Preprints.org

Article

Not peer-reviewed version

Glutaminase Inhibition as a Novel Radiosensitizer in Triple Negative Breast Cancer

Mohammad Zahid Kamran [†], Anny Ho [†], Brittany A. Simone [*]

Posted Date: 6 May 2026

doi: 10.20944/preprints202605.0311.v1

Keywords: triple-negative breast cancer; glutamine metabolism; radiosensitivity

Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC, OpenAlex.

Copyright: This open access article is published under a Creative Commons CC BY 4.0 license, which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

Article

Glutaminase Inhibition as a Novel Radiosensitizer in Triple Negative Breast Cancer

Mohammad Zahid Kamran †, Anny Ho † and Brittany A. Simone *

Department of Radiation Oncology, SUNY Upstate Medical University, Syracuse, NY, United States

* Correspondence: simoneb@upstate.edu

† These authors contributed equally to this work.

Abstract

Triple-negative breast cancer (TNBC) is the most aggressive subtype and accounts for 15-20% of breast cancer cases. TNBC lacks expression of the estrogen, progesterone, and human epidermal growth factor receptor 2/neu (HER2/neu) receptors. Due to the lack of these receptors, targeted therapies are virtually ineffective. In addition, due to their aggressive nature, standard therapy options are limited by the development of resistance making TNBC very challenging to treat, highlighting the need for new therapeutic approaches. TNBCs undergo metabolic alterations to support growth and survival, one of which is glutamine addiction. TNBCs have been shown to demonstrate increased levels of *GLS* mRNA, which correlates with their dependence on exogenous glutamine for growth and survival. This study examined whether inhibiting glutamine metabolism enhances radiotherapy (RT) efficacy against TNBC. In two TNBC cell lines (MDA-MB-231 and 4T1), glutamine deprivation and the glutaminase (GLS) inhibitor CB-839 combined with ionizing radiation (IR) reduced colony formation in the combination treatment was significantly more effective than either treatment alone. In a murine model of TNBC, this combination significantly decreased mammary fat pad tumor growth. These findings demonstrate that inhibiting glutamine metabolism combined with RT represents a promising therapeutic strategy that could improve treatment outcomes in TNBC patients who currently have limited effective treatment options.

Keywords: triple-negative breast cancer; glutamine metabolism; radiosensitivity

1. Introduction

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer that accounts for approximately 10-15% of all breast cancers and disproportionately affects African American women [1,2] (PMID: 16757721, 17671126). This form of breast cancer metastasizes quickly and is fatal when it does. Its characteristic lack of expression of the estrogen, progesterone, and human epidermal growth factor receptor 2/neu (HER2/neu) receptors is what contributes to the difficulty in treating TNBC, as there are no efficacious targeted therapies [3,4] (PMID: 33413427/ 29978332). Immunotherapy has been evaluated in addition to cytotoxic chemotherapy, and this treatment strategy has shown some promise in TNBC, but the median overall survival for women with metastatic TNBC remains dismal at 18-25 months [5,6] (PMID: 28572258/ 37079257). Many women with metastatic TNBC will receive tumor-directed radiotherapy (RT) as a component of their care, whether the intent is palliative or ablative [7,8] (PMID: 40647452/ 29240541). Additionally, many patients receiving RT will have their systemic therapy held during RT out of concern for undue toxicity. This highlights the need for improved targeted therapies that can be safely combined with RT in the setting of TNBC.

Often, TNBCs lack phosphatase and tensin homologue (PTEN), which is a tumor suppressor gene, and this loss of PTEN leads to an innate dependence on glutamine metabolism for tumor growth and progression (PMID: 23000897). PTEN loss in breast cancer is also associated with more aggressive disease and worse outcomes. In particular, PTEN deficiency occurs more frequently in TNBC, which are not responsive to targeted cancer therapies (PMID: 26051240/28410191). In addition,

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

TNBCs have been shown to demonstrate increased levels of *GLS* mRNA, which correlates with their dependence on exogenous glutamine for growth and survival. The glutaminase (GLS) inhibitor CB-839 (Telaglenastat) has been shown to have antitumor activity in preclinical models of TNBC [9] (PMID: 24523301). By inhibiting the conversion of glutamine to glutamate, which cancer cells require for the tricarboxylic acid (TCA) cycle (PMID: 24047273), these cells have a lower metabolic capacity for repair of damage due to cytotoxic therapies.

Given the lack of effective therapy options and inability to deliver systemic therapies concurrently with radiation due to toxicity, combining metabolic inhibitors with radiation could provide a novel and safer approach for radiosensitization in TNBC. In addition, the metabolic dependencies of TNBC and the energy requirements of DNA repair suggest that combining metabolic inhibitors with radiation could offer a new and safer approach to enhance radiosensitivity in TNBC. Therefore, we hypothesized that CB-839 in combination with tumor-directed RT would improve the efficacy of RT in TNBC.

2. Materials and Methods

Cell lines and culture conditions: Murine triple-negative breast cancer (TNBC) cells 4T1 (CRL-2539) and human TNBC cells MDA-MB-231 (HTB-26) were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, #11875-093), with 10% fetal bovine serum (FBS) (Gibco, #A56707-01) and 1% penicillin/streptomycin (Gibco, #15140-122) to prevent bacterial growth/contamination and were maintained at 37°C in a humidified atmosphere with 5% CO_2. Glutamine deprivation was completed by incubating cells in glutamine-free Dulbecco's modified Eagle medium (DMEM) (Gibco, #A14430-01), supplemented with dialyzed FBS (Corning Life Sciences, #35-071-CV).

Radiation Exposure: Radiation experiments used a MultiRad 225 x-radiation unit (Precision X-Ray, North Branford, CT) operating at 225 kV/17 mA at a source-to-surface distance of 55 cm, with a 0.5-mm copper beam hardening filter in place, delivering a dose rate of ~1.2 Gy/min. Daily dose quality assurance and dose-rate calibrations were performed in air using an integrated Farmer-type ionization chamber at 37 cm source-to-surface distance. Cultured cells were exposed at ambient temperature, with sham irradiated control plates transported to the radiation facility but not exposed.

