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The role of β-Hydroxy-β-methyl butyrate (HMB) in glioma growth suppression in mice

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Abbreviations

1. **GBM**: Glioblastoma Multiforme
2. **GBA:** Gut-Brain Axis
3. **HMB:** Beta Hydroxy Beta Methyl Butyrate
4. **LGGs**: low-grade gliomas
5. **HGGs**: high-grade gliomas
6. **TICs:** tumor-initiating cells
7. **WHO**: World Health Organization
8. **GSC**: Glioblastoma Stem Cells
9. **IDH1**: Isocitrate Dehydrogenase 1
10. **RTK**: Receptor Tyrosine Kinase
11. **Ras**: Rat Sarcoma
12. **MAPK**: Mitogen-Activated Protein Kinase
13. **ERK**: Extracellular Signal-Regulated Kinase
14. **PI3K**: Phosphoinositide 3-Kinase
15. **AKT**: Protein Kinase B
16. **EGFR**: Epidermal Growth Factor Receptor
17. **VEGFR**: Vascular Endothelial Growth Factor Receptor
18. **PDGFR**: Platelet-Derived Growth Factor Receptor
19. **HGFR/c-MET**: Hepatocyte Growth Factor Receptor
20. **FGFR**: Fibroblast Growth Factor Receptor
21. **IGF-1R**: Insulin-Like Growth Factor 1 Receptor
22. **PTEN**: Phosphatase and Tensin Homolog
23. **mTOR**: Mechanistic Target of Rapamycin
24. **p53**: Tumor Protein p53

25. **DNA**: Deoxyribonucleic Acid

26. **TME**: Tumor Microenvironment

27. **ATP**: Adenosine Triphosphate

28. **ROS**: Reactive Oxygen Species

29. **CNS**: Central Nervous System

30. **BBB**: Blood-Brain Barrier

31. **GFAP**: Glial Fibrillary Acidic Protein

32. **Cx43**: Connexin 43

33. **IL-10**: Interleukin 10

34. **IFN-β**: Interferon-β

35. **NF-κB**: Nuclear Factor Kappa B

36. **TGF-β**: Transforming Growth Factor-β

37. **MHC-II**: Major Histocompatibility Complex Class II

38. **EGF**: Epidermal Growth Factor

39. **PDGF**: Platelet-Derived Growth Factor

40. **MMPs**: Matrix Metalloproteinases

41. **RNA**: Ribonucleic Acid

42. **TCA**: Tricarboxylic Acid Cycle (Krebs Cycle)

43. **SCOT**: Succinyl-CoA: 3-Ketoacid CoA Transferase

44. **IGF-1**: Insulin-Like Growth Factor 1

45. **HIF-1**: Hypoxia-Inducible Factor 1

46. **TNF-α**: Tumor Necrosis Factor α

47. **NLRP3**: NOD-Like Receptor Family Pyrin Domain Containing 3

48. **MMP-2**: Matrix Metalloproteinase 2

49. **MMP-9**: Matrix Metalloproteinase 9

50. **AMPK**: AMP-Activated Protein Kinase

51. **UCP-2**: Mitochondrial Uncoupling Protein 2

52. **CSC**: Cancer Stem Cells

53. **BCAA:** branched-chain amino acid

Summary

Glioblastoma (GBM) represents one of the most aggressive malignancies of the central nervous system (CNS), characterized by limited therapeutic options and high recurrence rate. One of the factors contributing to the poor prognosis of glioblastoma is its highly invasive nature, which enables glioblastoma cells to migrate and infiltrate adjacent healthy brain tissues. This diffuse infiltration complicates efforts to achieve complete surgical resection, thereby limiting the effectiveness of surgical interventions and contributing to the tumor's recurrence and resistance to treatment. This invasive behavior is driven by a complex interplay of genetic, molecular, and environmental factors that regulate cytoskeletal dynamics, cell adhesion, and extracellular matrix degradation. Previous studies reveal that the gut microbiota plays a significant role in glioblastoma progression through the modulation of neurotransmitters and immune responses, and also gut microbiome can influence the growth of gliomas by altering the composition of microbes and metabolites, impacting tumor development. Previously we have shown that gut microbiota plays an important role in the gut-brain axis both in physiological and glioma conditions. Here we show that in the presence of glioma, fecal metabolite β-hydroxy-β-methylbutyrate (HMB) was significantly reduced compared to the same mice before tumor cell inoculation. Of note, HMB administration reduces tumor growth and proliferation in carcinoma cells. To understand whether HMB could be effective also against glioma cells, we first demonstrated in vitro that HMB directly inhibits proliferation in both mouse and human glioblastoma cell lines and inhibits glioma cell basal cell migration and FBS-induced invasion. In in vivo studies, oral gavage of HMB to glioma-bearing mice resulted in a significant reduction in tumour volume compared to control mice, suggesting that this leucine metabolite, which is produced in the gut, may have an anti-tumour role in glioma.

Keywords: Glioblastoma, Gut-Brain Axis, Metabolites, HMB, Proliferation, Migration

1. Gliomas

According to the Central Brain Tumor Registry of the United States (CBTRUS), brain cancers are broadly classified into two main categories: gliomas and non-glioma cancers (c) as shown in **Figure 1.** Gliomas, a diverse group of brain tumors originating from glial cells, are classified based on the cell type they arise from and their histological features (Oliveira et al., 2017) Astrocytomas, arising from astrocytes, exhibit a broad range of aggressiveness from slow-growing to highly malignant forms such as glioblastoma. These tumors can occur at any age, but high-grade astrocytomas like glioblastoma multiforme (GBM) are more frequently diagnosed in adults (Hirtz et al., 2020).

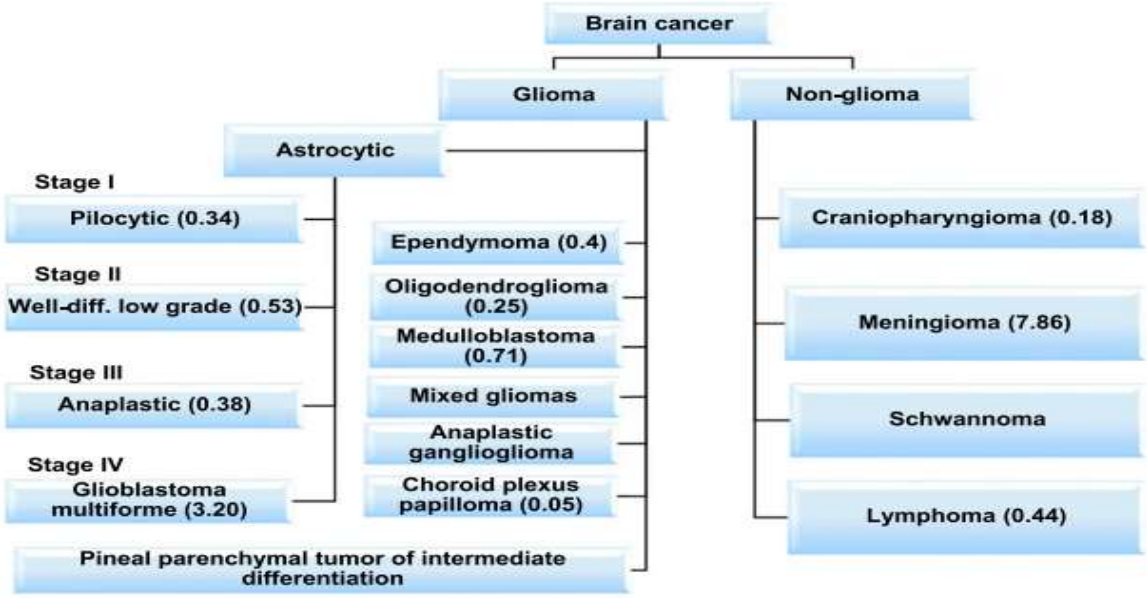


Figure 1: Classification of brain tumors based on data from the Central Brain Tumor Registry of the United States (CBTRUS) (Shergalis et al., 2018)**.**

1.1 Classification of Gliomas

The previous World Health Organization (WHO) classification of astrocytomas divided them into four grades: Grade I (pilocytic astrocytoma) slow-growing and often curable with surgery (Salles et al., 2020); Grade II (diffuse astrocytoma) infiltrative and likely to progress to higher grades (Osada et al., 2022); Grade III (anaplastic astrocytoma) malignant and aggressive (Grimm et al., 2016); and Grade IV (GBM) the most lethal, characterized by rapid growth and extensive invasiveness

(Alexander et al., 2017). The new (2021) WHO classification for gliomas includes three categories of adult-type diffuse gliomas: isocitrate dehydrogenase (IDH)-mutant astrocytoma; IDH-mutant, 1p/19q-codeleted oligodendroglioma; and IDH wild-type glioblastoma as shown in figure 2. In summary, in adults we found astrocytoma or oligodendroglioma expressing IDH-mutant that can be 2, 3 or 4 grade and GBM expressing IDH-wt that is always 4 grades. Grade 1 is associated to paediatric age or to the new class of gliomas named the "circumscribed astrocytic glioma".

Tumor Type	CNS WHO Grade
Adult-type diffuse gliomas	
Astrocytoma, IDH-mutant	2, 3, 4
Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted	2, 3
Glioblastoma, IDH-wildtype	4
Pediatric-type diffuse low-grade gliomas	
Diffuse astrocytoma, MYB- or MYBL1-altered #	1
Angiocentric glioma	1
Polymorphous low-grade neuroepithelial tumor of the young #	1
Diffuse low-grade glioma, MAPK pathway-altered *#	-
Pediatric-type diffuse high-grade gliomas	
Diffuse midline glioma, H3 K27-altered	4
Diffuse hemispheric glioma, H3 G34-mutant #	4
Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype #	4
Infant-type hemispheric glioma *#	-
Circumscribed astrocytic gliomas	
Pilocytic astrocytoma	1
High-grade astrocytoma with piloid features *#	-
Pleomorphic xanthoastrocytoma	2, 3
Subependymal giant cell astrocytoma	1
Chordoid glioma	2
Astroblastoma, MN1 altered *	-
Ependymal tumors	

Newly recognized tumor types in 2021 WHO classification of CNS tumors. * Definitive CNS WHO grade not established.

Figure 2: Glioma classification according to World Health Organisation (WHO) 2021(Z. Lan et al., 2024)

Despite the classification based on the glial cells, in particular astrocytic origin for gliomas, recent studies suggest that GBMs may originate from neural stem cells rather than glial cells, with mutations at different stages of neural development leading to tumour-initiating cells (TICs) that possess stem cell-like properties (Loras et al., 2023).

1.2 Histopathology of glioblastoma

Glioblastoma (GBM) is characterized by a range of histopathological features including intense mitotic activity, extensive vascular proliferation, necrosis, and cellular pleomorphism, which denotes the diverse shapes and sizes of its cells. This pleomorphism, alongside necrotic cores encircled by microvascular proliferation and pseudopalisading cells, underscores the tumor's aggressive nature and its high recurrence rates due to its invasive properties (Ohgaki & Kleihues, 2013); (Demuth & Berens, 2004).

1.2.1 Glioblastoma parenchymal invasion

In **Figure 3**, the key mechanisms by which **glioblastoma (GBM)** invades and reshapes the tumor microenvironment are shown. One of the primary drivers of GBM's aggressive invasion is its ability to penetrate surrounding brain tissue through **perivascular spaces** and **white matter tracts**. This invasive capacity is largely mediated by changes in the **extracellular matrix (ECM)**, which provides structural support to tissues. GBM cells disrupt the ECM by overexpressing **matrix metalloproteinases (MMPs)**, particularly **MMP-2** and **MMP-9**, enzymes that degrade ECM components. The degradation of the ECM allows GBM cells to invade and spread more easily through brain tissue (Ulrich et al., 2009)(Chintala et al., 1996)(Mariño et al., 2014)

These MMPs not only facilitate the breakdown of structural barriers, but also create a more permissive environment for tumor cells to proliferate and migrate, contributing to GBM’s highly invasive behavior. This invasive behavior is compounded by the tumor’s high mitotic activity, which leads to rapid and uncontrolled growth. The high cellular density and necrotic areas, surrounded by proliferative pseudopalisading cells, reflect the tumor’s adaptation to hypoxic environments and its aggressive proliferation, making surgical resection and treatment particularly challenging (Malhotra et al., 2022)(Martínez-González et al., 2012). Furthermore, GBM cell migration is influenced by the tumor microenvironment and involves a complex interplay of chemoattractants, pro-migratory

signals, and cytoskeletal reorganization, which allows cells to navigate and invade deeper into brain tissue, especially under hypoxic conditions. This migration is a response to environmental cues and a survival mechanism, which facilitates the tumor's spread and evasion of treatment (Freese, 1971); (Tamai et al., 2022)(Domènech et al., 2021)(Gilard et al., 2021). Understanding these interconnected processes of proliferation, invasion, and migration is crucial for developing effective therapies to manage GBM and improve patient outcomes.

