reference genome (accessed from gnomAD v3.1.2), rs number (reference SNP cluster ID), and amino acid location numbers and identities of the original and coded replacement. Populations are defined by Karczewski 2020 (15): African/African American (AFR/AA), East Asian (E ASN), non-Finnish European (EUR), Latino/Latina (LAT), Middle Eastern (MID E), and South Asian (S ASN). The number of affected transcripts listed include total transcripts (first number) and transcripts with missense mutations (in parentheses) that contain the gene variant, but do not include transcripts of any overlapping genes.





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nonsynonymous variants unique to the Asian population, namely 2-21006289-G-A (rs144467873, MAF = 0.001253 and 0.0003594 in East and South Asians, respectively, but < 0.00008 for all other populations (gnomAD v2.1.1) and 2-21029662-G-A (rs13306194, MAF = 0.1343 in East Asians, MAF < 0.007 in all other populations) were evaluated for their association with lipid profiles, metabolic syndrome and risk of diabetes in a large Taiwan Biobank study (237). Both variants were independently associated with total, LDL, and non-HDL cholesterol levels, whereas rs144467873 (Arg3527Trp) was associated with elevated lipid levels and metabolic syndrome, while rs13306194 (Arg532Trp) was linked with serum triglyceride levels.

### 3.2.4 Dihydroorotate dehydrogenase

Dihydroorotate dehydrogenase (DHODH), which catalyzes the initial and rate-limiting step of the *de novo* pyrimidine pathway, is positioned on the inner mitochondrial membrane (330). DHODH has been a therapeutic target for the treatment of rheumatoid arthritis, psoriasis, autoimmune disorders, and Plasmodium, bacterial and fungal infections (241). For over five decades, elevated DHODH expression has been known to promote tumor progression. *De novo* pyrimidine synthesis becomes essential during increased demands for nucleic acid precursors in rapidly dividing cells making cancer cells highly dependent on DHODH and suggesting that this enzyme is a strategic target for cancer therapy (245). Recently, DHODH was also shown to protect against mitochondrial ferroptosis by preventing the lipid peroxidation that triggers this phenomenon (244). Notably, cancer cells exhibit low levels of glutathione peroxidase 4 (GPX4) and inhibition of DHODH hinders respiration, boosts glycolysis and enhances GLUT4 translocation to the plasma membrane (246). This is further supported by the activation of the tumor suppressor p53, which elevates the levels of GDF15/MIC1 (another candidate listed in Table 4), a cytokine known for its appetite-reducing effects and ability to extend lifespan. DHODH inhibition that depletes pyrimidine ribonucleotides is also thought to be responsible for reduced RNA virus replication and decelerated growth in rapidly dividing cells, such as activated T cells and, as just mentioned, cancer cells (243). Interestingly, uridine, a pyrimidine nucleoside present in RNA, has been shown to modulate insulin activity and glycogen synthesis through its interaction with uridine diphosphate (UDP)-glucose (247). The base sequence of the *DHODH* gene is remarkably conserved, with one exception being a prevalent Lys7Gln missense polymorphism (16-72008783-A-C, rs3213422) found in its first exon (248). This variant is found in 75% of individuals in East Asia vs. 34% of individuals in the Middle East (gnomAD) and has been linked with drug (leflunomide) response to rheumatoid arthritis (248–250).

## 3.3 Population-enriched variants with unknown/uncharacterized function

No known effect on gross phenotype or evidence of association with disease has yet been reported among the population-enriched variants identified with the 20 genes listed in Table 6. However, a newly released resource, GWAS Central (457), was accessed to provide phenotype associations with a subset of variants in Table 6. Further, disease disparities related to the parent gene and/or other variants of the gene were identified and/or the predicted impact of a population-enriched variant on the coded change in protein function were evaluated and listed in Table 6.