Clonogenic survival assays: Cells were seeded at a density of 500 and 1000 cells/well in 6-well culture dishes and allowed to adhere for 24 hours. After 24 hours, cells were then either subjected to glutamine deprivation by maintaining them in glutamine-free medium or treated with 2 μM of GLS inhibitor (CB-839, Telaglenastat, Cayman Chemicals, #22038) or 0.01% dimethyl sulfoxide (DMSO) as a vehicle control (Fisher, #BP231-100) for 16 hours. After 16 hours, all cell lines were exposed to a single radiation fraction (2 or 4 Gy) and left undisturbed for 10 to 15 days, depending on the doubling time of the cells. To facilitate colony counting, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline at pH 7.4, stained with 0.5% crystal violet for 15 minutes, and subsequently washed in deionized water. Colonies of more than (≥) 50 cells were counted. All results were normalized to the non-irradiated and 0.01% DMSO controls, and the percentage of colonies was calculated as the number of colonies formed in the treatment groups relative to the untreated control (defined as 100%).

Mito Stress Test assay: 4T1 and MDA-MB-231 cells were seeded at a density of 2×10^5 cells/mL and were plated in at least 6-12 replicates on 96-well Seahorse Cell Culture (Agilent) plates. Cells were either subjected to glutamine deprivation by maintaining them in glutamine-free medium for 16 hours or treated with 2 μM CB-839 or DMSO vehicle control. Following treatment, cells were irradiated with 2 or 4 Gy. For glutamine deprivation experiments, cells were replenished with complete medium after irradiation and analyzed 72 hours post-irradiation. For CB-839 experiments, analysis was performed 30 minutes post-irradiation. Prior to measurement, cells were subjected to Seahorse analysis. Cells were washed twice with phosphate-buffered saline (PBS) (Gibco, #10010-023) and replenished in Seahorse DMEM medium, pH 7.4 (Agilent, #103575-100) supplemented with 1 mM pyruvate (Agilent, #103578-100), 2 mM glutamine (Agilent, #103579-100), and 5 mM glucose

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

(Agilent, 103577-100) and incubated at 37 °C in non-CO_2 incubator for 60 minutes. Experimental protocols were set up with Seahorse XFe 96 Flux Analyzer Controller software (Agilent). The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using a Seahorse XFe 96 Flux analyzer (Agilent) according to the manufacturer's instructions. The OCR and ECAR values were obtained at baseline and after injections of Seahorse XF Mito Stress Test Kit (Agilent, #103015-100) reagents: oligomycin (1.5 μM), FCCP (1.0 μM), and rotenone/antimycin (0.5 μM). All measurements were taken using Seahorse Analytics Wave Pro software (Agilent). After the assay was completed, a BCA assay was performed to obtain protein-normalized OCR and ECAR values.

Western blot: Cells were seeded in a 24 mm flask or 6-well plates. Cells were either subjected to glutamine deprivation by maintaining them in glutamine-free medium for 6 or 16 hours or treated with 2 μM CB-839 or DMSO vehicle control for 16 hours. After irradiation, cells were replenished with a complete medium. Cells were harvested with trypsin-EDTA (Gibco, 25300054) at different time points post irradiation (30 minutes, 6 hours, 24 hours, and 72 hours). Cells were lysed with RIPA buffer (Thermo Scientific, #89900) supplemented with 1× HALT protease/phosphatase inhibitor cocktail (Thermo Scientific, 78442); total cell lysate was collected, and protein estimation was carried out using bicinchoninic acid assay (BCA) assay (Thermo Scientific, #23227); 30μg of protein was resolved on 4 -15% gradient gel (Bio-Rad, #4561084). Proteins were transferred to 0.2μm PVDF membrane (Bio-Rad, #1704156) and probed with Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb (1:2000, Cell Signaling, #9718), and β-tubulin (1:3000, Cell Signaling, #2146) antibody overnight at 4 °C. Membrane was washed with 1X Tris-buffered saline (Cell signaling, #12498) with Tween 20 (TBST) (Bio-Rad, #1706531) buffer three times and probed with HRP-conjugated anti-rabbit secondary antibody (1:3000, Cell Signaling, #7074) for 1 hour at room temperature. Protein detection was performed using SuperSignal West Femto chemiluminescence substrate (Thermo Scientific, #34096), imaged using Chemidoc XRS+ with Image Lab 6.0.1 software (Bio-Rad).

Efficacy of CB-839: *In vivo* experiment: Six to eight-week-old female BALB/c mice were purchased from the Jackson Laboratory and maintained in our institution's animal facility. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. $0.25x10^6$ 4T1 cells in PBS were implanted subcutaneously into the right flank of mice. When tumors reached approximately 100 mm^3, mice were randomized into four groups. Vehicle control (DMSO), CB-839, radiation, and CB-839 + radiation. Each group has 5. The vehicle for CB-839 was 25% (w/v) hydroxypropyl-β-cyclodextrin in 10 mmol/L citrate, pH 2. CB-839 was administered at 20mg/kg [10] through intraperitoneal (IP) injection a day before and 4 hours prior to administration of a single dose of 8 Gy radiation. The tumor-bearing right flank was focally irradiated while the remainder of the body was shielded with a custom-designed lead shield. The radiation was delivered using Faxitron Multirad 225 X-ray irradiator operated at 225/17 kV/mA with a 0.5 mm Cu filter. Source-to-shelf distance was 55.0 cm. CB-839 treatment continued for 14 days. Tumor sizes were measured using a precision caliper every alternate day, and tumor volume was calculated using the formula $0.5\ ab^2$, where "a" is the length of the tumor and "b" is the width of the tumor.

3. Results

Exogenous glutamine deprivation inhibits the cellular proliferation of TNBC and sensitizes them to radiation

As a proof of concept, we examined the effect of glutamine deprivation in vitro with and without ionizing radiation in TNBC cell lines. The effect of exogenous glutamine deprivation on cellular proliferation of MDA MB-231 and 4T1 cells was evaluated via colony formation assay. Glutamine-deprived MDA-MB-231 cells demonstrated decreased colony formation by 70% as compared to control (Figure 1A). Treatment of cells with 2 Gy and 4 Gy of IR reduced 45% and 69% colonies, respectively, as compared to the untreated control. Glutamine deprivation with 2 Gy of radiation reduced colony formation by 88%, whereas glutamine deprivation with 4 Gy of radiation inhibited 98% colonies as compared to control (p-value). 4T1 cells demonstrated a similar response with 66%

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

decreased colony formation with exogenous glutamine deprivation (Figure 1C). Radiation alone at 2 Gy and 4 Gy reduced 4T1 colony formation by 45% and 75% respectively, as compared to the control. Glutamine deprivation in combination with 2 Gy or 4 Gy inhibited 92% or 99% of 4T1 colonies, respectively, as compared to control (p-value).