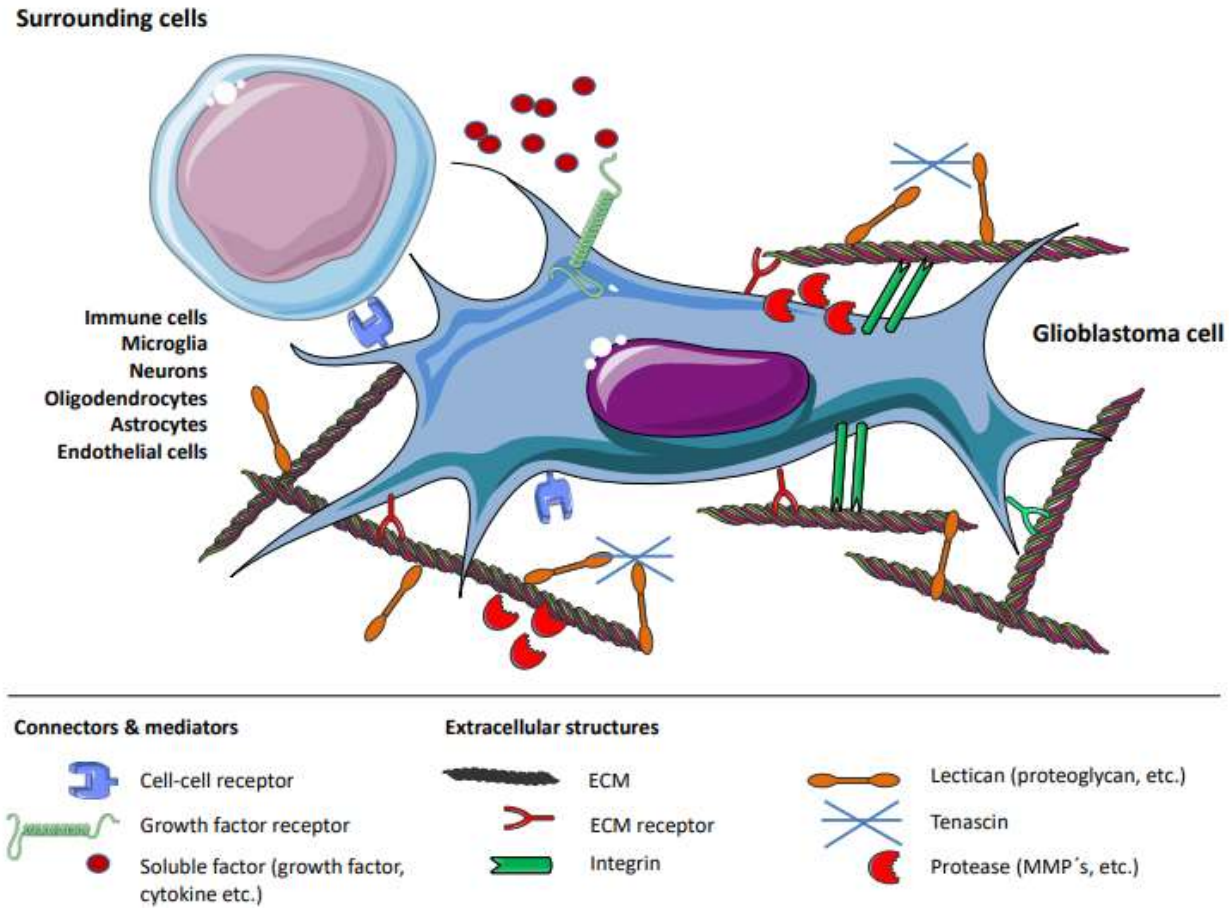


Figure 3: key mechanisms by which GBM cells invade and reshape the tumor microenvironment. These include ECM remodeling, integrin signaling, MMP secretion, growth factor and cytokine release, angiogenesis, immune modulation, and hypoxia response—collectively enhancing tumor cell migration, invasion, and survival.(Vollmann-Zwerenz et al., 2020)

1.3 Diagnosis of Glioblastoma

An accurate diagnosis of glioblastoma (GBM) necessitates the use of advanced imaging techniques to precisely locate the tumor and differentiate it from lower-grade lesions. Computed tomography (CT) is valuable for its rapid imaging capability, especially in emergencies, allowing for the detection of major mass effects, such as brain swelling or haemorrhages. However, magnetic resonance imaging (MRI) is the gold standard for detailed brain tumor assessment due to its superior soft tissue contrast. MRI provides crucial information about the tumor's borders, extent, and its relationship with surrounding brain structures. Advanced MRI sequences, including contrast-enhanced imaging, are particularly useful in identifying areas of abnormal vascularity and tumor infiltration characteristic of GBM. Additionally, magnetic resonance spectroscopy (MRS) complements these imaging techniques by offering a biochemical profile of the tumor, highlighting specific metabolic changes such as increased choline and decreased N-acetyl aspartate, which are indicative of malignant activity. Together, CT, MRI, and MRS provide a comprehensive diagnostic approach, with CT for initial assessment, MRI for detailed anatomical information, and MRS for biochemical characterization, thereby enhancing diagnostic accuracy and guiding treatment strategies (Gilard et al., 2021).

1.4 Current Therapies for Glioblastoma

The management of glioblastoma (GBM) typically involves a multimodal approach combining surgical resection, radiation therapy, and chemotherapy to optimize treatment outcomes. Surgical resection aims to remove as much of the tumor as possible while preserving neurological function. Despite the challenges posed by the tumor's infiltrative nature and its location in critical brain areas, debulking the tumor can alleviate symptoms and enhance the effectiveness of subsequent treatments (Roh & Kim, 2023). Following surgery, radiation therapy is employed to target any remaining tumor cells. This technique uses high-energy rays to damage the DNA of cancer cells, thereby inhibiting their proliferation. While effective, radiation therapy can lead to adverse effects such as cognitive

impairment and damage to healthy brain tissue (Baskar et al., 2012). Chemotherapy has historically included nitrosourea drugs like carmustine (BCNU), lomustine, and procarbazine, which were chosen for their ability to cross the blood-brain barrier (BBB). However, the introduction of temozolomide (TMZ) has marked a significant advancement in GBM treatment (Wu et al., 2021). TMZ, a prodrug converted into its active form, 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC), methylates DNA guanine residues, leading to apoptosis in tumor cells. Despite its efficacy in extending median survival from 12 to 14.6 months, cell chemoresistance remains a challenge. This resistance is partly due to the enzyme O6-alkylguanine DNA alkyltransferase (AGTor MGMT), which repairs TMZ-induced DNA damage, and other factors such as overexpression of anti-apoptotic proteins, EGFR, and p53 alterations (Singh et al., 2021). The BBB continues to pose a significant barrier to effective drug delivery, and resistance to TMZ, whether intrinsic or acquired, complicates treatment further (Gbm et al., 2023). Thus, while surgical resection, radiation, and TMZ-based chemotherapy form the cornerstone of GBM treatment, ongoing research is crucial to overcoming resistance and developing new therapies to improve patient outcomes (Ashby et al., 2016).

1.5 Signaling pathways in gliomas

Glioblastomas (GBMs) are marked by extensive gene expression abnormalities that drive their aggressive behavior, including key processes such as cellular invasion, angiogenesis, immune cell infiltration, and extracellular matrix remodeling(Mehta et al., 2018). A crucial aspect of GBM pathology is the deregulation of several signaling pathways, which play pivotal roles in tumor growth, survival, and therapeutic resistance. Among these, the **Receptor Tyrosine Kinase (RTK) family** is significantly implicated in the development and progression of glioblastomas.

As illustrated in Figure 4, RTK pathways are involved in multiple aspects of glioblastoma biology, including cell proliferation, invasion, and migration. Members of the RTK family, such as the **Epidermal Growth Factor Receptor (EGFR)**, **Platelet-Derived Growth Factor Receptor**

(PDGFR), and **Vascular Endothelial Growth Factor Receptor (VEGFR)**, are often overexpressed or mutated in GBMs. These aberrations result in the continuous activation of downstream signaling cascades that fuel tumorigenic processes.

PI3K/Akt/mTOR Pathway: This pathway regulates cell proliferation, survival, and apoptosis. It is activated by growth factor receptors like PDGFR and EGFR, leading to Akt phosphorylation and activation (He et al., 2021). Akt promotes cell survival and growth by inhibiting apoptosis and enhancing protein synthesis. In GBMs, mutations in PTEN, which normally dephosphorylates PIP3 back to PIP2, lead to persistent Akt activation and tumor aggressiveness (Cheung & Rando, 2013). Approximately 80% of GBMs show Akt hyperactivation, promoting oncogenic processes and therapeutic resistance (Tilak et al., 2021).**NF-κB Pathway**: NF-κB is a transcription factor involved in inflammation, cell survival, and immune response. Normally sequestered in the cytoplasm by IκB proteins, NF-κB is released upon IκB degradation triggered by stimuli like TNFα and IL-1β (Léger et al., 2022). In GBMs, persistent NF-κB activation promotes inflammation and tumor progression, supporting an aggressive cancer phenotype (Jacob et al., 2023).

Ras/Raf/ERK Pathway: Also known as the MAPK pathway, this cascade regulates Raf and ERK kinases. ERK1/2 activation leads to phosphorylation of various targets, including transcription factors like NF-κB (Matallanas et al., 2011). Dysregulation of this pathway contributes to glioma aggressiveness and resistance to therapy.

PKC Pathway: Protein Kinase C (PKC) is involved in numerous cellular processes. PKC isoforms are categorized into conventional (e.g., PKC-α), novel, and atypical types based on their activation requirements (Cosentino-Gomes et al., 2012). PKC-α, a conventional isoform, regulates tight junctions and cellular permeability. Increased PKC-α activity leads to disrupted tight junctions, promoting tumor invasion and growth (Nehme et al., 2023). Phorbol 12-myristate 13-acetate (PMA) is used to activate PKC in research to study its role in cancer (Batarseh et al., 2008).

JAK/STAT Pathway: This pathway transduces signals from cytokines and growth factors to the nucleus. Ligand binding activates JAKs, which phosphorylate STAT proteins. Phosphorylated STATs then regulate gene transcription related to proliferation and survival (Hu et al., 2023). Persistent STAT3 activation in GBMs promotes tumor progression, angiogenesis, and immune evasion (Gong et al., 2015).

mTOR Signaling Pathway: mTOR, downstream of PI3K/Akt, is involved in cell growth and metabolism. It exists in two complexes, mTORC1 and mTORC2. mTORC1 regulates protein synthesis and cell growth, while mTORC2 affects cell survival and cytoskeleton organization (Dibble et al., 2015). Dysregulation of mTOR signaling contributes to GBM progression and resistance to therapy (Tian et al., 2019).

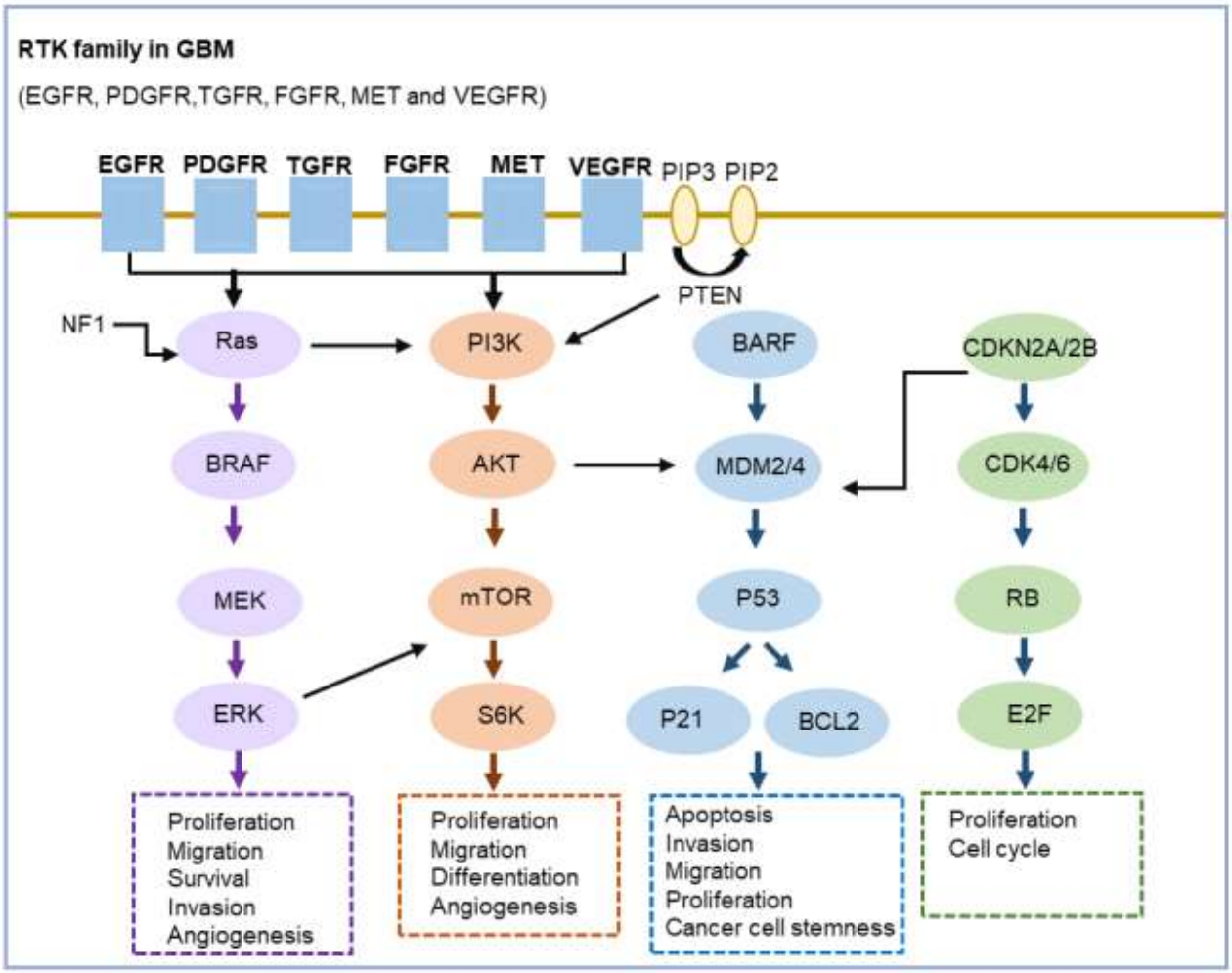


Figure 4: This schematic illustrates the main signaling pathways dysregulated in glioblastoma (GBM), highlighting their roles in promoting tumor growth, survival, invasion, and resistance to therapy (Z. Lan et al., 2024)

2. Microbiota

The term microbiota refers to microbial populations thrive on and within the human body, encompassing a diverse array of organisms including bacteria, archaea, fungi, protozoa, and viruses (Shukla et al., 2017). The terms "microbiota" and "microbiome" are used interchangeably, but the former refers to the community of microorganisms in a particular environment, while the latter refers to the complete set of genetic material of those microorganisms (Joos et al., n.d.)Bacteria are the predominant constituents, comprising over 99% of the human microbiota (Falk et al., 2019; Sender et al., 2016). The human microbiota, often considered an additional organ due to its vast microbial cell count—comparable to the number of human cells—plays a crucial role in maintaining health and influencing disease (Bäckhed, 2012). This complex ecosystem harbors approximately 10 million unique protein-encoding genes, vastly surpassing the 20,000 genes in the human genome (Ursell et al., 2012). Specifically, the gut microbiota alone contains around 3.3 million genes, approximately 150 times more than the human genome (Falk et al., 2019; Sender et al., 2016).

Despite the 99.9% genetic similarity among humans, microbiota composition varies significantly between individuals, with differences of 80%-90% in the hand or gut microbiome, making each microbiota unique, akin to a fingerprint. The microbiota is categorized by location, including the stomach, skin, mouth, urogenital tract, and respiratory system (Ursell et al., 2012)Bequero and Nombela (2012) termed the microbiome our "last organ" due to its profound health impact and species-specific genetic influence. Gonzalez et al. (2011) highlighted that, similar to Mendelian inheritance, the microbiome preserves intra-personal variation across generations within families. The intestinal microbiota is the most extensively studied component of the human microbiota. The gastrointestinal mucosa, covering approximately 200 m^2, provides a diverse habitat for commensal microorganisms and is essential for shaping and sustaining the body's microbial population

2.1 Gut Microbiota

The gut microbiota is a dynamic and complex community of microbes residing in the human gastrointestinal tract (GIT). This ecosystem includes over 3 million genes and approximately 100 trillion bacteria. While the microbial composition at the phylum level is similar across individuals, significant variability exists in the diversity and abundance of these microorganisms (Saffrey, 2014)(Ursell et al., 2012)Gut bacteria play a crucial role in various physiological processes, including metabolic phenotype shaping, innate immunity development, and epithelial regulation. They produce metabolites essential for numerous biological functions, highlighting their importance in maintaining physiological homeostasis (De Looze et al., 2023) (O'Hara & Shanahan, 2006).