### 3.3.1 Understudied genes SIPA1L2 and TVP23C

Among the 20 genes in Table 6, six of these remain understudied, including the exosomal CCDC105/TEKTL1, the putative protein disulfide isomerase CRELD2, the FAM131C protein with unknown function, the putative immune checkpoint ITPRIPL1 membrane protein, the presumptive neural GTPase activator SIPA1L2, and the putative vesicular protein transporter TVP23C. Notably, evidence of an impact on function does exist for one of two population-enriched variants of *SIPA1L2* and one of three population-enriched variants of *TVP23C*. In the case of *SIPA1L2*, both characterized and uncharacterized variants occur at the same high frequency (MAF = 0.48) in East Asians, but Gly1639Ser increases the number of potential phosphorylation sites, whereas Thr1322Ala reduces them, which may result in different functional outcomes (e.g. changes in activation status and/or protein-protein interactions). In both *SIPA1L2* variants, eight of nine possible transcripts code for missense mutations, whereas with *TVP23C*, only in the canonical transcript does the variant result in a missense mutation among five (Ser256Arg) or twelve (Trp202Arg and Ser199Thr) possible isoforms, some of which are read-through fusions with CDRT4 (CMT1A Duplicated Region Transcript 4). It is likely that the *TVP23C* Trp202Arg and Ser199Thr variants commonly co-occur, given their proximity to one another on the gene and their matching frequency distribution, as both have MAFs that range from 0.54 in East Asians to 0.28 in South Asians. Thus, one might speculate that the unknown functional impact of Ser199Thr matches that of Trp202Arg, which was found in a choriocarcinoma patient (458). Notably, choriocarcinoma shows a geographical disparity as it occurs at a ten-fold greater frequency in Southeast Asia than in the West (reviewed in 439). The third *TVP23C* variant Ser256Arg is most common among Africans/African Americans (MAF = 0.24) and involves the loss of a potential phosphorylation site about 50 amino acid residues downstream of the other two *TVP23C* variants.

### 3.3.2 Additional representative genes of interest

The remaining 14 genes in Table 6 are better characterized; notably, many have pleiotropic functions beyond the functions initially attributed to them. ATPase Phospholipid Transporting 10D (*ATP10D*) codes for the catalytic subunit of a glycoslyceramide flippase complex at the endoplasmic reticulum (ER), nucleoplasm, and plasma membrane. DnaJ Heat Shock Protein Family (Hsp40) Member B11 (*DNAJB11*) codes for an ER-resident and secreted co-chaperone of BiP/GRP78/HSPA5. Desmocollin 1 (*DSC1*) codes for an adhesive glycoprotein cadherin family member. The Immunoglobulin Like Domain Containing Receptor 1 protein (ILDR1) maintains structural

TABLE 6 Geographic Ancestral Variants with Unknown/Uncharacterized Function.