Similarly, CB-839-treated MDA-MB-231 cells demonstrated decreased colony formation by approximately 47 % as compared to control (Figure 1B). CB-839 with 2 Gy of IR reduced colony formation by approximately 90%, whereas CB-839 with 4 Gy of IR reduced colony formation by 95% as compared to the control (p-value). 4T1 cells showed approximately 31% decreased colony formation with CB-839 treatment (Figure 1D). In the CB-839 experiments, IR alone at 2 Gy and 4 Gy reduced 4T1 colony formation by approximately 32% and 82%, respectively, as compared to the control. CB-839 in combination with 2 Gy or 4 Gy reduced colony formation by approximately 90% or 95% respectively, as compared to control (p-value).

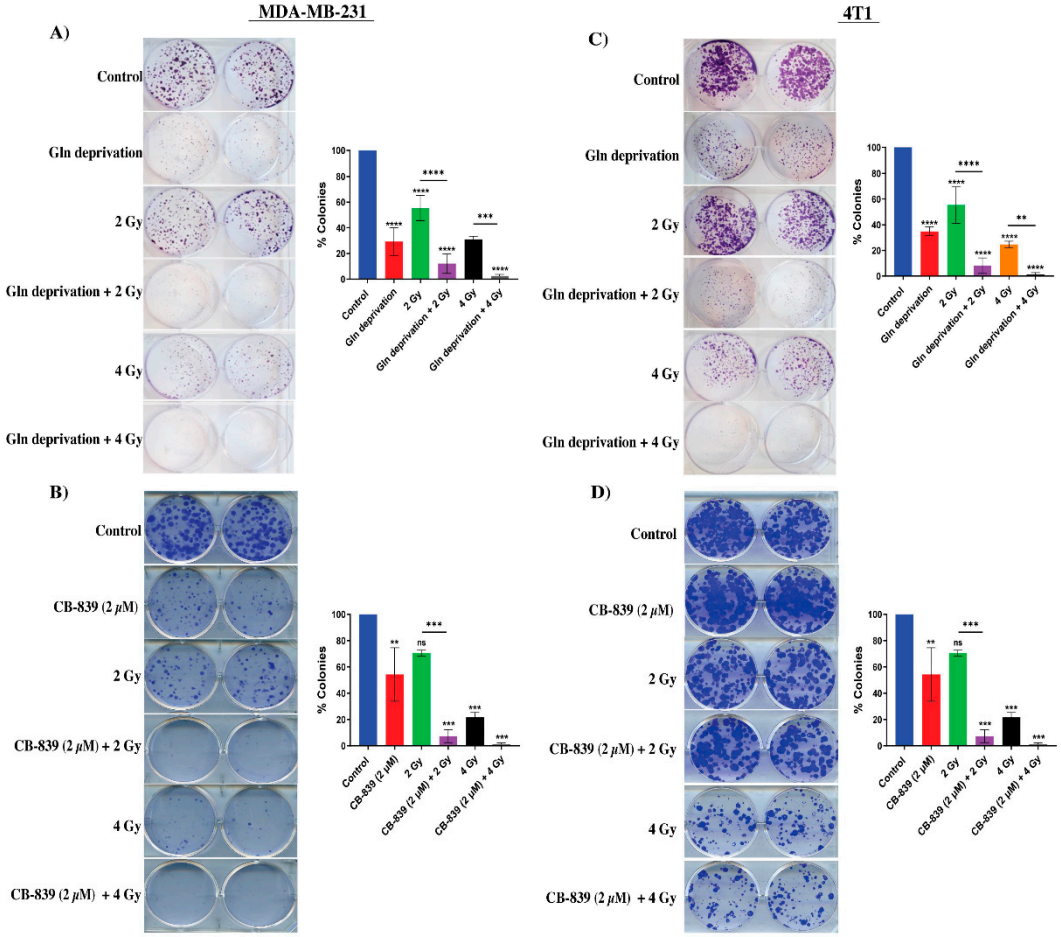

Figure 1. ***Inhibting glutamine metabolism enhances radiation-induced cell death in TNBC.*** TNBC cell lines were subjected to either glutamine deprivation or CB-839 (2 μM) treatment for 16 h, followed by irradiation with either 2 Gy or 4 Gy. Colony formation assay with glutamine deprivation: (A) MDA-MB-231 and (C) 4T1. Colony formation assay with CB-839 treatment: (B) MDA-MB-231 and (D) 4T1. Values are expressed as the mean ± standard deviation (SD). Statistical significance was determined by appropriate statistical tests: asterisks above bars indicate comparisons to control; asterisks with lines show comparisons between treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns = not significant).

Glutamine deprivation decreases the DNA damage repair capacity in TNBC

To repair damaged DNA, cells need a continuous nucleotide supply, and glutamine is the major source of carbon and nitrogen for nucleotide synthesis. To examine whether glutamine deprivation

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

influences the DNA damage repair capacity of cells, we measured the level of γH2AX, a marker of DNA double-strand break. In MDA-MB-231, we observed that radiation at 2 Gy (Figure 2A) or 4 Gy (Figure 2B) increased γH2AX expression as early as 30 minutes post-irradiation and then rapidly decreased, suggesting very quick and efficient DNA repair. Glutamine deprivation alone did not have any effect on γH2AX expression. Further, glutamine deprivation in combination with radiation did not have a significant effect on γH2AX expression 30 minutes postirradiation as compared to radiation treatment alone. γH2AX expression was decreased 24h post-irradiation in the cells treated with radiation alone (Figure 2C). However, cells that were treated with a combination of glutamine deprivation and radiation had increased γH2AX expression up to 24h as compared to cells treated with radiation alone (Figure 2D). In 4T1 cells, radiation alone at 2 Gy (Figure 2G) or 4 Gy (Figure 2H) increased γH2AX expression at 30 minutes post-irradiation, with decreasing expression by 24 hours. Along with glutamine deprivation, combined with radiation resulted in persistent γH2AX expression at both 30 minutes and 24 hours post-irradiation (Figure 2I,J). Similarly, in MDA-MB-231 cells, CB-839 combined with radiation showed persistent γH2AX expression compared to radiation alone (Figure 2E,F) In 4T1 cells, CB-839 treatment compared to radiation also showed persistent γH2AX expression compared to radiation alone (Figure 2K,L). These data suggest that targeting glutamine metabolism in combination with radiation decreases the DNA repair capacity of the cells.