Beyond the gut, humans host microbiota in other organs such as the skin, vagina, lungs, and mouth, forming a "holobiont" that describes the complex relationship between hosts and their microbial communities (Simon et al., 2019)The gut microbiota is the most diverse, encompassing bacteria, viruses, fungi, and protozoa (Ursell et al., 2012)The hologenome theory of evolution suggests that holobionts rely on cooperative microbial interactions for stability, though these interactions can also be antagonistic (Rosenberg & Zilber-Rosenberg, 2018)

2.1.1 Gut microbiota development

Gut microbiota is acquired postnatally, with initial colonization influenced by delivery mode—vaginally born infants acquire maternal microbes, while those born by caesarean section are exposed firstly to environmental microbes (Boggess et al., 2006)

Microbial communities evolve over time, with the gut microbiota expanding significantly in the first six weeks of life and continuing to develop with dietary changes and other factors (Tamburini et al., 2016)(Williams et al., 2014)

During early life, the gut microbiota is predominantly composed of Actinobacteria and Proteobacteria. By around 2.5 years of age, the microbiota undergoes significant maturation, resulting in a composition that closely resembles that of an adult microbiota (Koenig JE et al., 2015). This period of development is crucial for the stabilization of the gut flora, although its composition remains susceptible to influences such as nutrition, environmental factors, diseases, and socioeconomic status (Dethlefsen & Relman, 2011).

At different stages of human life, gut microbiota composition depends on the presence of specific species that can outcompete others. Meta-transcriptomic studies indicate that the ability of microbial members to metabolize simple carbohydrates is vital for the growth and adaptation of the ileal microbiota. This adaptability allows the microbiota to respond to varying nutritional conditions across different parts of the gut (Donaldson et al., 2015). Dietary fibers that contain microbiota-accessible carbohydrates (MACs) can significantly alter the composition of the gut microbiota, leading to substantial changes in microbial diversity and function during lifelong(Zoetendal et al., 2012)(David et al., 2014). Bile acids, produced in both the small and large intestines, play a critical role in the regulation and development of the gut microbiota. Taurocholate, a common bile acid, is particularly important for addressing intestinal dysbiosis. It aids in restoring a healthy microbial balance by promoting spore germination and serving as a homing signal for beneficial microorganisms (Ridlon JM et al., 2014(Browne et al., 2016).

The immune system is crucial in managing the gut microbiome by compartmentalizing and stratifying microbial populations, thereby preventing opportunistic infections and tissue invasion. This defence mechanism is heavily reliant on antimicrobial proteins (AMPs) produced by the host. These proteins work by enzymatically disrupting the cell walls or inner membranes of pathogenic bacteria, which helps to control microbial proliferation and support gut heal Ley R et al 2006)(Hooper & MacPherson, 2010). Understanding the interplay between nutrition and the microbiota is essential for

comprehending how gut flora influences disease processes. Metabolites produced by gut bacteria, for instance, play a significant role in the development of colon cancer (Hooper & MacPherson, 2010).

Antibiotic use, whether for therapeutic purposes or within the food chain, can disrupt the microbial ecosystem and lead to long-term imbalances. Intravenous beta-lactam antibiotics such as ampicillin, sulbactam, and cefazolin can significantly impact microbial ecology by inhibiting the production of essential metabolites like acetyl phosphate and acetyl-CoA, which are necessary for various cellular functions (Ley R et al., 2008; Ferrer M et al., 2014).

2.2 The Relevance of Gut Microbiota in Host Health, Metabolism, and Disease

The gut microbiota plays a pivotal role in maintaining host health and energy balance through a symbiotic relationship with the host. Beneficial gut bacteria assist in metabolizing indigestible substances such as dietary fibers and contribute to forming a protective barrier against pathogens while synthesizing essential nutrients and vitamins (Roberfroid et al., 1995) (Hou et al., 2022)

A key function of the gut microbiota is the production of short-chain fatty acids (SCFAs) like acetic, propionic, and butyric acids, derived from dietary disaccharides and other unabsorbed sugars. These SCFAs, with daily concentrations of 50-100 mmol/L, are crucial for gastrointestinal motility, inflammation regulation (figure 5), and energy extraction (Den Besten et al., 2018) (B. Wang et al., 2017)microbes also synthesize essential nutrients, including folates, biotin, riboflavin (B2), cobalamin (B12), and vitamin K, and deconjugate bile salts while metabolizing xenobiotics (Bergman et al., 1990; Hill et al., 1997; Koppel et al., 2018,(Hou et al., 2022).

Recent studies reveal that gut microbes produce neurotransmitters and neuromodulators affecting gut motility and sensory perception (Lesniewska et al., 2006). Variations in microbiota composition can impact brain function, behavior, and cognition (A. H. Smith et al., 2015)SCFAs like butyrate not only serve as an energy source for colonocytes but also act as histone deacetylase (HDAC) inhibitors,

influencing cancer-related signaling pathways and liver function through mechanisms such as miR-22 upregulation (Donohoe et al., 2011)(Pant et al., 2017).

The gut microbiota's composition is influenced by diet, disease, stress, genetics, and environmental factors. Dysbiosis, or microbial imbalance, is associated with various health issues, including metabolic disorders, immune dysfunction, and neurological conditions (Kho & Lal, 2018) (Li et al., 2018; Rogers et al., 2016,)(Hou et al., 2022). For instance, obesity is linked to increased Firmicutes and Actinobacteria, while type 2 diabetes is associated with decreased butyrate-producing bacteria like Prevotella and Clostridium coccoides ((Koliada et al., 2017)(J. Wang et al., 2012). Autoimmune and immune-mediated disorders such as multiple sclerosis and rheumatoid arthritis have also been connected to dysbiosis (Li et al., 2018) (Y. Fan & Pedersen, 2021)

In gastrointestinal inflammatory diseases like inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), there is an overgrowth of pathogens such as Enterobacteriaceae and Escherichia coli, and a reduction in butyrate-producing bacteria (Machiels et al., 2014)(Vatanen et al., 2016). Recent research has also explored the gut microbiome's influence on brain tumors, noting that microbial community alterations can affect tumor development and treatment outcomes (Y. Wang & Kasper, 2014). Studies have shown that treatment with drugs like temozolomide can significantly alter the microbiome, impacting immune suppression and enhancing the effectiveness of cancer immunotherapies (Patrizz et al., 2020)(Dees et al., 2021).

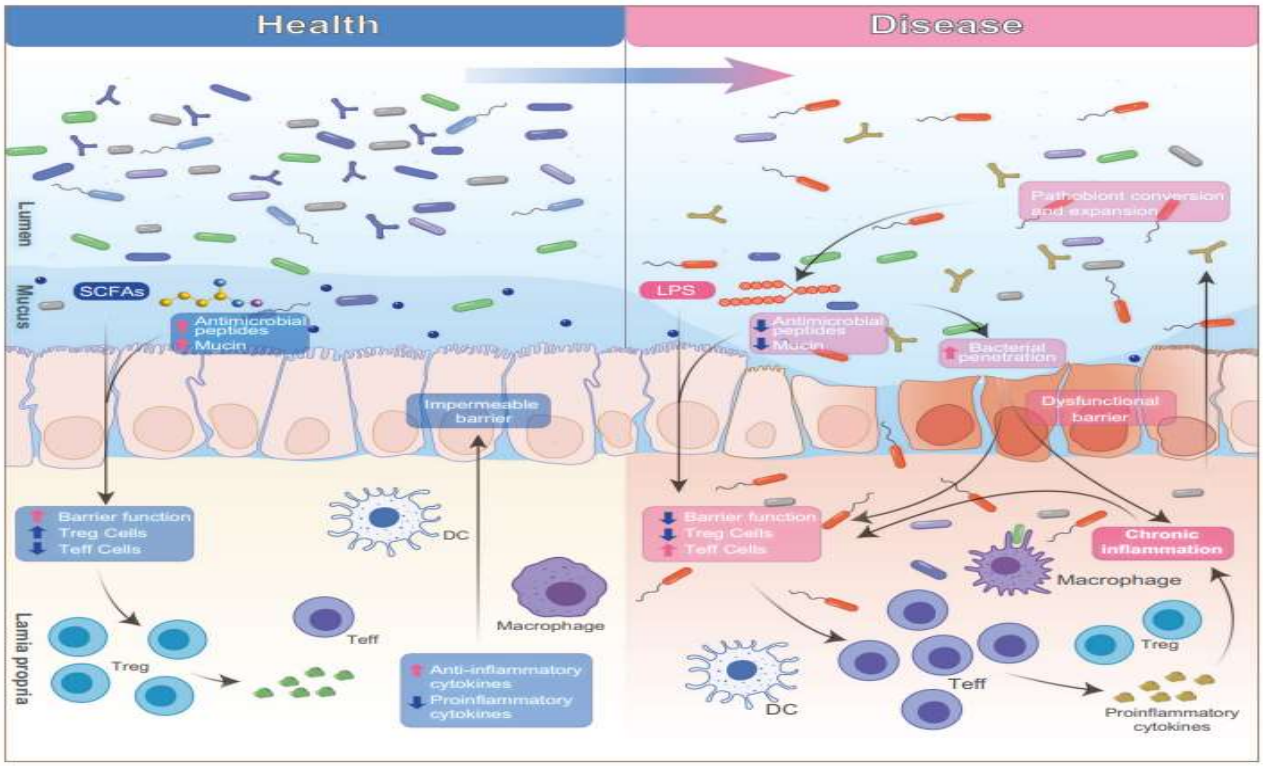


Figure 5: This schematic demonstrates the factors affecting microbiota-associated chronic inflammation in healthy and diseased states (Hou et al., 2022)**).**

2.3 Gut-brain axis

The gut-brain axis is a bidirectional communication network connecting the central nervous system (CNS) and the enteric nervous system (ENS), integral for linking gut microbiota with neurological health. Figure 6 shows the bidirectional communication between the gut and brain (Mayer eta al 2022). While early research concentrated on gut microbiota's effects on gastrointestinal disorders like irritable bowel syndrome (IBS) (Mayer et al., 2015)recent studies have expanded to show that dysbiosis also impacts CNS function and behavior (Xuelian et al., 2009;(Cryan & Dinan, 2012).Preclinical studies using wild-type and germ-free (GF) mice reveal that imbalances in gut microbiota can alter brain signaling and behavior, leading to memory impairments, reduced neurotransmitter receptor expression, and changes in neuronal excitability after probiotic treatments (Gareau et al., 2011)(Kennedy et al., 2012). However, the unique conditions of GF mice limit the direct applicability of these findings to humans, where microbiota variability adds complexity to clinical translations.

Recent advances in brain imaging have linked microbial ecology with neural network activity. For example, manipulating the gut microbiome with antibiotics has been shown to improve brain connectivity and cognitive function in patients with mild hepatic encephalopathy (Mayer et al., 2015)(Dono et al., 2022b). This research underscores the gut-brain axis's crucial role in connecting gastrointestinal health with neurological outcomes.

The gut-brain axis functions through neuroendocrine, immunological, and autonomic pathways, with the autonomic nervous system (ANS) playing a key role. The vagus nerve, a major component of the ANS, transmits signals from the gut to the brain's nucleus tractus solitarius (NTS), influencing brain function and behavior (Deplancke & Gaskins, 2003)(Cryan & Dinan, 2012). Gut microbiota produces metabolites like short-chain fatty acids (SCFAs), which are important for modulating neuroendocrine and immune responses. SCFAs found in cerebrospinal fluid and brain tissue affect immune regulation and neurotransmitter levels, highlighting the significant impact of gut microbiota on CNS health (Clemente et al., 2012)(L. Wang et al., 2022).

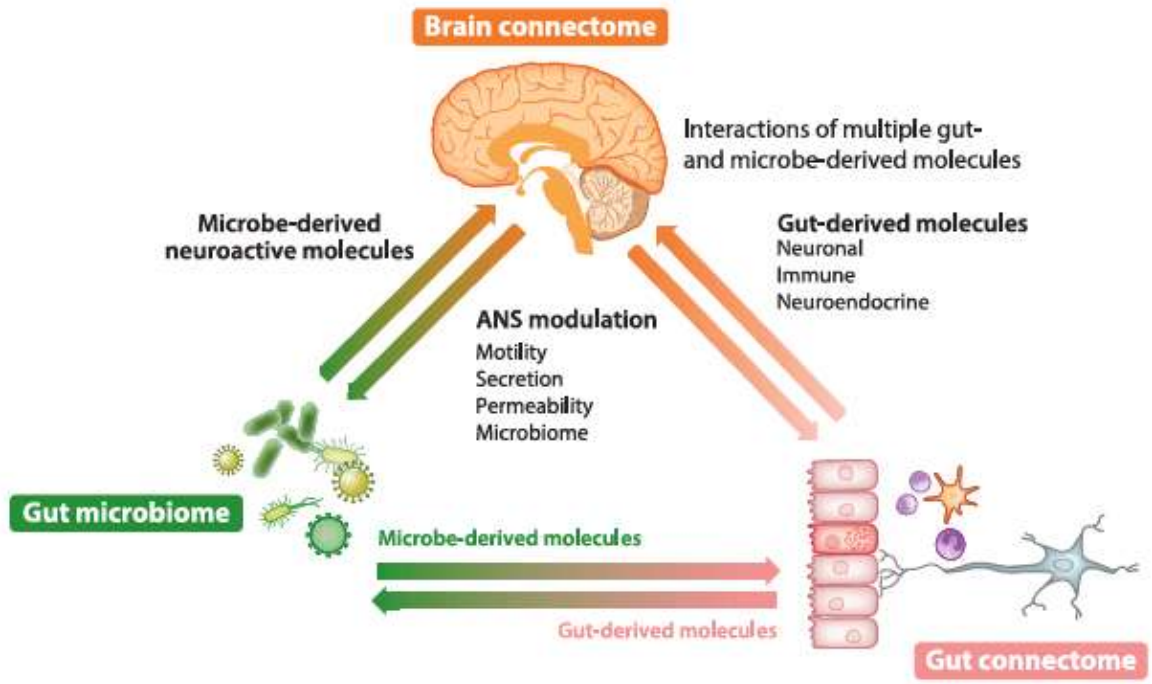


Figure 6: The gut-brain–microbiome (GBM) network is a complex system with three interconnected nodes: the brain connectome, the gut connectome, and the gut microbiome. These nodes communicate bidirectionally through feedback loops. The gut microbiota can influence the brain via signaling molecules or the gut–brain axis, while the brain can modify the microbiome directly or by altering the gut environment. The autonomic nervous system (ANS) plays a key role in this communication, maintaining the system's balance. (Mayer et al., 2022)

2.3.1 Pathways involved in the gut-brain axis

The interaction between gut microbes and brain cells involves a complex network of neurological, endocrine, immunological, and metabolic processes, reflecting a bidirectional connection between gut microbiota and brain function (Y. Wang & Kasper, 2014)(De Looze et al., 2023)

Immune Pathway:

Gut microbiota influences immune cell activity, affecting both innate and adaptive immune responses. Disruptions in microbial signaling can trigger immune activation, leading to inflammation and autoimmune diseases (Y. Wang & Kasper, 2014)(Cryan & Dinan, 2012)Commensal bacteria produce metabolites like short-chain fatty acids (SCFAs) and neuromodulators, which modulate immune responses by releasing cytokines, chemokines, and neurotransmitters. These messengers can enter the bloodstream, impacting the central nervous system (CNS) via the vagus and spinal nerves (Fülling et al., 2019)(De Looze et al., 2023)

Endocrine Pathway:

The gut microbiota regulates the hypothalamic-pituitary-adrenal (HPA) axis, crucial for stress responses and gut physiology, including motility and secretion;(Zhou et al., 2023) (Dinan, 2017)Enteroendocrine cells (EECs) integrate microbial signals to maintain gut homeostasis (Y. Wang & Kasper, 2014)(Cryan & Dinan, 2012) L cells release hormones influencing hunger and energy balance, while enterochromaffin cells produce serotonin from dietary tryptophan, influenced by gut bacteria (Ursell et al., 2012)(Dinan, 2017)

Metabolic Pathway:

Gut commensals produce SCFAs, key metabolites from dietary fiber fermentation that support energy provision, mucosal barrier maintenance, and immune regulation. SCFAs may indirectly influence the CNS by activating receptors or inducing epigenetic changes (Dinan, 2017)Gut bacteria also produce neurochemicals like GABA, serotonin, and catecholamines, which likely affect CNS activities

through the vagus nerve or enteric nervous system (ENS) (Mazzoli & Pessione, 2016) (Cryan & Dinan, 2012)(Fülling et al., 2019).