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***ATP10D*** | | ATPase, class V, type 10D | | sphingolipids assoc w/ innate immune response | 331 | lung cancer | 332 | controls circulating sphingolipids responsible for atherosclerosis, T2D | 333 |
| | | transports glucosylceramide, a sphingolipid | | downregulated by TGFb in eosinophils | 334 | colorectal cancer | 335 | | |
| 4-47514685-C-T (rs33995001) | 3 (2) | Thr43Ile | 0.44 LAT to 0.16 E ASN | | | | | | |
| 4-47582029-G-A (rs1058793) | 4 (1) | Val1240Ile | 0.58 E ASN to 0.16 EUR | found in Bulgarian centenarians | 336 | | | | |
| 4-47591266-G-C (rs4145944) | 3 (1) | Ser1389Thr | 0.62 AFR/AA to 0.12 E ASN | | | | | serum cholesterol | 323 |
| | | **Disease Disparity:** Sphingolipid levels are elevated in lupus [337] and hepatocellular carcinoma [338], two diseases with known disparities based on geographic ancestry [339 and 340, respectively] | | | | | | | |
| ***CCDC105*** | | coiled-coil domain containing 105, tektin like 1, TEKTL1 | | HBV infection | 341 | colon, lung cancer | 342, 343 | interacts with MESD [344], part of WNT pathway in cancer and cardiovascular disease | 345, 346 |
| 19-15020518-G-A (rs35352238) | 1 (1) | Val245Met | 0.54 E ASN to 0.11 AFR/AA | | | | | | |
| 19-15023114-C-A (rs8112667) | 1 (1) | Pro499Thr | 0.53 E ASN to 0.18 MID E | serum fibrinogen | 323 | | | | |
| | | **Disease Disparity:** interacts with MAGEA11 [347], a biomarker for stomach cancer [348], which shows racial and geographic disparities [reviewed in 349] | | | | | | | |
| ***CRELD2*** | | cysteine rich with EGF like domains 2 | | marker in joint infection | 350 | multiple cancers | 350 | cardiometabolic disease | 350 |
| 22-49921715-C-A (rs8139422) | 10 (5) | Asp182Glu | 0.51 AFR/AA to 0.03 EUR | age-related macular degeneration | 351 | | | | |
| | | **Disease Disparity:** breast [352] and prostate [353] cancers, reviewed in [60] | | | | | | | |
| ***CSF2RB*** | | IL3RB, CD131, IL5RB | | colony stimulating factor 2 receptor beta surfactant homeostasis | 354 | variant assoc w leukemia variant assoc w breast cancer | 355 356 | peptide agonists of EPOR/CD131 heteroreceptor are anti-atherosclerotic | 357 |
| 22-36930401-G-C (rs16845) | 4 (4) | Glu249Gln | 0.21 AFR/AA to 0.00 E ASN | | | | | | |
| | | **Disease Disparity:** breast cancer [60, 352] | | | | | | | |

*(Continued)*

TABLE 6 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| ***DNAJB11*** | | ER-associated DnaJ Hsp40 member B11 | | immune infiltration in thyroid | 358 | liver, breast, pancreatic cancer | 358 | diabetes | 119 |
| 3-186583914-A-G (rs8147) | 3 (2) | Ile264Val | 0.47 AFR/AA to 0.15 E ASN | rheumatoid arthritis | 359 | | | | |
| | | **Disease Disparity:** context-dependent breast cancer [60, 352] | | | | | | | |
| ***DSC1*** | | desmocollin 1 | | reduced in pediatric pneumomia | 360 | head and neck, ovarian, anal | 361–363 | prevents HDL biogenesis | 360 |
| 18-31140184-C-T (rs17800159) | 2 (2) | ValIle460Ile | 0.48 E ASN to 0.03 AFR/AA | | | | | | |
| | | **Disease Disparity:** ovarian cancer [364] | | | | | | | |
| ***FAM131C*** | | family with sequence similarity 131 member C | | autoimmune target in ApoE KO mice | 365 | associated with cancer survival | 366 | upregulated in high fat diet | 367 |
| | | | | upregulated in M1 macrophage-rich adipose | 368 | | | | |
| 1-16058636-C-A (rs1832151) | 2 (1) | Ser215Ile | 0.33 AFR/AA to 0.0013 E ASN | | | | | | |
| 1-16060000-C-T (rs71510977) | 2 (1) | Arg107Gln | 0.78 E ASN to 0.10 AFR/AA | | | | | | |
| 1-16062531-T-C (rs2863458) | 2 (1) | Lys48Glu | 0.38 AFR/AA to 0.01 E ASN | | | | | waist-hip ratio | 324 |
| | | **Diseaese Disparity:** interacts with VSNL1 [369], which is associated with colon cancer [370, 371] and gastric cancer [372], both cancers that show ethnic disparities [373] | | | | | | | |
| ***ILDR1*** | | Ig-like domain containing receptor 1 | | flu virus replication | 374 | gastric cancer marker | 375 | diet-induced obesity and hyperglycemia | 376 |
| 3-121993958-G-C (rs3915061) | 5 (3) | Pro264Arg | 0.49 S ASN to 0.22 E ASN | | | | | | |
| | | **Disease Disparity:** gastric cancer [reviewed in 349, 373] | | | | | | | |
| ***ITPRIPL1*** | | inositol triphosphate interacting protein like 1, KIAA1754L | | immune checkpoint inhibition of T-cell activation | 377 | gene methylation assoc w breast cancer | 378 | diabetic nephropathy | 379 |
| 2-96328019-C-T (rs2279105) | 4 (4) | Thr463Met | 0.69 S ASN to 0.21 E ASN | HbA1c | 323 | | | | |
| | | **Disease Disparity:** breast cancer [60, 352] | | | | | | | |
| ***PDIA6*** | | protein disulfide isomerase A6, ERP5 | | lymphoid and myeloid development | 380 381 | NSCLC, breast, bladder, gastic, oral, | 382–387 | diabetes | 119 |