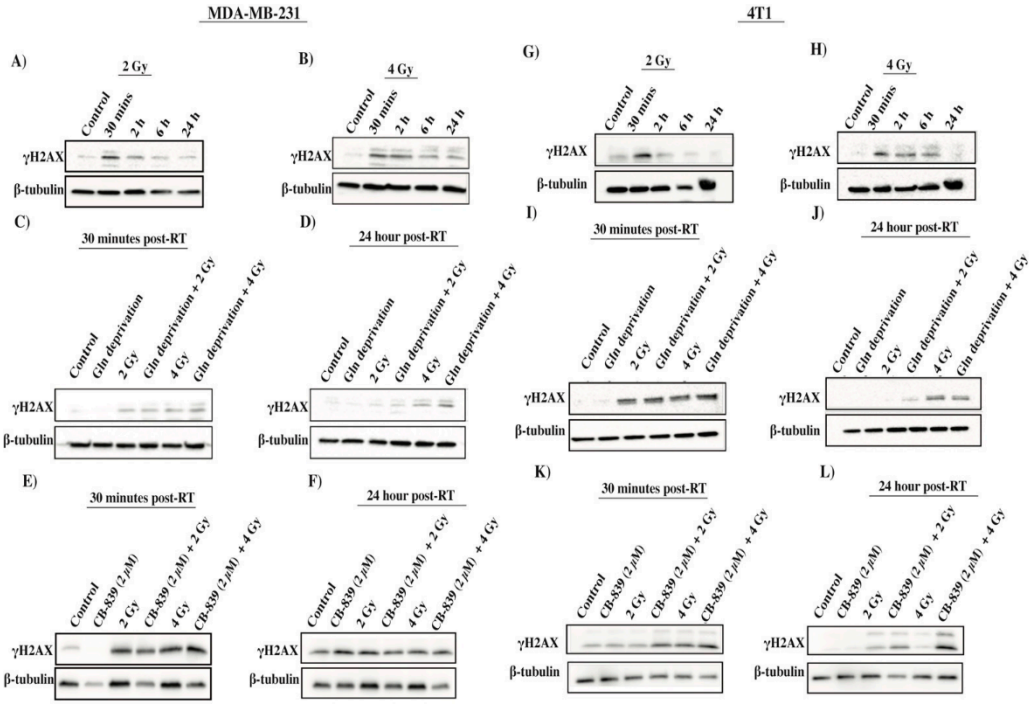

Figure 2. ***Inhibiting glutamine metabolism decreases DNA damage repair capacity in TNBC.*** Western blot analysis of γH2AX expression following glutamine deprivation or CB-839 treatment and radiation. Time course analysis of γH2AX levels after (A) 2 Gy and (B) 4 Gy radiation in MDA-MB-231 cells, and (G) 2 Gy and (H) 4 Gy radiation in 4T1 cells at the indicated time points (control, 30 minutes, 2h, 6h, 24h). Effects of glutamine deprivation (MDA-MB-231; C-D, 4T1; I-J) and CB-839 (MDA-MB-231; E-F, 4T1; K-L) combined with radiation at 30 minutes and 24 hours post-irradiation. β-tubulin serves as a loading control.

Glutamine deprivation inhibits mitochondrial respiration and ATP production in TNBC

To elucidate the mechanism behind the observed synergistic effect of glutamine deprivation with radiation in TNBC, we evaluated mitochondrial respiration in vitro using the Seahorse metabolic flux analyzer. We observed that, in MDA-MB-231 cells, glutamine deprivation alone decreased basal respiration, maximal respiration, and ATP respiration by 39.77%, 35.17% and 36.17%, respectively. There was no significant decrease in mitochondrial respiration and ATP respiration

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

when the cells were irradiated with 2 Gy; however, 4 Gy inhibited basal respiration, maximal respiration, and ATP respiration by 41.56%, 37.59% and 39.63%, respectively (Figure 3A). Importantly, when the cells were subjected to the combination of glutamine deprivation and radiation at 2 Gy or 4 Gy, the effect on mitochondrial respiration and ATP production was more pronounced compared to either treatment alone. There was a 43.27% decrease in basal respiration, 40.25% in maximal respiration, and a 29.10% decrease in ATP respiration as compared to control when the cells were exposed to the combination of glutamine deprivation and 2 Gy of radiation. Glutamine deprivation, combined with 4 Gy of radiation, decreased basal respiration, maximal respiration, and ATP respiration by 68.76%, 72.26%, and 67.26%, respectively, compared to the control.

Similarly, in 4T1 cells, glutamine deprivation alone or in combination with radiation at 2 Gy or 4 Gy decreased mitochondrial respiration and ATP respiration. Glutamine deprivation decreased basal respiration, maximal respiration, and ATP respiration by 52.66%, 53.29% and 50.12% respectively, as compared to control. 2 Gy of radiation exposure did not have a significant effect on mitochondrial respiration and ATP respiration. However, 4 Gy of radiation exposure inhibited basal respiration, maximal respiration, and ATP respiration by 30.50%, 16.40% and 32.46% respectively, as compared to control. The combination of glutamine deprivation and 2 Gy of radiation inhibited 70.73% of basal respiration, 70.29% of maximal respiration, and 68.63% of ATP respiration as compared to the control. Glutamine deprivation in combination with 4 Gy of radiation exposure decreased basal respiration, maximal respiration, and ATP respiration by 88.55%, 90.18% and 87.27% respectively, as compared to control.

We next evaluated the effects of the GLS inhibitor CB-839 on mitochondrial respiration. In MDA-MB-231 cells, CB-839 (2 μM) alone decreased basal respiration, maximal respiration, and ATP respiration by approximately 29.56%, 36.69% and 27.05% respectively (Figure 3B). CB-839 combined with 2 Gy radiation decreased basal respiration, maximal respiration, and ATP respiration by approximately 36.70%, 43.97% and 32.80% respectively. CB-839 combined with 4 Gy radiation showed the most pronounced effects, decreasing basal respiration, maximal respiration, and ATP respiration by approximately 61.34%, 70.23% and 59.07% respectively, as compared to the control. In 4T1 cells, CB-839 treatment alone decreased basal respiration, maximal respiration, and ATP respiration by approximately 40.40%, 35.43% and 37.63% respectively (Figure 3D). CB-839 combined with 2 Gy radiation inhibited basal respiration, maximal respiration, and ATP respiration by approximately 56.31%, 47.67% and 50% respectively. The combination of CB-839 with 4 Gy radiation resulted in the greatest metabolic disruption, decreasing basal respiration, maximal respiration, and ATP respiration by approximately 67.60%, 61.78% and 70.14% respectively, as compared to control.