Neural Pathway:

The neural pathway involving the vagus nerve and ENS is vital for gut-brain communication. The ENS regulates gastrointestinal activities and communicates with the CNS via parasympathetic and sympathetic routes (Cryan & Dinan, 2012). Gut microbiota affects the ENS through microbial metabolites and peptides(Y. Wang & Kasper, 2014) (Cryan & Dinan, 2012)The vagus nerve, a key parasympathetic component, facilitates bidirectional gut-brain communication, impacting body homeostasis (Fülling et al., 2019)(Ma et al., 2019). Research shows that gut microbes can alter brain processes and behavior, as demonstrated by the influence of Lactobacillus rhamnosus on anxiety and depression through vagus nerve-mediated communication (Bravo et al., 2011)(Browning et al., 2017)

2.4 Gut metabolites

2.4.1 Beta Hydroxy Beta Methyl Butyrate (HMB)

Among the plethora of microbial metabolites, β-Hydroxy-β-methylbutyrate (HMB or 3H3MB or 3-Hydroxyisovaleric acid), a metabolite generated from the catabolism of the essential amino acid leucine, is known to be crucial for muscle metabolism and maintenance. Indeed, endogenous synthesis of HMB occurs by the reversible transamination of leucine to α-ketoisocaproic acid (KIC) (Duan et al., 2016), catalyzed by branched-chain amino acid (BCAA) transaminase, which is largely found in skeletal muscle. β-Hydroxy-β-methylbutyrate (HMB) is increasingly recognized for its therapeutic potential in mitigating muscle loss and weakening associated with various clinical conditions, including HIV, critical illness, aging, and cancer (Wilson et al., 2013).

As shown in figure 7, 3-Hydroxyisovaleric acid (3-HIVA, red square) is a significant intermediate also in the bacterial catabolism of the essential L-leucine amino acid. During the degradation of L-leucine, 3-HIVA (or HMB) is produced through several enzymatic reactions, primarily facilitated by specific bacterial enzymes, including 3-methylcrotonyl-CoA carboxylase and 3-hydroxyisobutyryl-CoA hydrolase, which are crucial for the metabolic pathways that lead to the production of various secondary metabolites and energy (Beemer et al., 1983). This catabolic process not only aids in the efficient utilization of L-leucine but also results in the generation of valuable byproducts that can serve as energy sources for bacteria or contribute to the synthesis of other essential compounds. The ability of bacteria to convert L-leucine into 3-HIVA and other metabolites highlights their metabolic versatility and adaptability, allowing them to thrive in various environments and contribute to the overall biochemical cycles within ecosystems.

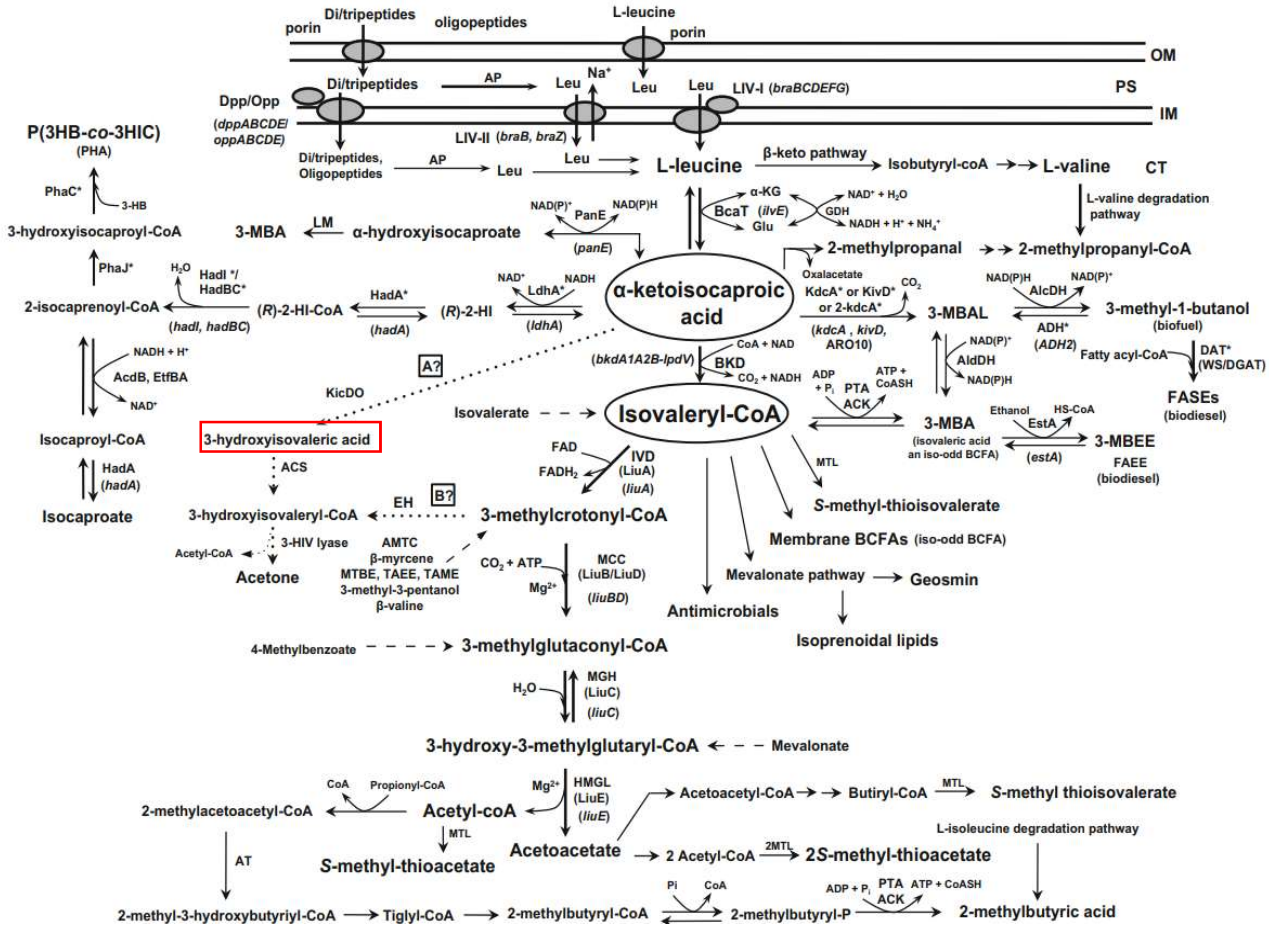


Figure 7: The bacterial L-leucine catabolism pathway involves several key steps and enzymes, leading to various secondary metabolites(Díaz-Pérez et al., 2016)

It has been shown that β-hydroxyisovaleric acid (or HMB), a branched-chain hydroxy acid (BCHA) produced by gut microbiota, has significant antimicrobial activity, promoting the growth of beneficial bacteria like **Lactobacillaceae** and **Bifidobacteriaceae**, while inhibiting harmful bacteria such as **Enterobacteriaceae** and **Bacteroides fragilis**(Hwang et al., 2024). These probiotic bacteria play a critical role in regulating gut pH, strengthening the intestinal barrier, and producing antibacterial peptides, which are essential for maintaining gut health (Hwang et al., 2024)Meanwhile, a previous study on **Lactobacillaceae** and **Bifidobacteriaceae** found that their administration induced antitumoral effects, prolonging survival in glioma models by inhibiting the MEK/ERK cascade and enhancing gut microbiota diversity (H. Fan et al., 2024)

3-HIVA or HMB has also been linked to various metabolic disorders, such as 3-methylcrotonyl-CoA carboxylase deficiency, where its elevated levels in biological fluids can indicate underlying metabolic dysfunctions (Packman et al., 1982). Furthermore, the detection of 3-HIVA is not only pertinent in understanding microbial metabolism but also serves as a biomarker for certain genetic conditions and provides insights into prenatal diagnostics, particularly in identifying disorders related to biotin-responsive multiple carboxylase deficiency (Wilkinson et al., 1979). Overall, the study of 3-HIVA's role in bacterial metabolism and its implications for human health underscores its significance as both a metabolic intermediate and a potential diagnostic marker.

Beta-hydroxy-beta-methylbutyrate (HMB) has emerged as a promising nutritional supplement with multifaceted effects on cancer, particularly in its role as an antitumoral agent. Research indicates that HMB supplementation can influence various aspects of cancer pathology, including tumor growth, cachexia, and patient quality of life during treatment. For instance, in Walker 256 tumor-bearing rats, HMB supplementation at a dose of 320 mg/kg body weight per day significantly extended lifespan, underscoring its potential to improve outcomes in cancer models (Nunes et al., 2008)

HMB's antitumoral effects are believed to be closely tied to its ability to modulate key signaling pathways, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. This modulation is crucial, particularly in conditions characterized by muscle wasting, as HMB has been shown to reduce protein breakdown in MAC16 tumor-bearing mice(Caperuto et al., 2007). By inhibiting the NF-κB pathway, HMB not only preserves skeletal muscle but may also exert direct effects on tumor cells, thus positioning it as a valuable intervention in managing cancer-related cachexia and sarcopenia(Prado et al., 2022).

In addition to its role in muscle preservation, HMB enhances muscle protein synthesis and inhibits proteasome activity, contributing to a reduction in inflammation and tumor formation (Mochamat et al., 2017). These attributes indicate HMB's potential for use in multi-modal strategies to combat

cancer morbidity. Specifically, HMB's ability to mitigate TNFα-induced interleukin-6 (IL-6) production by inhibiting NF-κB activation is particularly relevant in managing stress responses associated with cancer treatments and surgical interventions (Miyake et al., 2019).

Moreover, HMB has demonstrated efficacy in alleviating adverse effects of cancer treatments. For example, in patients undergoing treatment for advanced hepatocellular carcinoma, HMB reduced sorafenib-related hand-foot skin reactions (HFSR) when used in conjunction with standard care (Naganuma et al., 2019)While HMB may not directly improve survival, its role in enhancing the quality of life during cancer therapy is significant.

HMB's impact extends to autophagy regulation, particularly in contexts of muscle atrophy induced by pharmacological stimuli such as dexamethasone. HMB has been shown to normalize aberrant autophagy pathways, reduce lysosomal proteolysis, and promote autophagosome formation via the Akt/FoxO signaling axis, essential for cellular homeostasis (Mochamat et al., 2017). This regulation may provide therapeutic avenues for not only cancer but also metabolic disorders like diabetes, where maintaining balanced autophagy is vital to minimizing detrimental effects such as excessive cell death.

2.5 Role of the microbiota in oncology

Microbial metabolites significantly influence both cellular and immune pathways, playing a central role in cancer development and progression. Genotoxic metabolites, such as colibactin and reactive oxygen species (ROS), induce DNA damage, facilitating tumor initiation.(Lopez et al., 2021) In contrast, short-chain fatty acids (SCFAs) like butyrate regulate cell proliferation and apoptosis by inhibiting histone deacetylases (HDACs). Secondary bile acids and hydrogen sulfide activate oncogenic pathways, including NF-κB and Wnt/β-catenin, further promoting tumorigenesis.(Dong et al., 2023) Dysbiosis-induced metabolic alterations contribute to these effects, particularly in

metabolic diseases like brain tumors, which often coexist with imbalances in gut microbiota, exacerbating tumor growth, migration, and progression.(Kandpal et al., 2022)

On the immune front, microbial metabolites also exhibit diverse effects. Lipopolysaccharides (LPS) activate Toll-like receptor 4 (TLR4), inducing pro-inflammatory responses that promote chronic inflammation, which is closely linked to cancer.(Zheng et al., 2020) (Aljarrah et al., 2024)Conversely, SCFAs enhance anti-inflammatory regulatory T cells (Tregs) and cytotoxic T cell activity, while other metabolites recruit myeloid-derived suppressor cells (MDSCs) to suppress immune responses, thereby facilitating tumor immune evasion(Goldmann & Medina, 2024). These interactions can also influence the effectiveness of immunotherapies. For instance, metabolites from *Akkermansia muciniphila* have been shown to enhance the efficacy of immune checkpoint inhibitors, suggesting that modulation of the microbiota could be a promising therapeutic approach(Xia et al., 2024).

The role of microbiota in cancer development extends through both **contact-dependent** and **contact-independent** interactions. Contact-dependent interactions occur locally at the mucosal surface or within primary and secondary lymphoid organs, such as the bone marrow, thymus, GALT, lymph nodes, spleen, and tumor microenvironment (TME)(Zhao et al., 2023). For example, gut microbes can directly interact with the gastrointestinal tract mucosal surface, leading to genotoxic effects, epithelial cell proliferation, loss of cellular polarity, and intestinal metaplasia. Additionally, microbiota can stimulate hematopoiesis in the thymus and bone marrow, particularly through RIG-IFN-1 signaling, which may provide a radioprotective effect in radiotherapy(Kaur et al., 2022).