*(Continued)*

TABLE 6 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | TXNDC7 thioredoxin domain containing 7 | | platelet aggregation and activation | | pancreatic cancers | | | |
| 2-10790777-T-C (rs4807) | 6 (6) | Lys214Arg | 0.39 E ASN to 0.125 AFR/AA | serum IgE, HbA1c, rheumatoid arthritis | 323, 359 | | | age-related macular degeneration | 351 |
| | | **Disease Disparity:** breast [60, 352] and gastric cancer [reviewed in 349, 373] | | | | | | | |
| ***RB1*** | | retinoblastoma 1 transcriptional co-repressor | | associated with Treg infiltration in bladder cancer | 388 | tumor suppressor in multiple cancers | 389 | negative association with BMI and insulin resistance | 390 |
| 13-48599402-T-C (rs1887154) | 1 (1, non-cannonical) | Leu99Ser (1 transcript) | 0.79 AFR/AA to 0.28 MID E | | | | | | |
| | | **Predicted Impact:** potential phosphorylation site introduced (+Ser): RB1 phosphorylation inactivates this tumor suppressor and promotes tumor progression [389], impacts several regulatory pathways and protein-protein interactions [391] | | | | | | | |
| ***RPAIN*** | | RPA interacting protein, nuclear transporter, HRIP | | variants assoc w/ influenza A virus (RNA) pathogenesis | 392 393 | alternate splice variants in colon cancer, glioblastoma | 394 395 | gene expression is associated with BMI | 396 |
| 17-5422825-C-G (rs12761) | 16 (11) | Asn103Lys | 0.82 E ASN to 0.22 AFR/AA | | | | | BMI | 397 |
| | | **Disease Disparity:** colon cancer [398] | | | | | | | |
| ***SEMA6D*** | | sematophorin D6 | | regulates late phase CD4+ T cells response, anti-inflammatory macrophage polarization | 399 400, 401 | lung cancer, chemoresponse in breast cancer | 151, 402 | cardiomyocyte development, immune cell metabolism | 401 403 |
| 15-47764022-A-G (rs3743279) | 9 (9) | Asn307Ser | 0.24 AFR/AA to 0.00 EUR | skin pigmentation | 404 | | | | |
| 15-47765874-G-A (rs532598) | 9 (8) | Ser478Asn | 0.59 E ASN to 0.34 MID E | partial epilepsies, asthma | 405, 406 | | | | |
| | | **Disease Disparity:** SEMA6D expression is associated with survival in triple negative breast cancer [407], which occurs disproportionately among women of African descent [352] | | | | | | | |
| | | **Predicted Impact:** both variants may alter phosphorylation status (+/- Ser) with the potential to alter activity, stability and/or protein-protein interactions | | | | | | | |
| ***SIPA1L2*** | | signal induced prolif assoc 1 like 2, SPAR2, SPAL2, KIAA1389 | | assoc w/ H2O2 release from healthy Caucasian lymphoblastoids | 408 | metastatic clear cell kidney carcinoma (EUR) | 409 | identified by bioinformatics in type 2 diabetes | 410 |
| | | | | inactivates RAP1 (involved in inflammatory response) | 410 | varying correlation with 23 cancers | 411 | gene expression assoc with NAFLD | 412 |