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

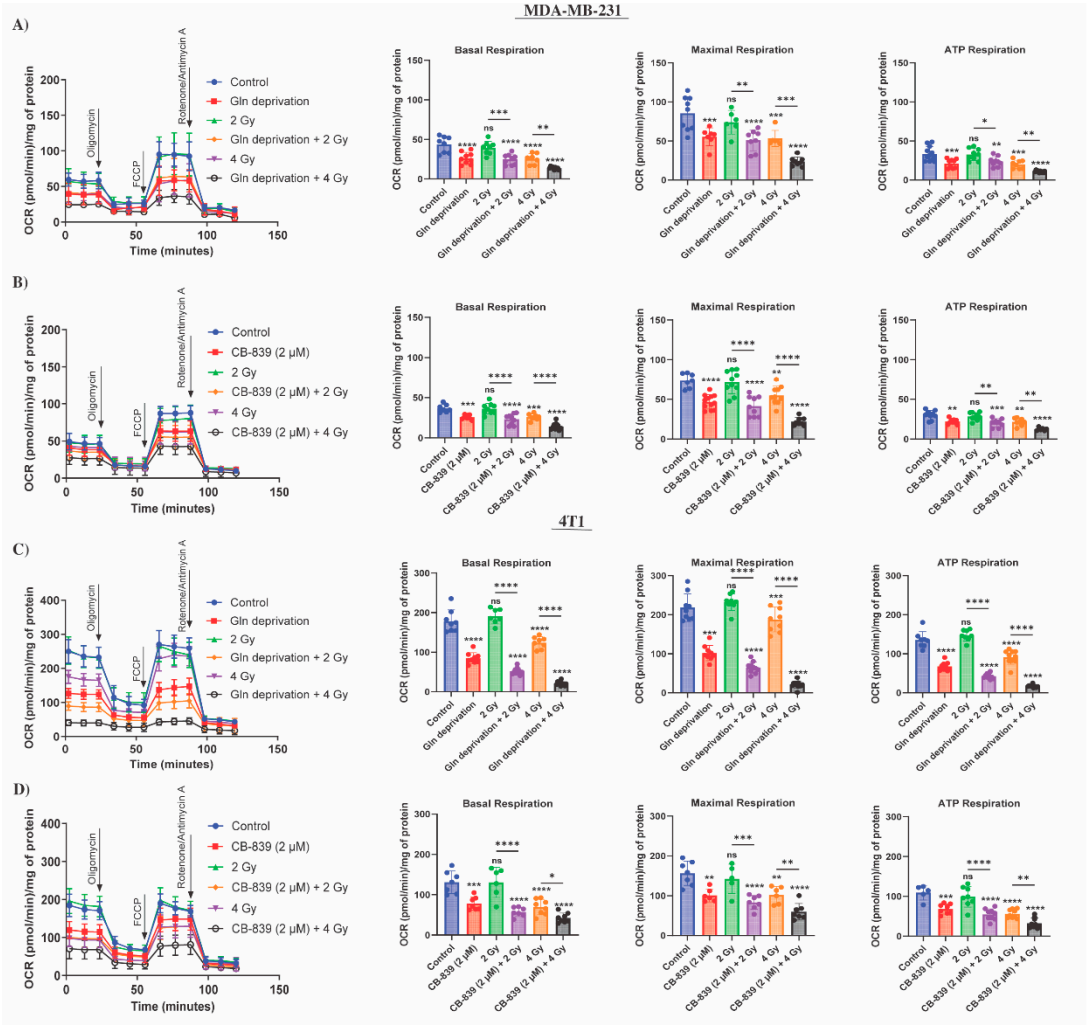

Figure 3. ***Inhibiting glutamine metabolism decreases mitochondrial respiration in TNBC.*** 4T1 and MDA-MB-231 cell lines were treated with glutamine deprivation or glutaminase inhibitor CB-839 (2 μM) for 16 hours, followed by irradiation of 2 Gy or 4 Gy. Mitochondrial stress assay was performed using Seahorse XF analyzer 72 hours post-irradiation for glutamine deprivation and 30 minutes post-irradiation for CB-839 treated cells. Representative oxygen consumption rate (OCR) and quantification of basal respiration, maximal respiration, and ATP respiration for (A) MDA-MB-231 glutamine deprivation, (B) MDA-MB-231 CB-839, (C) 4T1 glutamine deprivation, and (D) 4T1 CB-839. OCR values were normalized to total protein content. Data are expressed as mean ± SD. Statistical significance was determined by appropriate statistical tests: asterisks above bars indicate comparisons to control; asterisks with lines show comparisons between treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns = not significant).

Glutaminase inhibitor, CB-839, radiosensitizes TNBC in vivo

Since we observed that glutamine deprivation and GLS inhibitor CB-839 inhibited cellular proliferation of TNBC *in vitro* and sensitized them to radiation, we explored the radiosensitization efficacy of GLS inhibitor CB-839, *in vivo*. 4T1 murine TNBC cells were implanted on the right flank of BALB/c female mice and were treated with either CB-839, a single dose of radiation (8 Gy), and a combination of CB-839 with a single dose of radiation (8 Gy) (Figure 4A). CB-839 treatment for 14 consecutive days (from day 7 to day 20 post-cell implantation) significantly decreased tumor burden. At day 20, CB-839 treatment reduced tumor burden by 44.21% as compared to mice treated with vehicle control. However, as treatment was stopped, tumor growth resumed, and the delay in tumor growth was not statistically significant. Primary tumor growth delay was seen in mice irradiated with 8 Gy of radiation. At day 36, there was a 49% decrease in tumor volume of mice treated with 8 Gy of radiation as compared to control mice. When mice were treated with a combination of CB-839 and

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

radiation, a decrease in primary tumor burden was more significant than either treatment alone. At day 36, there was a 53.83% and 64.36% decrease in tumor volume of the mice treated with a combination of CB-839 and radiation as compared to mice treated with only radiation or CB-839, respectively. Final tumor weights confirmed these findings, with the combination treatment showing the greatest reduction in tumor burden (Figure 4B,C).