Contact-independent interactions, mediated by microbial metabolites and outer membrane vesicles (OMVs), circulate systemically and influence immune responses. Microbial metabolites and OMVs interact with the GALT, lymph nodes, and spleen, modulating T cell and dendritic cell activity through pathways such as enhancing TH17 responses, IFN production, antigen presentation, and signaling via IFN-1, IL-12, and TLR4. In the TME, microbes can exert both immunostimulatory

effects, such as presenting microbial-specific antigens to T cells, and immunosuppressive effects by modulating the balance between Tregs and tumor-infiltrating lymphocytes (TILs). (Zhao et al., 2023)Furthermore, microbial-secreted metabolites and OMVs can influence the innate immune response in the TME by attracting and activating immune cells like neutrophils, which produce TNFα and ROS to combat tumorigenesis, and modulate adaptive immune responses by co-stimulating T cells(Cullin et al., 2021).These complex interactions demonstrate how microbiota and their metabolites influence cancer progression and immunology through both direct contact-dependent mechanisms and systemic, contact-independent pathways. Understanding these multifaceted roles provides a foundation for developing microbiota-targeted strategies in oncology

2.6 Gut-brain axis and glioblastoma

Emerging research indicates that the gut microbiota may significantly affect glioblastoma progression and treatment outcomes. In glioma mouse models, tumor growth is linked to gut dysbiosis, marked by changes in the Firmicutes-to-Bacteroidetes ratio and an increase in the Verrucomicrobia phylum, particularly *Akkermansia* (Patrizz et al., 2020). The chemotherapeutic agent temozolomide (TMZ) can counteract these microbial alterations, with similar microbial changes observed in glioma patients, suggesting potential relevance to human disease (Dono et al., 2022a).Further studies have shown that tumor progression in these murine models of glioma is associated with reduced levels of essential short-chain fatty acids (SCFAs) and neurotransmitters such as norepinephrine and 5-HIAA, with glioma patients also having lower levels of these neurotransmitters compared to healthy controls (Dono et al., 2022a). Notably, antibiotic treatment in glioma-bearing mice accelerated tumor growth and altered immune cell activity, underscoring the potential role of the gut-brain axis in modulating brain tumor immunity (D'Alessandro et al., 2020) and also induced tumor vasculogenesis and affects the glioma microenvironment in mouse (Rosito et al., 2024). These findings suggest that the gut microbiome could influence the glioblastoma microenvironment, warranting further investigation into the underlying mechanisms (Dono et al., 2022a)

Table 1. Summary of studies evaluating the role of the gut microbiome in glioma

Study	Study subjects	Summary findings
D'Alessandro et al.[16]	Glioma-bearing mice	Antibiotic treatment of glioma-bearing mice promoted tumor growth and changed the NK cell subsets and microglia phenotype.
Dono et al.[15]	Glioma-bearing mice and glioma patients	Norepinephrine and 5-HIAA were decreased in mice and humans with glioma, compared to control. Additionally, glioma-bearing mice had decreased levels of SCFAs.
Patrizz et al.[14]	Glioma-bearing mice and glioma patients	An increased in *Verrucomicrobiota* and *Bacteroidetes* and decrease in *Firmicutes* was observed in mice and humans. In mice, TMZ administration abrogated the microbial taxa changes.
Dees et al.[76]	Humanized glioma-bearing mice	Humanized gut microbiome mouse models responded differently to PD-1 inhibitors. Taxa comparison between glioma-bearing mice that responded to anti-PD-1 revealed high abundance of *Bacteroides cellulosilyticus*.

5-HIAA, 5-hydroxyindoleacetic acid; NK, natural killer cells; PD-1, programmed cell death protein 1; SCFA, short-chain fatty acids; TMZ, temozolomide.

Figure 8: Summary of studies evaluating the role of the gut microbiome in glioma (Dono et al., 2022b)

2.6.1 Gut bacterial metabolites as therapeutics in treating Glioblastoma

The importance of gut microbiota in both health and disease has been thoroughly examined, revealing its critical role in various physiological processes and its potential influence on cancer. Metabolites produced by gut bacteria significantly impact glioblastoma (GBM) biology, affecting processes such as angiogenesis, apoptosis, and cell proliferation. These metabolites can modify immune responses, alter the tumor microenvironment, and influence dysregulated signaling pathways within GBM cells (Trejo-Solis et al., 2023). For example, butyrate, a fermentation byproduct from dietary fibers, is crucial for colon cell health and has been extensively studied for its anti-inflammatory and anticancer properties. Produced predominantly by Firmicutes species like Lachnospiraceae and Faecalibacterium prausnitzii, butyrate serves as an energy source for colonocytes, regulates cell proliferation and apoptosis, and inhibits histone deacetylase (HDAC) activity, which helps prevent cancer cell growth (Casanova et al., 2018)(Rowland et al., 2018) Sun J et al., 2024).

In addition to butyrate, other gut-derived substances also exhibit anticancer effects. For instance, the probiotic strain Propionibacterium freudenreichii produces short-chain fatty acids (SCFAs) such as propionate, which have demonstrated cytotoxic effects against several cancer cell lines (Casanova et al., 2018). Metagenomic studies reveal that variations in gut bacteria composition, such as the balance between Ruminococcaceae and Peptostreptococcaceae, may significantly impact gut health and disease outcomes (Rusling et al., 2024)(Yan et al., 2024)

One on the first evidence that the gut microbiota-derived metabolites could influence GBM progression, concerned compound K. This microbial metabolite decreased phosphorylation of PKCα and ERK1/2, expression of MMP9 and MMP2, and subsequent cell migration in glioma cells(Yan et al., 2024). Tryptophan metabolites, by activating the aryl hydrocarbon receptor (AHR) pathway, can contribute to tumor cell proliferation in various glioma models (Hezaveh et al., 2022)

Furthermore, metabolites such as acetate and glucose influence the tricarboxylic acid (TCA) cycle and key glioma markers like isocitrate dehydrogenase (IDH), thereby affecting tumor cell proliferation and survival (Mashimo et al., 2014). Alterations in the gut microbiota can influence the brain's Foxp3 expression and the likelihood of glioma development (Fan Y et al., 2022). Gut bacteria also affect neurotransmitter levels and interactions with the blood-brain barrier, which can impact the efficacy of glioma treatments (D'alessandro et al., 2021)(Vivarelli et al., 2019) Additionally, Bifidobacterium and Lactobacillus contribute to folic acid production, which is vital for cell development and maintenance, and can affect glioma prognosis by modulating pathways like MEK/ERK (Fan H et al., 2024). Finally, high-glucose diets may also alter gut microbiota, affecting immune responses and potentially inhibiting GBM growth (Kim et al., 2023)

2.6.2 Glioblastoma-associated gut bacteria

The gut microbiota plays an increasingly recognized role in the development and progression of glioblastoma (Lyu et al., 2022), the most aggressive form of brain cancer, with certain gut bacteria potentially influencing tumor growth through mechanisms such as immune modulation, DNA damage, and epigenetic changes(Liu et al., 2022). Recent studies have identified specific gut bacteria, including *Ruminococcaceae*, as protective factors that may reduce the risk of GBM by producing immune-priming metabolites like glucomannan polysaccharides, which enhance immune responses, especially when the blood-brain barrier (BBB) is compromised (Sakanaka et al., 2022). Similarly, *Faecalibacterium*, known for producing butyrate—a short-chain fatty acid with anti-tumor properties—reduces tumor necrosis factor (TNF) secretion and induces cancer cell apoptosis through histone deacetylase (HDAC) inhibition (Zhou et al., 2023). Other beneficial bacteria such as *Anaerostipes* and *Lachnospiraceae UCG004* also exhibit anti-inflammatory and immunomodulatory effects that may further regulate the immune response during GBM development (Zhou et al., 2023). Conversely, harmful bacteria like *Eubacterium brachy group* have been associated with GBM risk, potentially through pro-inflammatory pathways (Ju et al., 2021). Microbial dysbiosis—an imbalance

in gut bacteria—can promote a tumor-friendly environment characterized by increased immunosuppressive activities, such as reactive oxygen species (ROS) production and inhibition of glioma cell apoptosis, leading to immunosuppression within the tumor (Asseri et al., 2023). The impact of gut microbiota extends to GBM treatment, as dysregulated gut flora can alter the tumor microenvironment and influence responses to therapies like temozolomide(Guo et al., 2020) . Metabolites produced by gut bacteria, such as butyrate and tryptophan, are known to affect tumor growth, immune cell function, and inflammation, with butyrate inducing regulatory T-cell differentiation to modulate immune responses (Guo et al., 2020)in GBM. The integration of Mendelian Randomization (MR) analysis has helped establish a causal link between specific gut bacteria and GBM, highlighting the protective roles of *Ruminococcaceae* and *Faecalibacterium*, while also indicating the harmful potential of *Eubacterium* (S. Wang et al., 2023). This emerging research underscores the importance of investigating the detailed mechanisms by which gut microbiota influences GBM, suggesting that bacteria belonging to the families of *Ruminococcaceae* and *Faecalibacterium* hold promise as therapeutic targets, while understanding the exact pathways involved is crucial for future studies;(Y. Lan et al., 2024) (S. Wang et al., 2023)Ultimately, the gut-brain axis represents a promising frontier for cancer research, offering new insights into potential treatments for GBM.

3. Aim of the Project

Glioblastoma multiforme (GBM) is an aggressive brain tumor characterized by rapid growth and poor prognosis, largely due to its cellular, molecular, and metabolic heterogeneity, which drives tumor recurrence(Vollmann-Zwerenz et al., 2020). A key challenge in treating GBM is its extensive infiltration into surrounding brain tissue, complicating surgical resection and allowing tumor cells to migrate across the brain, which also facilitates resistance to conventional treatments such as irradiation and chemotherapy (Gbm et al., 2023)Given the limited success of current treatments, there is growing interest in exploring combination therapies alongside standard approaches. Recent research highlights the significant impact of gut microbiota on cancer growth, prognosis, and treatment efficacy (Ishaq et al., 2024). Gut bacteria and their metabolites, such as β-hydroxy-β-methylbutyrate (HMB), influence the effectiveness of anticancer therapies by remodeling the tumor microenvironment and modulating tumor immunity(Z. Lan et al., 2024).

Our results using nuclear magnetic resonance (NMR) analysis of fecal samples from tumor-bearing mice revealed reduced levels of HMB in the feces compared to healthy mice. This finding is associated to existing literature demonstrating HMB's antitumor effects in various cancer models, including Walker 256, MAC16, hepatocellular carcinoma (HCC), and TE-1 cells, where it reduced tumor growth, inhibited proliferation, and improved survival outcomes (Caperuto et al., 2007; Prado et al., 2022; Miyake et al., 2019). This evidence prompted us to investigate the role of HMB also in glioblastoma. We have assessed its antitumor effects in glioma both in vitro and in vivo, and we examined how HMB influences glioma cell migration and invasion. This research may offer new insights into HMB as a potential therapeutic strategy for glioblastoma.

4. Materials and Methods

4.1 Materials

Cell culture medium, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin G, streptomycin, glutamine, amphotericin B, sodium pyruvate, goat serum, and Hoechst dye, were obtained from GIBCO Invitrogen (Carlsbad, CA, USA). Additional reagents such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS) tablets, Dulbecco's phosphate-buffered saline, hematoxylin, eosin, and sodium formate were sourced from Sigma-Aldrich (Milan, Italy). The paraformaldehyde solution (4% in PBS) and secondary antibodies were supplied by Santa Cruz Biotechnology.

4.2 Animals and Cell Lines

All experimental procedures described in this study were conducted in strict accordance with the ethical standards set forth by the European Community Council Directive (2010/63/EU) dated September 22, 2010, and the Italian legislative decree D. Leg. 26/2014. Approval for the experiments was obtained from the local animal welfare committee and the Italian Ministry of Health (authorization No. 231/2015PR). The study design adhered to the ARRIVE guidelines [42].

Efforts were maximized to minimize animal distress and to reduce the number of animals utilized per experimental condition. Sample sizes were determined a priori to ensure statistical validity while adhering to the principle of reduction. Five-week-old C57BL/6N mice were purchased (Charles River, Calco, Italy) and were randomly allocated to different experimental groups. Continuous monitoring of the microbiological status of both the animals and the facility was maintained throughout the study.

The murine GL261 glioma cell line, provided by Prof. Michela Matteoli from Humanitas (Milan, Italy). The human U87 MG glioma cell line obtained from the European Collection of Authenticated Cell Cultures (ECACC). SB28-Ohlfest cells were purchased from DSMZ Leibniz Institute, Germany (ACC-880). CT2a cell lines established by Thomas N. Seyfried. Human GL15 cells were kindly provided by Dr. E. Castigli, Perugia University, Italy. Cell lines were cultured in DMEM supplemented with 20% (GL261) or 10% heat-inactivated fetal bovine serum (FBS). The culture medium also contained 100 IU/ml penicillin G, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 and were sub-cultured upon reaching confluency.

4.3 MTTAssay

GL261, U87MG, CT2A, GL-15 and SB-28 cells were plated in 96-well plates at a density of 5000 cells per well in case of SB28 we only seed 1500 cells in each well and treated with two dosages of HMB (Beta Hydroxy Beta Methyl Butyarate) (100, and 250µM) to determine cell viability. The MTT assay was used to measure cell viability. After treatment, add 500 µg/ml MTT solution to each well and incubate for 1.5 hours at 37°C with 5% CO_2. The resulting formazan crystals were dissolved in DMSO. Optical densities (OD) were then measured at 570 nm with a Tecan Infinite F Nano+ plate reader, and the results were processed using Tecan iControl 2.0 software. Cell viability was measured by comparing the change in absorbance over time with OD values at 570 nm.

4.4 Wound healing assay

A wound-healing assay was employed to assess the migratory capacity of GBM cells under both control and HMB treatment. Equal numbers of cells (5×10^4 cells/ml) were seeded into the inner chambers of cell culture inserts (Ibidi, Germany) placed within a Petri dish. Once the cells adhered to the substrate, the inserts were carefully removed, creating a 500 µm cell-free gap where the cells could migrate. To differentiate between the effects of cell migration and proliferation on wound closure, the cell cycle inhibitor hydroxyurea (5 mM, Sigma-Aldrich) was added during the course of

the experiment. Cell migration was monitored by capturing images of the wound areas at specific time points using a CoolSNAP camera (Photometrics) attached to an ECLIPSE Ti-S phase-contrast microscope (Nikon). The images were subsequently analyzed with ImageJ software (NIH). The extent of wound closure was quantified using the following formula: [1 − (empty area at X hours/empty area at 0 hours)] × 100

4.4 Invasion Assay Protocol

Fluorescent GL261 and GL15-RFP cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin G, 100 µg/mL streptomycin, and 25 mM HEPES (pH 7.4). The invasion assay utilized FluoroBlok™ transwell inserts with an 8 µm pore size (Corning®) that were coated with the extracellular matrix (ECM) protein diluted in cold, serum-free DMEM. CXCL12 (100 nM) served as the chemoattractant in presence or absence of HMB. Following incubation, the transwell membranes were fixed with 4% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), and FluorSave™ reagent was used for mounting membrane face up invading cells on microscope slide with a coverslip. The RFP-labelled cells that had invaded through the membrane were visualized using a fluorescence microscope, and images were captured at a magnification of 20x. Invasion was quantified by counting the number of cells in at least 20 random fields of view per condition.