*(Continued)*

TABLE 6 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 1-232403473-C-T (rs2275303) | 9 (8) | Gly1639Ser | 0.48 E ASN to 0.00 AFR/AA | Alzheimer's in Asian population | 413 | | | | |
| 1-232439175-T-C (rs2275307) | 9 (8) | Thr1322Ala | 0.48 E ASN to 0.22 EUR | | | | | | |
| | | **Disease Disparity:** gene shows highest correlation with cancers with known ethnic disparities [411], including bladder [414], esophageal [415], hepatocellular carcinoma [416], and ovarian [364] | | | | | | | |
| | | **Predicted Impact:** both variants may alter phosphorylation status (+Ser and -Thr) with the potential to alter activity, stability and/or protein-protein interactions | | | | | | | |
| ***TBC1D4*** | | AS160 Akt substrate of 160 kD | | delivery to chlamydial inclusions | 417 | breast cancer, multiple myeloma | 418, 419 | nonsense variant confers insulin resistance and T2D in Greenlandic population | 420 |
| 13-75286865-A-G (rs557337) | 4 (4) | Val1275Ala | 0.49 AFR/AA to 0.00 E ASN | fibrinogen | 323 | | | | |
| 13-75481466-G-A (rs77685055) | 3 (3) | Ala101Val | 0.29 E ASN to 0.02 AFR/AA | RBC count mean corp. Hb, hematocrit | 421–423 | | | | |
| | | **Disease Disparity:** gene associated with cancers that show ethnic disparities, including breast [60, 352] and multiple myeloma [424] | | | | | | | |
| ***TESPA1*** | | HSPC257, thymocyte expressed, positive selection associated 1 | | development and maturation of T cells TCR regulation | 425 | pan-cancer prognostic | 426 | mito-assoc ER mb proteins are assoc w/ cardiovascular disease | 427 |
| 12-54950349-C-G (rs2171497) | 7 (2, non-canonical) | Leu103Phe | 0.64 E ASN to 0.05 AFR/AA | ulcerative colitis | 428 | | | BMI | 397 |
| 12-54961249-C-T (rs997173) | 8 (5) | Leu496Lys | 0.63 E ASN to 0.06 AFR/AA | Kuru and sCJD (prion diseases) | 429 | | | | |
| | | **Disease Disparity:** although TESPA1 expresssion is upregulated in several cancers, the most dramatic increase in expression occurs in acute myeloid leukemia (AML) [426], a cancer which shows ethnic disparities [430] | | | | | | | |
| ***TVP23C*** | | TGN vesicle protein 23 homolog C, FAM18B2 | | gene is an integration site for HBV in liver cancer | 431 | plasma protein assoc w/ colorectal cancer | 432 | bioinformatic feature gene assoc w/ ischemic stroke | 433 |
| | | | | platelet granule secretion, chronic immune thrombocytopenia | 434 | data mining prognostic marker for liver cancer | 435 | readthrough translation with CDRT4 downregulated in obese individuals | 436 |
| | | | | associated with CD4 Tex (exhausted T) cells | 437 | fusion CDRT4 found in pancreatic cancer | 438 | | |
| 17-15502927-A-C (rs73289533) | 5 (1) | Ser256Arg | 0.24 AFR/AA to 0.00 E ASN | | | | | | |