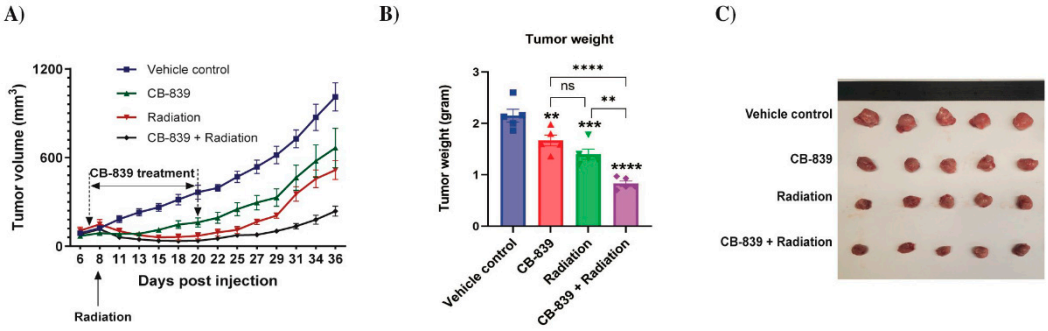

Figure 4. ***Glutaminase inhibitor (CB-839) radiosensitizes TNBC in vivo***. 4T1 cells were implanted subcutaneously into the right flank of female BALB/c mice. CB-839 was administered at 20mg/kg through intraperitoneal (IP) injection a day before and 4 hours prior to a single dose of 8 Gy radiation, and then continued for 14 days. (A) Tumor volume measured over time in the 4T1 xenograft model. Values are expressed as the mean ± SEM. (B) Individual tumor weights at study termination showing treatment effects across all groups (n=5). (C) Representative tumor images at study endpoint demonstrating visual differences in tumor size between treatment groups. Statistical significance: asterisks above bars indicate comparisons to vehicle control; asterisks with lines show comparisons between treatment groups (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

4. Discussion

Cancer cells undergo significant metabolic reprogramming to support rapid growth and proliferation, and survival [11] (PMID: 36994237). Our study demonstrates that disrupting glutamine metabolism can serve as a radiosensitizer for TNBC. TNBC represents one of the most challenging breast cancer subtypes to treat, with its lack of targeted therapeutic options and demonstrating resistance to conventional therapies further limiting treatment options. These findings demonstrate that disrupting glutamine metabolism not only impairs cancer cell proliferation but also alters energy biosynthesis needed for DNA repair following radiation-induced damage. By targeting glutamine metabolism in combination with ionizing radiation, it will disrupt mitochondrial metabolism and lead to impaired DNA repair capacity.

Glutamine Metabolism as a Therapeutic Target in TNBC

In addition to glucose, cancer cells also rely heavily on glutamine fueling the TCA cycle which is essential for energy production and biosynthesis [9,30] (PMID: 24523301/27492215). While many cancers rely on aerobic glycolysis, also known as the Warburg Effect, for energy [12] (PMID: 13298683), over the years, glutamine metabolism has emerged as another important role helping cells in therapy resistance. In previous studies, researchers have shown that cancer cells show higher dependence on glutamine for energy production, biosynthesis, and redox homeostasis [15–17] (PMID: 23999442/19881548/ 19703661). Glutamine is a non-essential amino acid that plays a key role in cancer cell metabolism and survival, specifically for rapid proliferation and stressful conditions [13,14] (PMID: 20570523/18177721). It has been reported that TNBC primary tumors and many cancer types have an overexpression of GLS and low expression of glutamine synthetase (GS), the key enzyme that converts glutamine from glutamate [18,19] (PMID: 24094812/ 21852960). The observed expression pattern may be linked to significant glutamine consumption and TNBC cell line survival and growth on exogenous glutamine, could indicate that TNBC would be sensitive to glutaminase inhibition.

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

For our first approach, we used a systematic approach to validate glutamine metabolism as a radiosensitization target. We used glutamine deprivation as a proof-of-concept method to demonstrate that targeting glutamine metabolism could enhance radiosensitivity in TNBC. To test this, we evaluated the effects of targeting glutamine metabolism on cellular proliferation using a colony formation assay. Glutamine deprivation treatments were studied in two TNBC cell lines (human; MDA-MB-231, and murine; 4T1), and resulted in decreased colony formation, indicating TNBC cells rely on glutamine metabolism for survival and are vulnerable to glutamine withdrawal. We found significant reductions in colony formation with glutamine deprivation alone (70% in MDA-MB-231 and 66% in 4T1 cells). In addition, there was a more significant synergistic effect when combining RT with glutamine deprivation which resulted in an 88% reduction when combined 2 Gy, and 98% reduction with 4 Gy in MDA-MB-231 and 92% reduction with 2 Gy and 99% reduction with 4 Gy in 4T1 cells.

Having established the proof-of-concept with glutamine deprivation treatment, we next evaluated the clinical translational potential. We used CB-839, a GLS inhibitor, which is a commercially available drug with promising results in various cancers and has a favorable safety profile and tolerability in clinical settings [20] (PMID: 34285061). CB-839 treatment has shown promising results in other cancer types (ref), however, has not been explored in combination with RT in TNBC. Our results showed that CB-839 treatment successfully enhanced radiosensitivity and had similar effects observed with glutamine deprivation treatment. In CB-839 treatment, we observed a reduction in colony formation by 47% in MDA-MB-231 and 31% in 4T1 cells. Similarly to glutamine deprivation, CB-839 achieved 90% inhibition with 2 Gy and 95% inhibition with 4 Gy in both cell lines compared to radiation alone.

Previous studies have reported that glutamine serves as a key nutrient for cancer cell survival and proliferation [13,15] (PMID: 20570523/23999442). Therefore, the reduced colony formation observed in TNBC cells treated with glutamine deprivation and CB-839 is consistent with the concept that cancer cells highly depend on glutamine. Through targeting glutamine metabolism, there were fewer colony formations, demonstrating that cells depend on glutamine for survival. Furthermore, what could be observed is that the difference between glutamine deprivation and CB-839 treatment may likely depend on the difference between removing complete nutrients removing glutamine production (with glutamine deprivation) versus GLS enzymatic inhibition (with GLS inhibitor, CB-839) [9,21] (PMID: 24523301/ 28393116). Unlike glutamine deprivation, which could be systemically toxic, with CB-839, through GLS inhibition it potentially protects glutamine function for normal tissues while selectively impacting glutamine-dependent cancer cells. This result demonstrates that CB-839 can be successfully used and replicated to achieve the radiosensitizing outcome seen with glutamine deprivation that is suitable for patient treatment.