4.5 Mice housing and treatment

C57BL/6N mice were housed two to four per cage and were maintained under a 12-hour light/dark cycle in standard cages, with autoclaved bedding and drinking water, and provided with sterilized standard chow ad libitum. The mice were randomly assigned to various experimental groups. All procedures were conducted in accordance with the ARRIVE guidelines (Kilkenny et al., 2010) and received approval from the local animal welfare body and the Italian Ministry of Health (authorization No 775/2020-PR), adhering to EC Council Directive 2010/63/EU and Italian legislative decree

d.lgs.26/2014. Efforts were made to minimize animal suffering and reduce the number of animals used, with sample sizes calculated prior to the commencement of experiments.

4.6 Tumor cells transplantation and HMB treatment

Mice were anesthetized with Rompun (20 mg/ml, 75 mg/kg, i.p.) and Zoletil (50/50 mg/ml, 20 mg/kg, i.p.) before being placed in a stereotaxic head frame. Stereotaxic injections of 5.0 × 10^4 GL261 cells and 5.0 × 10^3 SB-28 cells were performed. A median incision of approximately 1 cm was made, followed by drilling a burr hole in the skull. The cells were injected into the right striatum (−2 mm lateral and +1 mm anteroposterior from the bregma) at a depth of 3 mm. A total cell suspension of 4 µL in sterile PBS was administered using a Hamilton syringe at a 1 µL/min rate. Mice were treated with HMB at a dose of 76 mg/kg body weight per day, administered in PBS. The treatment began two weeks prior to tumor implantation and continued for three weeks post-implantation. HMB was administered daily via oral gavage, with each treated mouse receiving 200 µL of the HMB solution in PBS-/-. Control mice received 200 µL of PBS -/- alone, administered under the same conditions.

4.7 Tumor volume evaluation

Twenty-one days post-tumor cell inoculation, glioma-bearing mice were sacrificed, and their brains were isolated, fixed in 4% buffered paraformaldehyde (PFA), and snap-frozen. Coronal brain cryosections, 20 µm in thickness, were prepared using standard procedures and collected at 100 µm intervals. Tumor volume was assessed using hematoxylin-eosin staining, following the manufacturer's protocol. Briefly, stained brain slices were analyzed using Image Tool 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA). Tumor volume was calculated using the formula (volume = t x ΣA, where t = thickness and A = tumor area/slice

4.8 Stool collection and processing

Fresh stool samples were collected from the animals and stored for subsequent analyses. For metabolomic analysis, the samples were rapidly frozen by immersing the tubes containing the stool in liquid nitrogen and then stored at -80°C until further use.

4.9 Sample Preparation for NMR analysis

Frozen stool samples were combined with 1.2 mL of phosphate buffer (PBS)-D2O-NaN3 (0.5% v/v). The samples were thawed for 30 minutes at 25°C and then vortexed to achieve a homogeneous solution. The supernatant was separated from the solid phase by centrifugation at 10,000 × g for 25 minutes at 4°C and filtered through a 40 µm pore filter. After the addition of 200 µL of PBS-D2O with 0.3% NaN3, the samples were centrifuged again at 10,000 × g for 25 minutes at 4°C. Finally, 600 µL of the supernatant was mixed with 60 µL of PBS-D2O-TSP (2 mM final concentration).

4.10 NMR acquirement and processing

^{1}H experiments

The ^1H monodimensional NMR spectra were recorded at 25°C on a JEOL ECZR JNM spectrometer operating at a proton frequency of 600.13 MHz. For each sample, 128 scans were acquired using a calibrated 90° detection pulse length of 8.3 µs, 64k data points, and a spectral width of 15 ppm. Presaturation was employed to suppress the water signal, and the relaxation delay was set to 7.723 s, ensuring a total acquisition time of 15 s to allow for complete resonance relaxation between successive scans.The spectra were processed by applying an exponential window function with a line broadening factor of 0.3 Hz. Following Fourier transformation, the spectra were manually phased and baseline-corrected using the BCFR protocol. Metabolite quantification was performed by comparing the integrals of specific resonances with that of the internal standard, normalized by the number of protons. Metabolite concentrations were expressed as µmol/g for brain and liver tissues, and as nmol/g for fecal water metabolites.

Bidimensional NMR Experiments

To definitively identify metabolites in the biological samples, bidimensional NMR experiments, including ^1H-^1H Total Correlation Spectroscopy (TOCSY), ^1H-^13C Heteronuclear Single Quantum Correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC), were performed on selected samples. TOCSY experiments were conducted with a spectral width of 9,025 Hz in both dimensions, a data matrix of 8,192 × 256 points, a mixing time of 80 ms, and a relaxation delay of 2 s. HSQC experiments were carried out with spectral widths of 9,025 Hz and 37,764 Hz for proton and carbon, respectively, using a data matrix of 8,192 × 256 points and a recycle delay of 2 s. HMBC experiments were acquired with spectral widths of 9,025 Hz and 37,764 Hz for proton and carbon, respectively, utilizing a data matrix of 8,192 × 256 points, long-range ^nJ_C–H coupling constants of 4, 8, and 12 Hz, and a recycle delay of 3 s

Statistical analysis

All experiments were performed at least three times. All data are reported as mean ± SEM. GraphPad Prism 8.0 software was used for statistical analysis. Data were analyzed using an unpaired t-test when comparing two groups, or two-way ANOVA when comparing more than two groups.

5. Results

5.1 Glioma growth reduces fecal HMB

To investigate if tumor growth could induce changes in fecal metabolites, NMR-based metabolomic analysis were conducted in healthy mice before glioma cell inoculation and in the same mice three weeks after tumor implantation (figure 1 a). The analysis revealed distinct metabolic profiles between the two groups. Among them, the concentration of 3-hydroxy-3-methylbutyrate (HMB) was significantly lower in the feces of glioma-bearing mice compared to healthy controls (figure 1b). This observation was based on a sample size of 10 mice per group (Healthy, 3.362 ± 1.302 µgmol/g; Glioma, 0.2602 ± 0.02533 µgmol/g; **p = 0.0055 by unpaired t-test). These results suggest a metabolic shift associated with tumor presence.

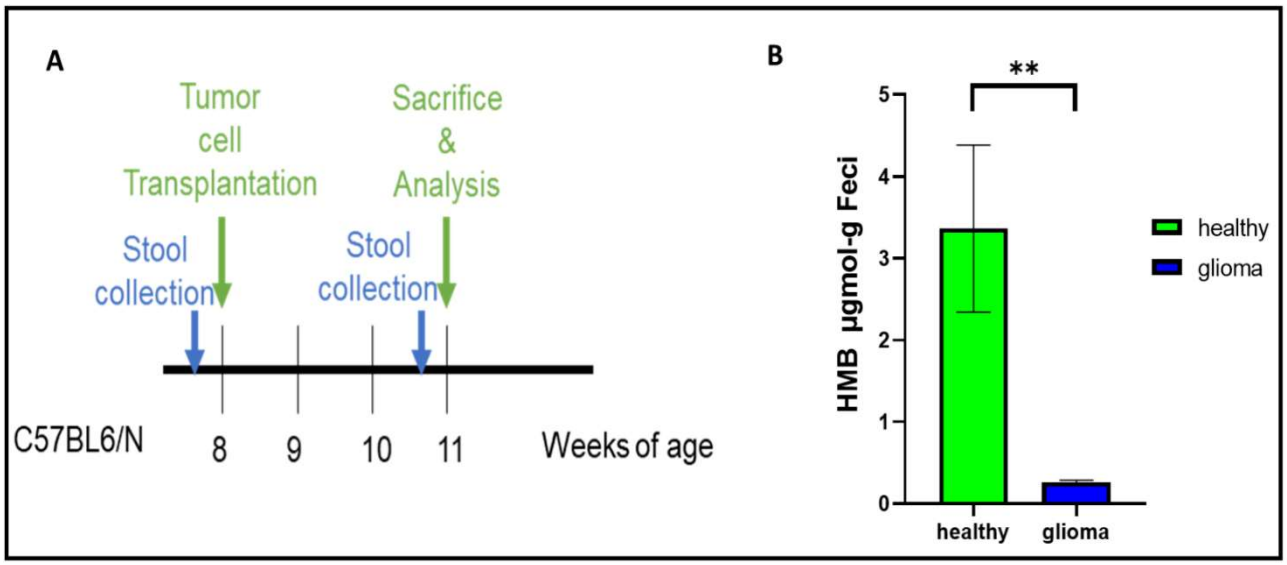


Figure1: Metabolomic Analysis Reveals Significant Alterations in Fecal Metabolites Between Healthy and Glioma-Bearing Mice. (A) Schematic of the Experimental Procedure. (B) 3-hydroxy-3-methylbutyrate (HMB) was observed higher in the faeces of healthy mice(green) as compared to mice with glioma(blue). Data are presented as mean±sem, n=10, $**p < 0.0055$ by Student's *t*-test.

5.2 HMB treatment reduces cell viability in both murine and human glioma cells

To evaluate the potential antitumor effects of β-Hydroxy β-Methylbutyrate (HMB) on glioma, both murine (GL261, CT2a, and SB-28) and human (U-87 MG, GL-15) glioma cell lines were cultured in 96-well plates, were exposed to two concentrations of HMB (100 μM and 250 μM) for up to 96 hours and checked for cell viability at four time points (24, 48, 72, and 96 hours) using the MTT assay.

In the murine glioma cell lines tested (GL261, SB28, and CT2a), HMB treatment led to a significant reduction in cell viability at 48, 72, and 96 hours compared to the control group (figure 2 A-C). Similarly, in the human glioma cell lines (U-87 MG and GL-15, figure 2D, E), HMB induced a significant reduction in cell viability from as early as 24 hours, with this effect persisting across all subsequent time points (48, 72, and 96 hours). The MTT assays for each cell line were repeated three times each in sextuplicate to ensure reproducibility. Statistical analyses, including All Pairwise Multiple Comparison Procedures (ANOVA) with Tukey post-hoc tests, were performed to assess glioma cell viability under all conditions tested. These findings suggest that HMB exerts potent anti-proliferative effects on both murine and human glioma cells. These results underscore HMB's potential as a therapeutic agent in glioma treatment, with further mechanistic studies warranted to understand its mode of action.

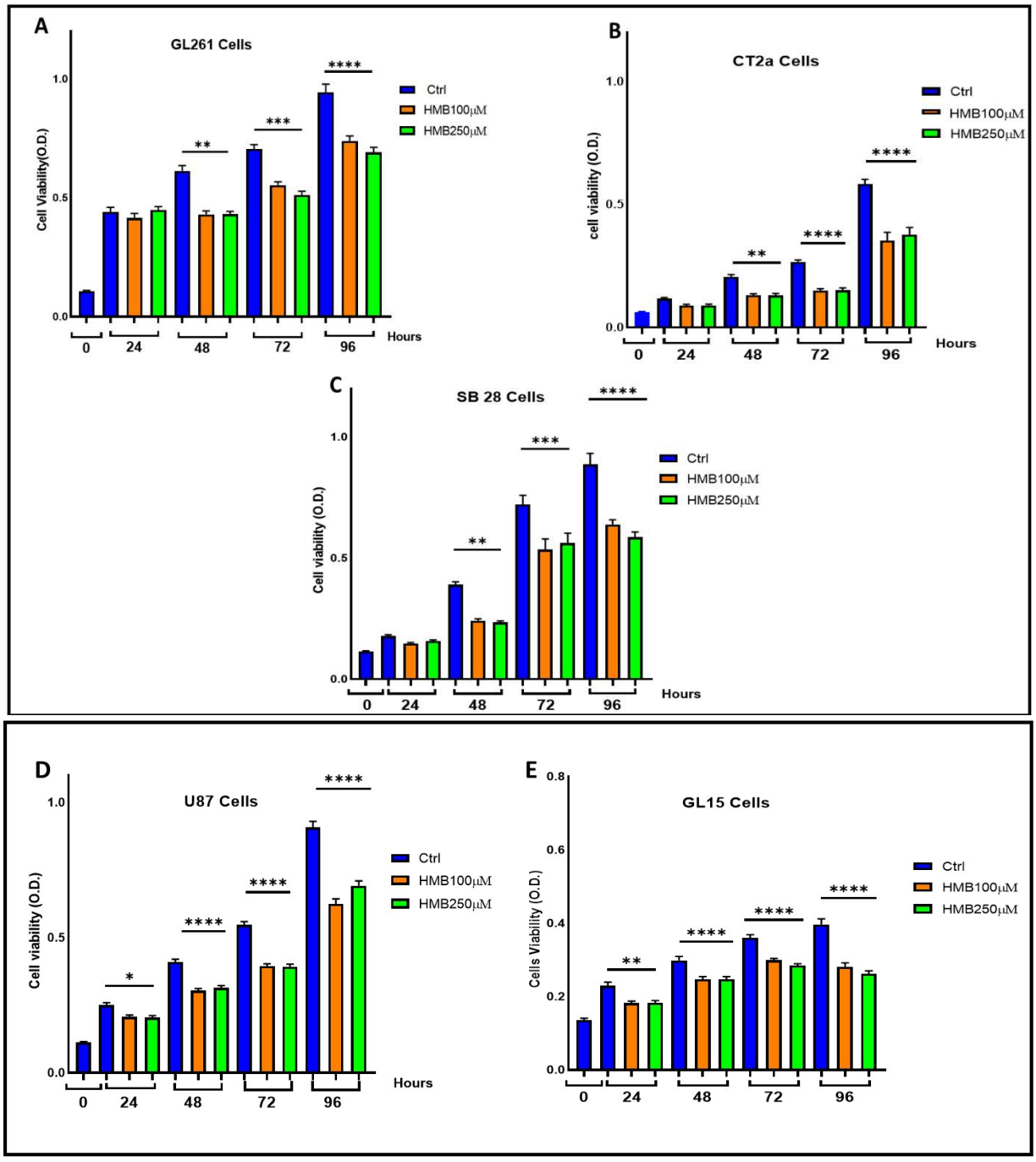
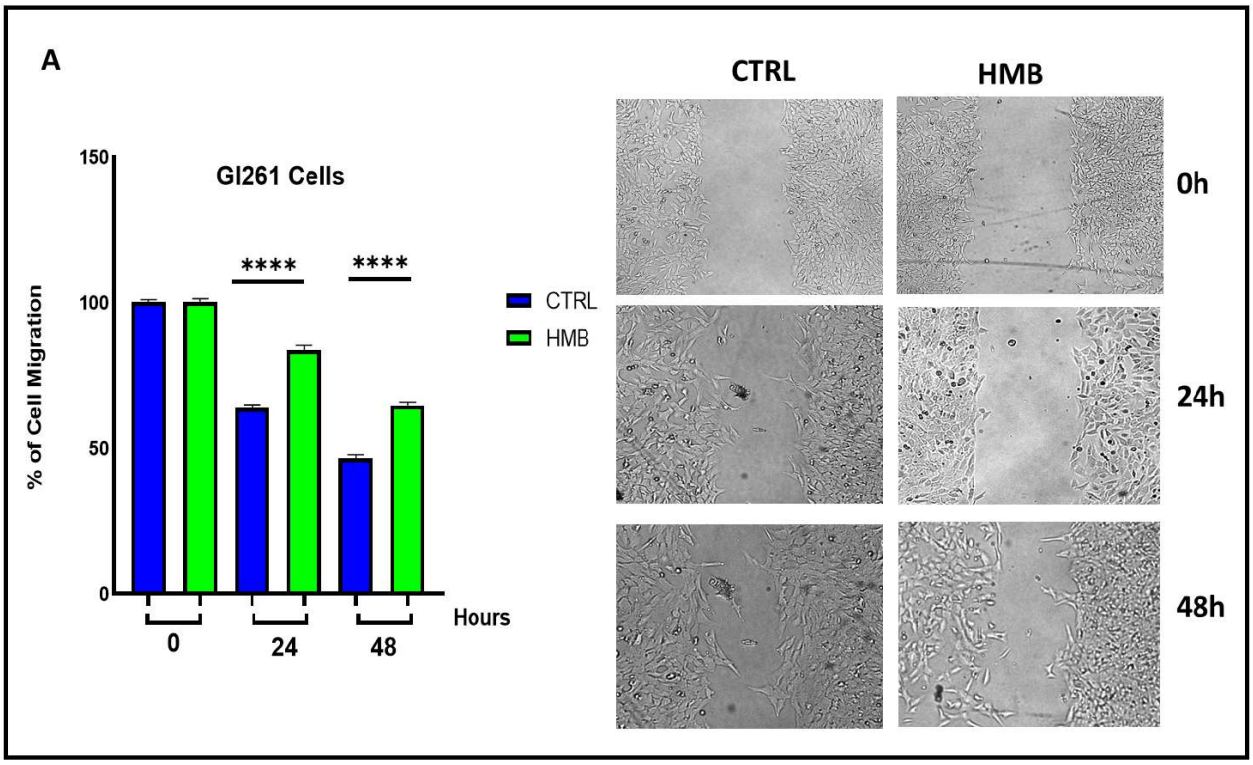