*(Continued)*

TABLE 6 Continued

| Gene (SNP) | Affected Transcripts | Name/ Function | Population MAF Range | Associated w/ Immunity | Ref | Associated w/ Cancer | Ref | Associated w/ Metabolic Disease | Ref |
|---|---|---|---|---|---|---|---|---|---|
| 17-15540420-A-G (rs200768112) | 12 (1) | Trp202Arg | 0.54 E ASN to 0.28 S ASN | | | choriocarcinoma | 439 | | |
| 17-15540428-C-G (rs2302252) | 12 (1) | Ser199Thr | 0.54 E ASN to 0.28 S ASN | | | | | | |
| | | **Disease Disparity:** Choriocarcinoma incidence rate 10-fold higher in Southeast Asia than in the West [439] | | | | | | | |
| ***ZNF23*** | | KOX16, ZNF359, ZNF612 | | correlation with pathogenic environment | 49 | downregulated in cancer | 440 | mitochondrial dysregulation in melanoma | 441 |
| 16-71453303-T-C (rs2070832) | 9 (7) | Ser28Gly | 0.94 AFR/AA to 0.29 E ASN | partial epilepsies | 405 | | | BMI | 323 |
| | | **Disease Disparity:** reduced expression of this tumor repressor gene in ovarian and endometrial cancers [440]**;** oviarian cancers show ethnic disparities [364] | | | | | | | |
| | | **Predicted Impact:** variant occurs in a putative N-terminal strong transcriptional repressor KRAB domain [442], loss of Ser may alter activity and/or binding interactions | | | | | | | |
| | | **Note:** ZNF23 KRAB domain is truncated and does not appear to alter repressor activity [440], however not all ZNF23 interactors (such as mitochondrial ATPAF2. keratin-associated KRTAP10-8, myelin-associated MOBP, growth factor signaling regulators SPRED1 and SPRY1, and TNFR associated adaptor TRAF1), are transcription factors | | | | | | | |
| ***ZNF267*** | | Zinc Finger Protein 267, HZF2 | | P. gingivalis infection | 443 | hepatic, colorectal cancer, B-cell lymphoma | 444–446 | liver disease (cirhhosis), NAFLD | 447, 448 |
| 16-31915298-G-A (rs3850114) | 2 (1) | Cys350Tyr | 1.0 E ASN to 0.61 AFR/AA | serum IgE | 323 | | | | |
| | | **Disease Disparity:** hepatic and colorectal cancers show ethnic disparities [340, 449] | | | | | | | |
| ***ZNF628*** | | Zinc Finger Protein 628, ZEC | | target gene protamine inhibits microbial infection | 450–452 | protamine 1 marker for leukemia and colorectal cancer | 453, 454 | protamine alters BP, mitochondrial function | 455 |
| 19-55481893-A-G (rs34864744) | 2 (2) | Thr234Ala | 0.93 AFR/AA to 0.45 E ASN | | | | | | |
| | | **Disease Disparity:** ethnic disparities observed in colorectal cancers [449] | | | | | | | |
| | | **Predicted Impact:** variant alters potential phosphorylation status (-Thr) in a disordered region of this transcription activator [344] between two zinc finger clusters of the canonical protein that bind DNA independently [456] | | | | | | | |

Genes listed have been associated with innate immunity/inflammation, cancer, and cardio-metabolic disease and have at least one variant in the human genome that occurs in at least 20% (Minor Allele Frequency (MAF) ≥ 0.2) of one or more populations. Missense variants are described by their location in the GRCh38 reference genome (accessed from gnomAD v3.1.2), rs number (reference SNP cluster ID), and amino acid location numbers and identities of the original and coded replacement. Populations are defined by Karczewski 2020 (15): African/African American (AFR/AA), East Asian (E ASN), non-Finnish European (EUR), Latino/Latina (LAT), Middle Eastern (MID E), and South Asian (S ASN). The number of affected transcripts listed include total transcripts (first number) and transcripts with missense mutations (in parentheses) that contain the gene variant, but do not include transcripts of any overlapping genes.

barriers in epithelia and auditory neurosensory hair cells (459), mediates fatty acid and lipoprotein-stimulated cholecystokinin secretion in the small intestine (460), regulates water homeostasis in kidney (461), and interferes with phospholipid scramblase (PLSCR1) anti-viral activity (374). Protein Disulfide Isomerase Family A Member 6 (PDIA6) inhibits intracellular aggregation of misfolded proteins and extracellular aggregation of platelets (381). Replication Protein A Interacting Protein (RPAIN) participates in