Metabolic Disruption Impairs DNA Repair

To understand how targeting glutamine metabolism enhances radiosensitivity, we examined DNA repair capacity by measuring γH2AX expression as a marker for DNA double-strand breaks (DSBs) [24,25] (PMID: 9488723/ 18772227). Radiation induces DSBs, which are lethal to cells if left unrepaired [26] (PMID: 19847258). Our mechanistic investigations reveal how targeting glutamine metabolism creates a therapeutic vulnerability that radiation can exploit. Our results demonstrated that while radiation alone induced temporary γH2AX expression that resolved by 24 hours, which indicates that there is efficient DNA damage repair occurring [27] (PMID: 21491423), with glutamine deprivation combined with radiation showed persistent γH2AX expression at 30 minutes and 24 hours post-irradiation. The persistent γH2AX expression observed in glutamine-deprived cells represents a critical finding that demonstrates a shift from repairable to irreparable DNA damage, fundamentally altering the cellular response to radiation.

The persistent DNA damage response indicates that glutamine metabolism impairs a cell's ability to effectively repair radiation-induced DNA lesions. Glutamine plays a role in many cellular biosynthesis processes, such as producing proteins, lipids, nucleotides, and amino acids [28] (PMID: 31040047). The depletion of nucleotides may reduce the available building blocks necessary for DNA

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

repair, resulting in unresolved DNA DSBs. Additionally, glutamine metabolism contributes to maintaining cellular redox balance through glutathione synthesis [29,30] (PMID: 11172416/ 22811024), and any disruptions can increase oxidative stress, especially after radiation exposure or stressful conditions, further impairing DNA repair mechanisms.

To further investigate, we examined mitochondrial function using the Seahorse XF Mito stress test to explore the link between glutamine metabolism and impaired DNA repair capacity. The results showed that glutamine deprivation significantly impaired mitochondrial respiration in both TNBC cell lines, confirming that these cancer cells heavily depend on glutamine to maintain mitochondrial function through the TCA cycle for energy production [9,31] (PMID: 24523301/27492215). Glutamine deprivation alone reduced ATP production by 36.17% in MDA-MB-231 cells and by 50.12% in 4T1 cells, highlighting the critical role of glutamine in TNBC metabolic disruptions, possibly causing energy production crises that inhibits cells from generating sufficient DNA damage responses [14,16,32] (PMID: 19881548/18177721).

The significant ATP depletion directly describes the impaired DNA repair response capacity that we observed with persistent γH2AX expression. DNA damage repair processes, mostly the repair of radiation-induced DNA DSBs, require significant ATP for protein recruitment, remodeling, and repair [33,34] (PMID: 25213441/17291544). Targeting glutamine metabolism has been shown to induce radiosensitivity in cancer cells by reducing cellular ATP levels, and as the cells lack sufficient energy to efficiently repair radiation-induced damage, this results in persistent DNA lesions and inhibiting cell survival and proliferation [35] (PMID: 39038517). This metabolic dependency likely comes from TNBC's aggressive phenotype and rapid proliferation demands, which need significant glutamine consumption for both energy production and biosynthesis processes [31,36] (PMID: 27492215 /35851845). Importantly, the finding that CB-839 had similar effects on DNA damage repair capacity as glutamine deprivation supports our approach and demonstrates that GLS inhibition could replicate the effects of nutrient-deprived conditions. These findings demonstrate a fundamental mechanistic insight into targeting glutamine. Targeting glutamine metabolism through glutamine deprivation or CB-839 creates a significant metabolic vulnerability that can be used as a therapeutic approach.

Clinical Validation and Therapeutic Potential

Since we observed that glutamine deprivation and CB-839 inhibited the proliferation of TNBC cells in vitro and made them more sensitive to radiation, we evaluated the effectiveness of the GLS inhibitor CB-839 in enhancing radiosensitivity in vivo. The results from the 4T1 model provide important translational evidence but also reveal significant limitations of monotherapy approaches. The murine 4T1 cell line is widely accepted as a suitable model for TNBC research and is commonly used in preclinical studies that closely resemble the characteristics and behavior of human TNBC [37,38] (PMID: 18432775/ 30860605). While CB-839 alone showed only brief anti-tumor effects, combining it with radiation resulted in better and more sustained tumor control. The 64% reduction in tumor volume compared to CB-839 alone demonstrates a clear synergistic benefit, implying that the metabolic stress caused by glutamine targeting impairs the adaptive responses that typically help cancer cells survive radiation therapy. Importantly, this strategy addresses a critical need in TNBC treatment. Currently, patients undergoing radiotherapy often have their systemic therapy stopped due to toxicity concerns. The favorable safety profile of CB-839 and the mechanistic rationale for combination therapy suggest that this approach could enable concurrent treatment, potentially improve outcomes while maintaining tolerability.

5. Conclusions

In conclusion, this study establishes that targeting glutamine metabolism can be a viable radiosensitization strategy to treat TNBC. By creating a metabolic vulnerability that radiation can exploit, this approach offers a mechanistically novel and clinically feasible path forward for improving radiation therapy outcomes in this challenging disease. The progression from proof-of-

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

concept glutamine deprivation to clinically relevant CB-839 treatment provides a clear translational pathway, while the mechanistic insights into metabolic-DNA repair interactions offer opportunities for further therapeutic development. Most importantly, this work demonstrates how understanding tumor metabolism can reveal new approaches to enhance existing therapies, potentially transforming treatment outcomes for TNBC patients.

Author Contributions: Conceptualization, B.A.S; methodology, M.Z.K, A.H and B.A.S.; validation, M.Z.K, A.H and B.A.S.; formal analysis, M.Z.K, and A.H; investigation, M.Z.K, A.H and B.A.S.; resources, B.A.S writing—original draft preparation, M.Z.K, A.H and B.A.S; writing—review and editing, A.H and B.A.S.; visualization, B.A.S.; supervision, B.A.S; project administration, B.A.S.; funding acquisition, B.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Upstate University Radiation Oncology Department at SUNY Upstate Medical University.

Institutional Review Board Statement: The animal study protocol was approved by the IACUC of SUNY Upstate Medical University (protocol number 476; approved 6/13/21).

Conflicts 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. .