Figure 2. HMB Treatment Reduces Cell Viability in Murine and Human Glioma Cells (A) GL261 and (B) CT2a (C) SB28 murine glioma cells, as well as (D) U-87MG, (E) GL15, and human glioblastoma cells were exposed to HMB (100-250 μM) over different time points (24, 48, 72, and 96 hours), and cell viability was assessed using the MTT assay. Error bars represent means ± SEM. Significant reductions in cell viability were observed: *$p < 0.05$ and ***$p < 0.001$ in GL261 and CT2a cells; ****$p < 0.0001$ in U-87MG, GL15, and SB28 cells vs Control.

5.3 HMB Suppresses Glioma Cell Migration in Both Murine Glioma and Human Glioblastoma Cells

To assess the anti-tumor efficacy of HMB, particularly its ability to inhibit glioma cell migration, we conducted wound healing assays using both murine (GL261, SB-28) and human (U-87 MG, GL-15) glioma cell lines. 5 mM hydroxyurea was used during the experiments to inhibit cell proliferation, thereby isolating the effects of HMB on cell motility. Wound closure was evaluated at 24 and 48 hours after seeding and following HMB treatment. In the GL261 murine glioma cell line, HMB treatment significantly delayed wound closure at both 24 and 48 hours compared to control cells (****$p < 0.0001$, one-way ANOVA). Similarly, in SB-28 cells, HMB treatment also caused a significant delay in wound closure at the same time points (****$p < 0.0001$, one-way ANOVA). These findings were consistent with results observed in human glioma cell lines. Indeed, in U-87 MG cells, HMB treatment led to a marked reduction in migration at both 24 and 48 hours compared to controls (****$p < 0.0001$, one-way ANOVA). A similar pattern was noted in GL-15 cells, where HMB significantly decreased cell migration at the same time points (****$p < 0.0001$, one-way ANOVA). Differences were determined using Tukey post-hoc tests, Overall, these results demonstrate that HMB exerts a strong inhibitory effect on glioma cell migration in both murine and human models, as shown by the wound healing assay.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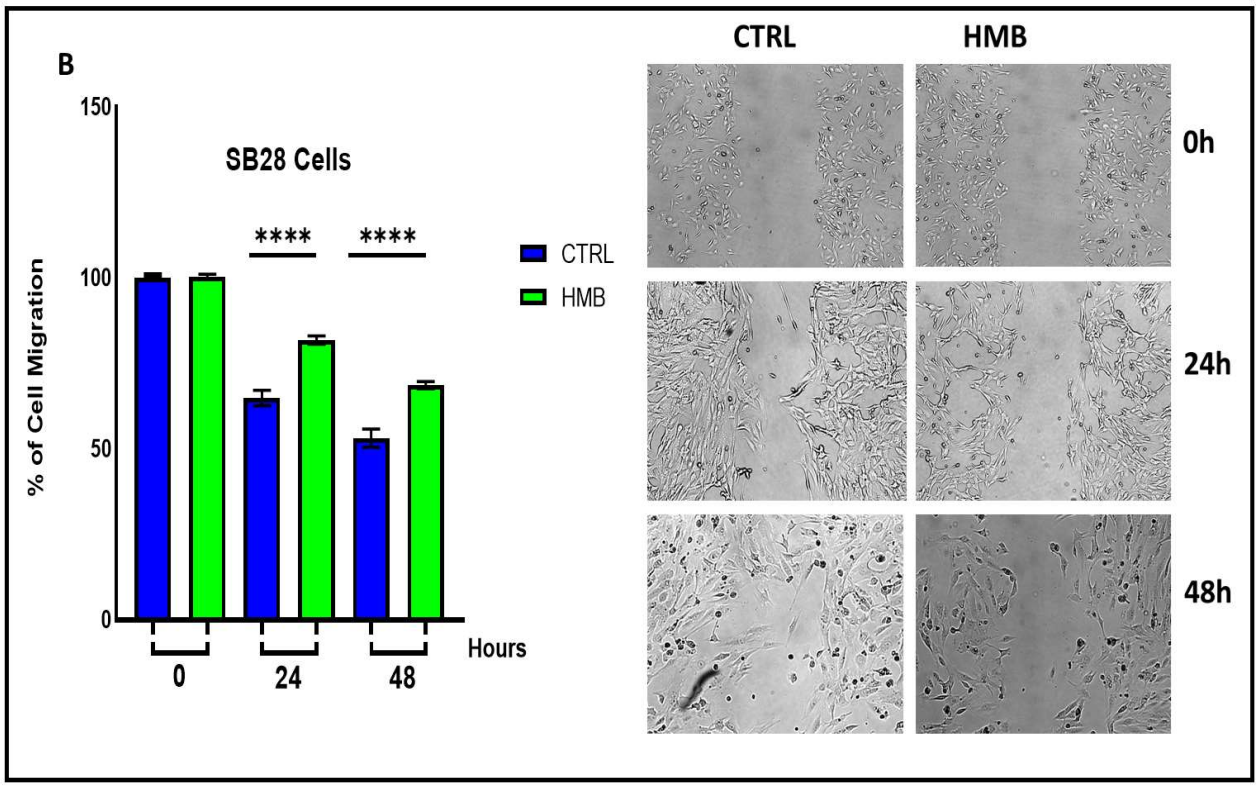
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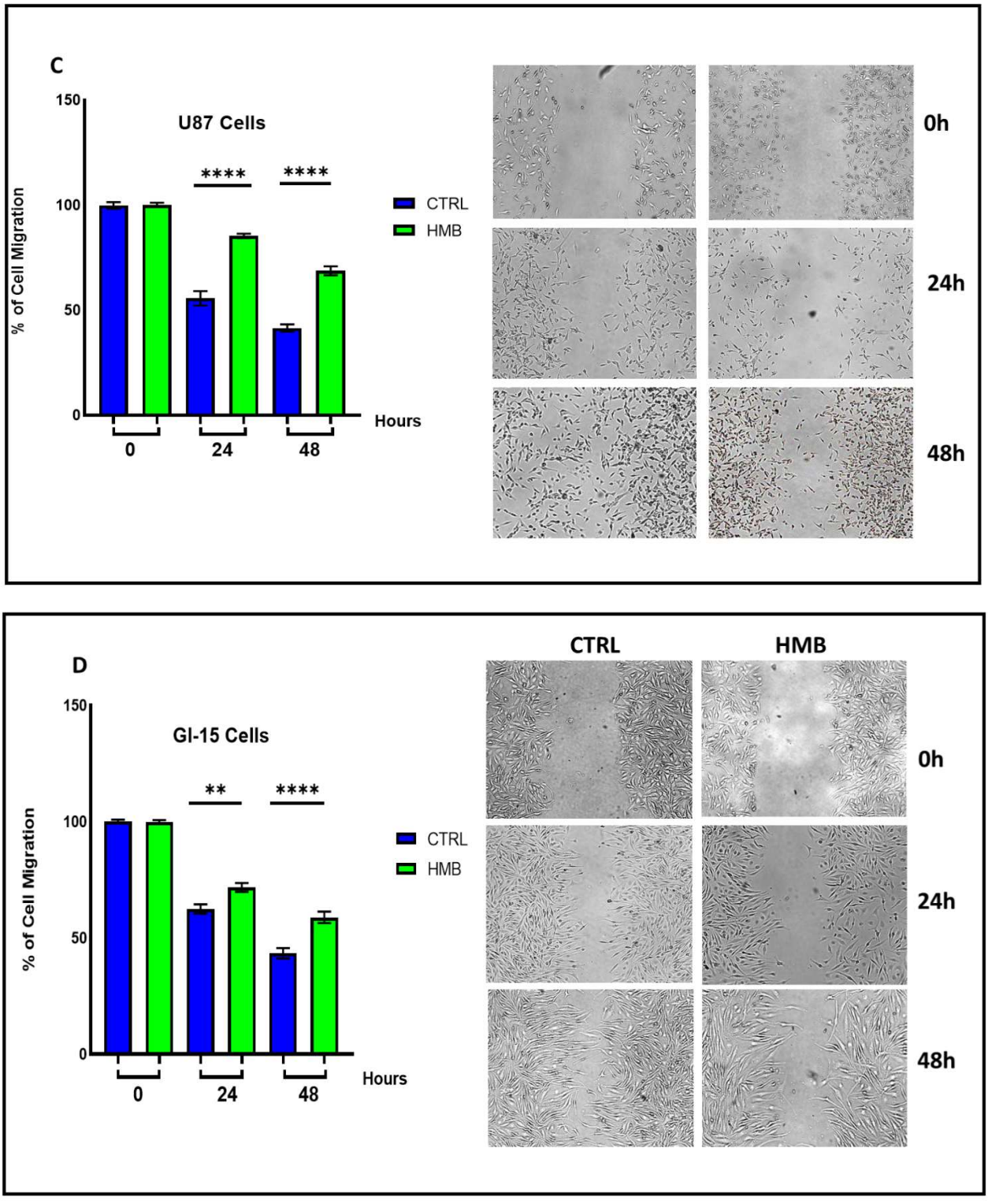

Figure 3: HMB Inhibits Glioma Cell Migration in Murine and Human Glioblastoma Cell Lines (A) GL261 (B) SB-28, murine glioma cells, along with (C) GL-15 and (D) U-87 MG human glioblastoma cell lines, were treated with HMB 500 µM Wound healing assays were performed to assess cell migration at 24- and 48-hours post-treatment. Data are expressed area as the mean percentage of wound closure relative to the 0-hour time point. and the results are displayed in the accompanying graphs. Error bars indicate mean ± SEM (****$p < 0.0001$**$p < 0.01$ vs CTRL), demonstrating that HMB significantly impedes glioma cell migration in both murine and human

glioblastoma models. Representative phase-contrast images were captured at each time point from four independent experiments. Wound areas were quantified using ImageJ software (NIH),

5.4 HMB Inhibits Glioma Cell Invasion in Murine and Human Glioblastoma Cell Lines

To further investigate the impact of HMB on glioma cell invasiveness, we conducted a Matrigel coated transwell invasion assay using fluorescent glioma cells. The results showed that HMB treatment (500 μM) effectively blocked FBS-induced tumor cell invasion in both murine (GL261, Ctrl = 41.47 ± 0.83 cells; HMB = 29.40± 0.58 cells , ****p < 0.0001 by unpaired t-test, figure 4A) and human glioblastoma cells (GL-15, Ctrl= 31.07 ± 0.51 cells; HMB= 24.44± 0.39 ****p < 0.0001 by unpaired t-test, figure 4 B) . These findings suggest that HMB not only impairs glioma cell chemokinesis but also inhibits stimulus-induced cell invasion, highlighting its potential as a therapeutic agent. The strong inhibitory effect of HMB on glioma cell invasion is depicted by the representative images showing the reduced GL-15-RFP number of treated cells passed through the pores (figure 4 C).

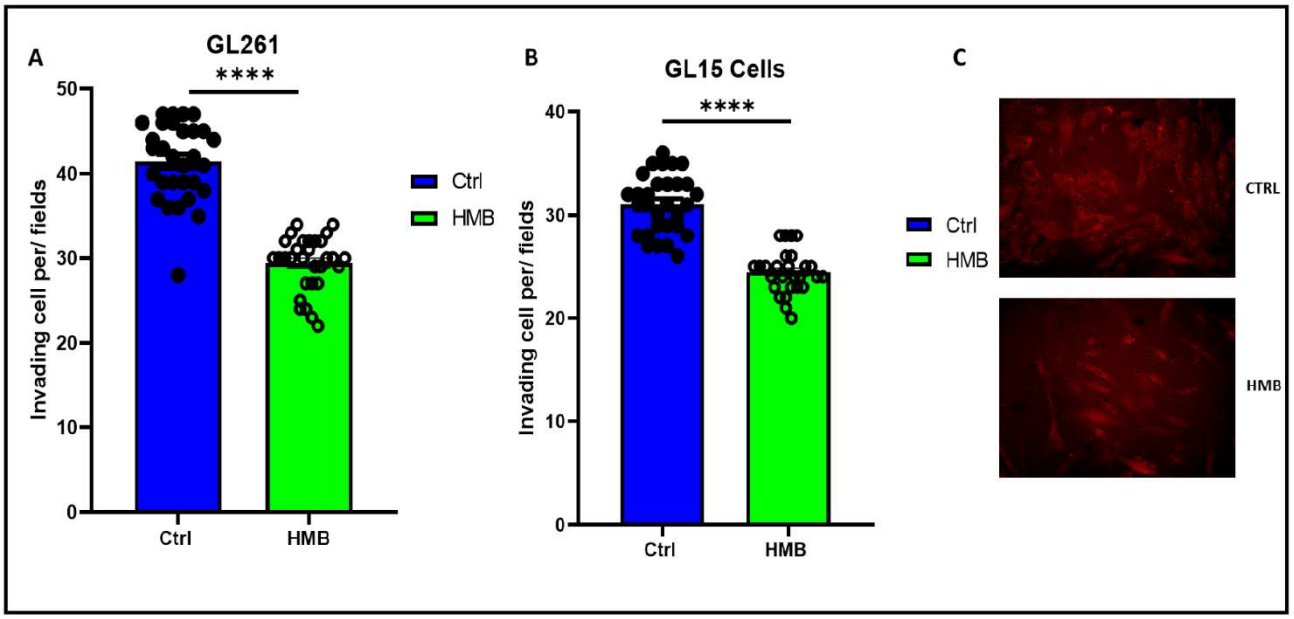

Figure4: HMB Inhibits Glioma Cell Invasion in Murine and Human Glioblastoma Cell Lines (A) GL261 murine glioma and (B) human glioblastoma GL15 quantification of cell invasion upon HMB (500 μM). Invasion was quantified as the mean number of invading cells per field of view. Data are shown as mean ± SEM, with n = 3 independent experiments and at least 10 fields of view analyzed per condition. Student's t-test revealed a highly significant reduction in invasion for both GL261 cells, demonstrating that HMB markedly inhibits glioma cell invasion in both murine and human glioblastoma models. (C) Representative image of GL-15-RFP invaded in control (upper) and in HMB (lower) condition (20x magnification)

5.5 HMB pre-treatment significantly reduces glioma volume in two murine glioma models

We investigated the therapeutic potential of β-hydroxy β-methylbutyrate (HMB) pretreatment in glioblastoma using a well-established murine glioma model in which tumour cells are inoculated into the striatum. This marks the first evaluation of HMB use for glioblastoma in vivo. Two distinct murine glioma cell lines, GL261 (5 × 10^4 cells) and SB-28 (5 × 10^3 cells) with different immunogenic impact in vivo, were implanted into C57BL/6N mice. HMB was administered via oral gavage at a dose of 76 mg/kg/day in 200 µL of phosphate-buffered saline (PBS), five times per week over a period of five weeks. Control groups received an equivalent volume of PBS alone.