DNA metabolism, nuclear import, and response to UV light. The Semaphorin 6D (*SEMA6D*) gene codes for an integral membrane protein member of the semaphorin family whose members collectively sculpt axonal paths, branches, conduction, and target selection; the distribution of nine *SEMA6D* transcript isoforms varies according to developmental stage and tissue type. Tre-2/BUB2/CDC16 (TBC) Domain Family Member 4 (TBC1D4, also referred to as Akt Substrate of 160 kD or AS160) is a Rab-GTPase activator with multiple transcript variants; isoform 2 promotes SLC2A4/GLUT4 presentation at the plasma membrane to increase cellular glucose uptake (344). Thymocyte Expressed, Positive Selection Associated 1 (*TESPA1*) interacts with COP9 and TCR signalsomes and participates in T cell differentiation and T cell receptor signaling. Three zinc finger (ZNF) proteins ZNF23, ZNF267, and ZNF628 localize to the nucleus and regulate transcription. Parent genes and the corresponding population-enriched variants of the common cytokine receptor beta chain *CSF2RB* and the transcription co-repressor *RB1* are both discussed below.

#### 3.3.2.1 CSF2RB

Colony stimulating factor 2 receptor beta (CSF2RB, CD131) forms dimers with the alpha receptor subunits for cytokines IL-3, IL-5, and GM-CSF (CSF2). As noted above, a population-enriched variant of the IL3RA subunit also exists, although the population distributions of these two variants are very different: the Val323Leu *IL3RA* variant is found least frequently among Africans/African Americans (MAF = 0.06, Table 4), whereas the Glu249Gln *CSF2RB* variant is more predominant in Africans/African Americans than any other population (MAF = 0.21).

*CSF2RB* is associated with pulmonary alveolar proteinosis (PAP), which involves the accumulation of surfactant and macrophage dysfunction in alveoli (reviewed in 462). Although studies so far have not suggested geographic or population differences in PAP occurrence, the most common PAP co-morbidities include cardiovascular disease, type 2 diabetes, and hypertension, all of which are unevenly distributed among populations. Further, a rare Arg461Cys *CSF2RB* variant (MAF< 0.001, not listed in Table 6) was found in individual patients with leukemia (355) and breast cancer (356). Notably, both of these cancers show racial and ethnic disparities [430 and 352 respectively].

#### 3.3.2.2 RB1

Retinoblastoma (RB1) was one of the first tumor suppressors to be identified. Alterations in the expression and sequence of the *RB1* gene have been implicated in several cancers besides retinoblastoma where they were originally characterized (reviewed in 391). More than 40 years of extensive research indicates that regulation of and by RB1 is highly complex, linked with multiple signaling pathways, and varies with context. Not surprisingly, the number of proteins shown to interact with RB1 is more than 30 as curated by UniProt (344) and more than 150 as curated in BioGRID (463) and IntAct (464). The functional diversity of the binding partners of RB1 is consistent with its pleiotropic effects, which extend beyond transcription and cell cycle control to include progenitor maturation, terminal differentiation, and immune evasion (391).

Five protein coding transcripts of *RB1* have been identified. These include 1) the MANE select (canonical) protein composed of 27 exons encoding a total of 928 aa residues; 2) a closely related transcript that is 5 as shorter and differs from the canonical protein by 18 of its last 19 C-terminal residues; and 3) three much shorter transcripts (coding for 53, 103 or 110 aa peptides) which include all or portions of only 2 or 3 exons of the canonical protein. Of these shorter transcripts, the two shortest are derived from the N-terminal portion of RB1. In contrast, the 110 aa non-canonical transcript codes for an unidentified N-terminal residue equivalent to the Ser501 residue of the canonical protein and then aligns with all canonical residues up through Ser565; the remaining non-canonical aa residues 66-110 are located downstream of the canonical C-terminal residue 928. It is in this extra-exonic portion of the non-canonical 110 aa *RB1* isoform that the Leu99Ser population-enriched variant, which introduces a potential phosphorylation site, is found. In spite of the high number of aa residues ($n \geq 105$) in the canonical RB1 protein that are known to be post-translationally modified, within the aa 501-565 residue range that overlaps with the first 65 residues of the 110 aa isoforms, only two potential ubiquitination sites have been identified in the vicinity of aa 550) (391).