Abbreviations

The following abbreviations are used in this manuscript:

DSBs	Double-stands breaks
GLS	Glutaminase
GS	Glutamine synthetase
HER2	Human epidermal growth factor receptor 2
IP	Intraperitoneal
IR	Ionizing radiation
PTEN	Phosphatase and tensin homologue
RT	Radiotherapy
TCA cycle	Tricarboxylic acid cycle
TNBC	Triple-Negative Breast Cancer

References

1. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006;295(21):2492-502.
2. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13(15 Pt 1):4429-34.
3. Zhang L, Mu C, Zhang T, Yang D, Wang C, Chen Q, et al. Development of targeted therapy therapeutics to sensitize triple-negative breast cancer chemosensitivity utilizing bacteriophage phi29 derived packaging RNA. J Nanobiotechnology. 2021;19(1):13.
4. Al-Mahmood S, Sapiezynski J, Garbuzenko OB, Minko T. Metastatic and triple-negative breast cancer: challenges and treatment options. Drug Deliv Transl Res. 2018;8(5):1483-507.
5. Vonderheide RH, Domchek SM, Clark AS. Immunotherapy for Breast Cancer: What Are We Missing? Clin Cancer Res. 2017;23(11):2640-6.
6. Geurts V, Kok M. Immunotherapy for Metastatic Triple Negative Breast Cancer: Current Paradigm and Future Approaches. Curr Treat Options Oncol. 2023;24(6):628-43.
7. LeVee A, Young H, Yoon S, Glaser S, Wu S, Mortimer J, et al. Efficacy of Metastasis-Directed Radiation Therapy to Prolong Systemic Therapy for Patients with Oligoprogressive Metastatic Breast Cancer. Cancers (Basel). 2025;17(13).

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

8. Ost P, Reynders D, Decaestecker K, Fonteyne V, Lumen N, De Bruycker A, et al. Surveillance or Metastasis-Directed Therapy for Oligometastatic Prostate Cancer Recurrence: A Prospective, Randomized, Multicenter Phase II Trial. J Clin Oncol. 2018;36(5):446-53.
9. Gross MI, Demo SD, Dennison JB, Chen L, Chernov-Rogan T, Goyal B, et al. Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. Mol Cancer Ther. 2014;13(4):890-901.
10. Zhong Y, Geng F, Mazik L, Yin X, Becker AP, Mohammed S, et al. Combinatorial targeting of glutamine metabolism and lysosomal-based lipid metabolism effectively suppresses glioblastoma. Cell Rep Med. 2024;5(9):101706.
11. Nong S, Han X, Xiang Y, Qian Y, Wei Y, Zhang T, et al. Metabolic reprogramming in cancer: Mechanisms and therapeutics. MedComm (2020). 2023;4(2):e218.
12. WARBURG O. On the origin of cancer cells. Science. 1956;123(3191):309-14.
13. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci. 2010;35(8):427-33.
14. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab. 2008;7(1):11-20.
15. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. J Clin Invest. 2013;123(9):3678-84.
16. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene. 2010;29(3):313-24.
17. Matés JM, Segura JA, Campos-Sandoval JA, Lobo C, Alonso L, Alonso FJ, et al. Glutamine homeostasis and mitochondrial dynamics. Int J Biochem Cell Biol. 2009;41(10):2051-61.
18. Timmerman LA, Holton T, Yuneva M, Louie RJ, Padró M, Daemen A, et al. Glutamine sensitivity analysis identifies the xCT antiporter as a common triple-negative breast tumor therapeutic target. Cancer Cell. 2013;24(4):450-65.
19. Kung HN, Marks JR, Chi JT. Glutamine synthetase is a genetic determinant of cell type-specific glutamine independence in breast epithelia. PLoS Genet. 2011;7(8):e1002229.
20. Harding JJ, Telli M, Munster P, Voss MH, Infante JR, DeMichele A, et al. A Phase I Dose-Escalation and Expansion Study of Telaglenastat in Patients with Advanced or Metastatic Solid Tumors. Clin Cancer Res. 2021;27(18):4994-5003.
21. Cluntun AA, Lukey MJ, Cerione RA, Locasale JW. Glutamine Metabolism in Cancer: Understanding the Heterogeneity. Trends Cancer. 2017;3(3):169-80.
22. Roux C, Riganti C, Borgogno SF, Curto R, Curcio C, Catanzaro V, et al. Endogenous glutamine decrease is associated with pancreatic cancer progression. Oncotarget. 2017;8(56):95361-76.
23. Ye Y, Yu B, Wang H, Yi F. Glutamine metabolic reprogramming in hepatocellular carcinoma. Front Mol Biosci. 2023;10:1242059.
24. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. 1998;273(10):5858-68.
25. Kinner A, Wu W, Staudt C, Iliakis G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res. 2008;36(17):5678-94.
26. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461(7267):1071-8.
27. Bourton EC, Plowman PN, Smith D, Arlett CF, Parris CN. Prolonged expression of the γ-H2AX DNA repair biomarker correlates with excess acute and chronic toxicity from radiotherapy treatment. Int J Cancer. 2011;129(12):2928-34.
28. Bernfeld E, Foster DA. Glutamine as an Essential Amino Acid for KRas-Driven Cancer Cells. Trends Endocrinol Metab. 2019;30(6):357-68.
29. Babu R, Eaton S, Drake DP, Spitz L, Pierro A. Glutamine and glutathione counteract the inhibitory effects of mediators of sepsis in neonatal hepatocytes. J Pediatr Surg. 2001;36(2):282-6.
30. Matés JM, Segura JA, Alonso FJ, Márquez J. Oxidative stress in apoptosis and cancer: an update. Arch Toxicol. 2012;86(11):1649-65.

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 May 2026 doi:10.20944/preprints202605.0311.v1

31. Altman BJ, Stine ZE, Dang CV. From Krebs to clinic: glutamine metabolism to cancer therapy. Nat Rev Cancer. 2016;16(10):619-34.
32. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci U S A. 2008;105(48):18782-7.
33. Hopfner KP. ATP puts the brake on DNA double-strand break repair: a new study shows that ATP switches the Mre11-Rad50-Nbs1 repair factor between signaling and processing of DNA ends. Bioessays. 2014;36(12):1170-8.
34. Osley MA, Tsukuda T, Nickoloff JA. ATP-dependent chromatin remodeling factors and DNA damage repair. Mutat Res. 2007;618(1-2):65-80.
35. Kim M, Hwang S, Jeong SM. Targeting cellular adaptive responses to glutaminolysis perturbation for cancer therapy. Mol Cells. 2024;47(8):100096.
36. Quek LE, van Geldermalsen M, Guan YF, Wahi K, Mayoh C, Balaban S, et al. Glutamine addiction promotes glucose oxidation in triple-negative breast cancer. Oncogene. 2022;41(34):4066-78.
37. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. Curr Protoc Immunol. 2001;Chapter 20:Unit 20.2.
38. Arroyo-Crespo JJ, Armiñán A, Charbonnier D, Deladriere C, Palomino-Schätzlein M, Lamas-Domingo R, et al. Characterization of triple-negative breast cancer preclinical models provides functional evidence of metastatic progression. Int J Cancer. 2019;145(8):2267-81.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

© 2026 by the author(s). Distributed under a Creative Commons CC BY license.