## 4 Conclusion

Population studies have traditionally focused on querying individual diseases or combinations of diseases, including cancer and cardio-metabolic disease, which frequently show disparate prevalence and/or severity in non-European populations. In this perspective, we have introduced a complementary approach that explores the intersection of innate immunity, cancer, and cardio-metabolic diseases. The effective elimination of disease disparities will involve not only addressing the profound social and behavioral determinants of health, but also identifying and treating the biological contributors of disease that include novel genes as well as previously characterized genes that participate in novel pathways.

We suggest that careful evaluation of population differences in conventional and unconventional innate immune genes and their related pathways will provide key insights into the underlying mechanisms that connect cancer and cardio-metabolic diseases. At the same time, the genes we have identified in this study that are associated with both cancer and cardio-metabolic diseases may play critical roles in under-appreciated facets of innate immunity and their contribution to disease disparities. Further, we predict that the geographic ancestral distribution of innate immune gene variants will match the geographical distribution of the environmental stressors (including but not limited to infectious agents) that they are designed to mitigate as described above for *HbS* and *DARC* variants with malaria (Section 1.3).

The genes we have identified serve as potential targets for diagnostics and/or therapeutic interventions. Notably, the development and clinical use of therapeutics targeting these candidate genes is likely to require a nuanced approach since variations in these genes across different global populations are likely to alter the activity and/or expression of their coded proteins, with the subsequent potential to impact therapeutic outcomes. Assessing the prevalence of specific target variants in one or more major populations and, more precisely, the presence of these specific target variants in individuals is a consequential step towards increasing the safety and effectiveness of emerging therapies. This perspective highlights the importance of 1) considering genetic diversity in identifying and developing treatments and 2) continuing to incorporate ongoing GWAS projects as they identify and characterize new or understudied genes and their population-enriched variants associated with complex and infectious diseases.

## Author contributions

SY: Conceptualization, Data curation, Writing – original draft. DH: Data curation, Writing – original draft. KW: Data curation, Writing – original draft. NL: Writing – review & editing. KSK: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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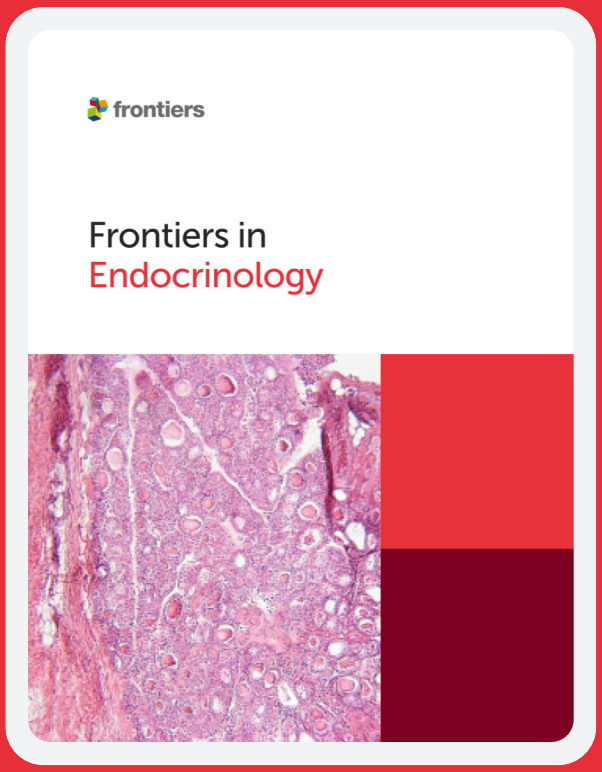


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