HMB treatment was initiated two weeks prior to tumor inoculation and continued for three weeks post-inoculation as shown in figure 5 A. At the conclusion of the five-week treatment period, the mice were sacrificed, and tumor volumes were carefully assessed. In the GL261 cell model, HMB treatment significantly reduced tumor volumes (HMB 29.32± 3.901 mm^3, n=10) compared to the control group (CTRL 45.20 ±4.19 mm^3, n=14 *p = 0.0135, unpaired Student's t-test). Similarly, in the SB-28 cell model, HMB-treated mice exhibited a significant reduction in tumor volume (HMB 21.00 ±3.91 mm^3, n=10) compared to controls (CTRL 38.20 ± 4.37 mm^3, n=13 *p = 0.0110, unpaired Student's t-test.) The relative reduction in tumor volume upon treatment was 20% in the GL261 model and 27% in the SB-28 model when compared to respective controls.

These findings strongly suggest that HMB exerts notable anti-tumor effects in murine models of glioblastoma, positioning it as a promising preventive agent. The observed reduction in tumor volume following HMB pre-treatment highlights its potential utility in preventing glioblastoma recurrence, warranting further exploration into its underlying mechanisms and clinical applicability.

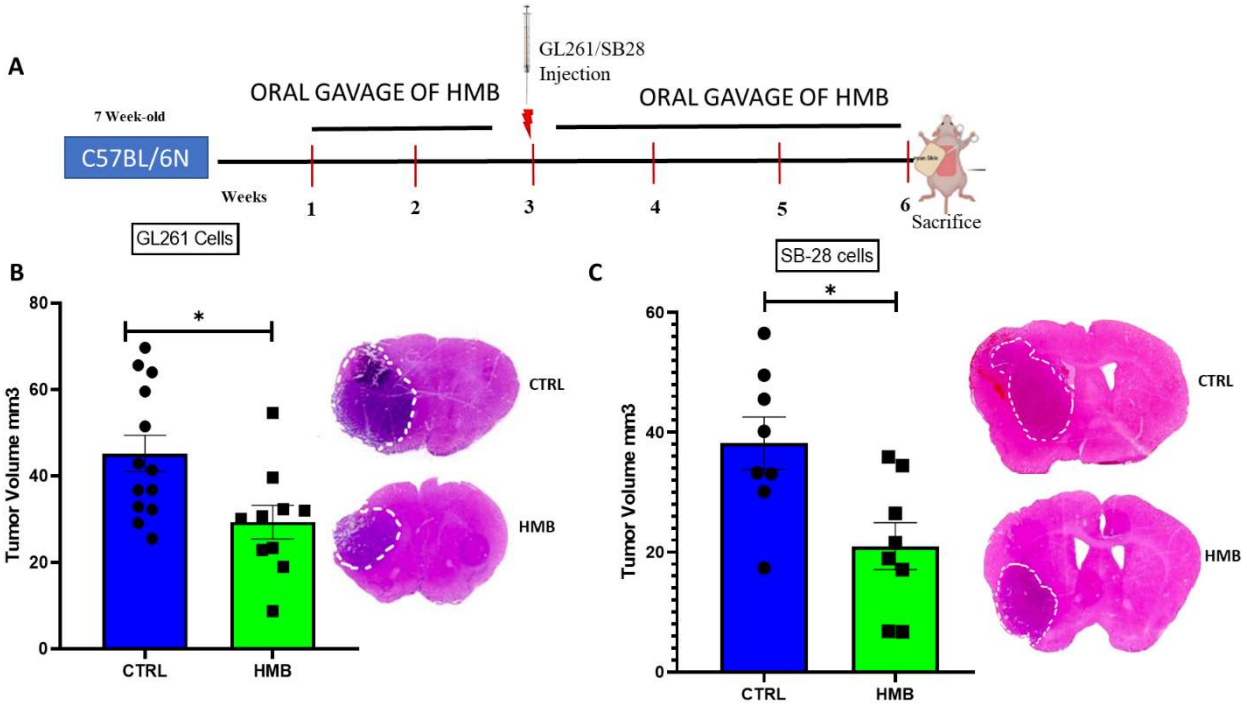

Figure 5: HMB Oral Gavage Significantly Reduces Glioma Volume (A) Schematic of the experimental design. C57BL/6N mice implanted with GL261 and SB-28 glioma cells were treated with HMB five times a week for five weeks. Treatment started two weeks before tumor implantation and continued for three weeks. Control mice received PBS alone. **(B)** Tumor volumes evaluation in GL261 glioma-bearing mice, treated with HMB or vehicle **(C)** Tumor volumes evaluation in SB-28 glioma-bearing mice, treated with HMB or vehicle An unpaired t-test was used for statistical analysis. Representative Hematoxylin & Eosin (H&E)-stained coronal brain slices illustrate the reduction in tumor size in HMB-treated mice and controls. Data are shown as mean ± SEM

5.6 HMB treatment significantly reduces tumor volume in two murine glioma models

Following our demonstration of HMB's antitumor effects as preventive agent, we aimed to further evaluate its potential as a therapeutic agent against glioblastoma. This investigation represents the first to assess HMB as a treatment for glioma using a well-established murine model. C57BL/6N mice were implanted with GL261 or SB-28 murine glioma cells and subsequently treated with HMB via oral gavage. The treatment regimen was initiated one week after tumor implantation, with HMB administered at a dose of 76 mg/kg/day in 200 µL of PBS, five times per week for two weeks as shown in figure 6 A. Control mice received the same volume of PBS without HMB.

Upon completion of the treatment period, the mice were sacrificed, and tumor volumes were measured to evaluate HMB's therapeutic efficacy. Results demonstrated a significant reduction in tumor volume in both murine models for the HMB-treated group compared to controls (GL261, HMB= 7.27 $\pm$0.16 mm³, n=6; CTRL= 11.90 $\pm$1.67 mm³, n=6 *p = 0.0202, unpaired Student's t-test and SB-28, HMB = 36.18 $\pm$3.77 mm³ n=7; CTRL= 68.52 $\pm$ 6.19 mm³, n=7 ***p = 0.0008, unpaired Student's t-test). The relative reduction in tumor volume was 28% in the GL261 model and 32% in the SB-28 model when compared to their respective controls. These findings underscore the therapeutic potential of HMB as an antitumor agent against glioma. The significant reduction in tumor volume observed in both glioma models suggests that HMB could serve as an effective therapeutic intervention in glioblastoma treatment. Further research is warranted to explore the underlying mechanisms of HMB's antitumor activity and to assess its applicability in broader clinical contexts.

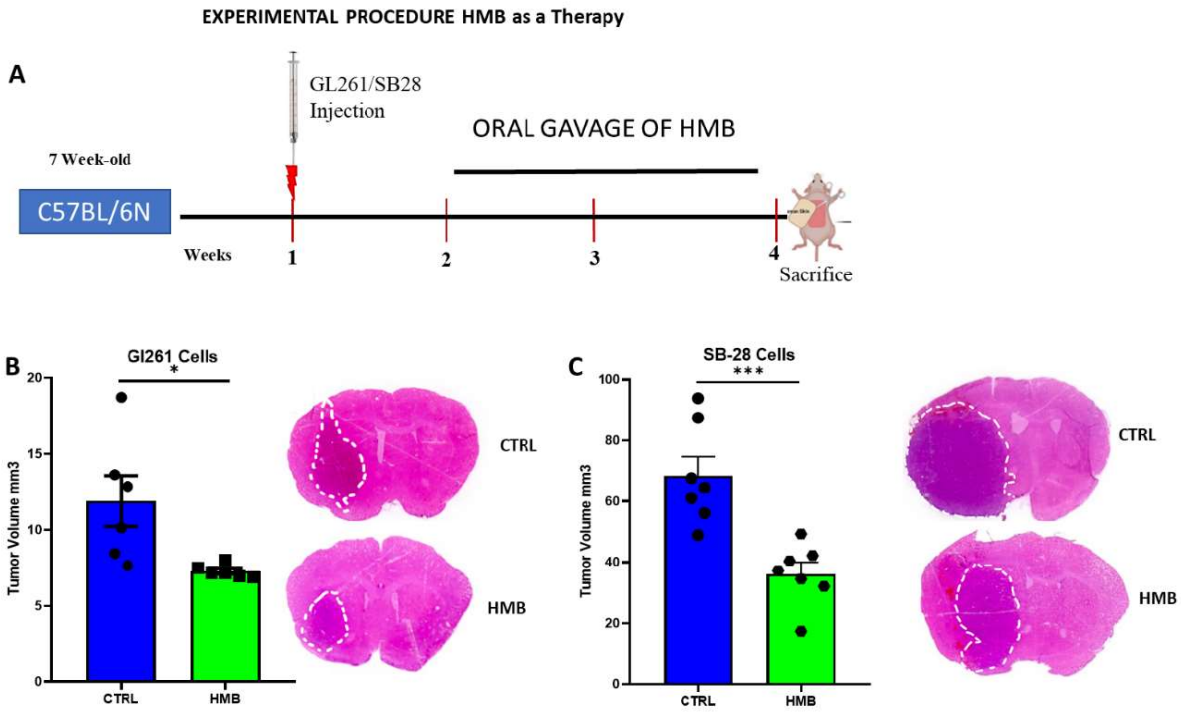

Figure 6: HMB Oral Gavage Significantly Reduces Glioma Volume (A) Schematic of the experimental design: C57BL/6N mice implanted with glioma cells were treated with HMB (76 mg/kg/day in 200 µL of PBS) via oral gavage, starting one week after tumor implantation. Treatment continued daily for two weeks, while control mice received PBS. **(B)** Tumor volumes after three weeks of HMB treatment in GL261 mice (N = 6 per group, *P = 0.0202 by unpaired t-test) **(C)** Tumor volumes after three weeks of HMB treatment in SB-28 mice (N = 7 per group, ***P = 0.0008). by unpaired t-test). Data are shown as mean ± SEM**. Representative Images:** respective H&E-stained coronal brain slices from HMB- or vehicle treated group.

Discussion

Glioblastoma multiforme (GBM) presents one of the most formidable challenges in oncology due to its aggressive nature and profound cellular, molecular, and metabolic heterogeneity, complicating the development of effective therapies (Lan et al., 2024). Current standard treatments—surgical resection, radiotherapy, and temozolomide-based chemotherapy—offer limited success, often failing to significantly improve patient outcomes (Liu et al., 2022). This therapeutic ineffectiveness has driven a growing interest in novel strategies, particularly those targeting cancer stem cells (CSCs), which reside in hypoxic and vascular niches and contribute to treatment resistance (Inda et al., 2014) Additionally, stromal cells in the GBM microenvironment play a pivotal role in supporting tumor progression, highlighting the complex interplay between tumor cells and their surroundings(Gimple et al., 2019). The dynamic relationships between cellular migration, proliferation, and invasion are fundamental to GBM's aggressiveness, directly affecting patient prognosis (Yin X et al., 2023).

Emerging evidence points to the gut microbiota and their metabolites as important factors influencing glioma, including GBM, particularly in relation to tumor progression and treatment response. Changes in the composition of gut microbiota have been linked to glioma development, providing potential diagnostic and prognostic biomarkers (Zhou et al., 2023).In light of this, our study introduces β-Hydroxy β-Methylbutyrate (HMB) as a promising therapeutic agent with notable antitumor activity in both murine and human glioma models. Our findings reveal that HMB inhibits glioma growth, reduces cell viability, and impairs migration and invasion of glioma cells. Notably, we observed a significant reduction in fecal HMB concentrations in glioma-bearing mice, as shown by NMR-based metabolomics, suggesting systemic metabolic shifts induced by gliomas, potentially affecting gut metabolism and nutrient absorption. Similar metabolic alterations have been observed with short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate, which are reduced in both glioma-bearing mice and GBM patients (Keane et al., 2024).

This metabolic shift likely reflects the tumor's high demand for leucine-derived metabolites, such as HMB, which are crucial for its rapid proliferation and survival. Leucine itself can cross the blood-brain barrier (BBB) and plays a critical role in various physiological processes, including neuroprotection. Studies have shown that leucine uptake is mediated by specific amino acid transporters like LAT1 and LAT4, which actively transport leucine across the BBB (Taslimifar et al., 2022). Experimental evidence further supports that intravenous administration of leucine leads to higher concentrations in the brain compared to blood, suggesting an active transport mechanism (Robinson et al., 2015). Additionally, leucine has been demonstrated to reduce BBB disruption during ischemia-reperfusion, potentially enhancing neuronal survival(Chi et al., 2023). Leucine also modulates the mTOR signaling pathway, which is vital for cognitive functions and protein synthesis (McColl & Clarke, 2024).

In the context of GBM, leucine plays a significant role through its metabolic pathways and regulatory proteins. The leucine-rich repeat-containing 4 protein (LRRC4) has been identified as a suppressor gene in gliomas, inhibiting GBM cell proliferation and invasion via microRNA regulatory loops (Deng & Wu, 2023)LRRC4 also acts as an autophagy inhibitor, thereby increasing GBM cell sensitivity to temozolomide (TMZ), a standard chemotherapy treatment (Feng et al., 2020) Leucine catabolism contributes to the metabolic needs of glioblastoma cells, and these cells can utilize leucine-derived metabolites for energy production (Gondáš et al., 2022). This reliance on leucine may help explain the low presence of its microbiota-derived catabolite, HMB, in glioma-bearing mice. These findings suggest that fecal HMB levels could serve as a non-invasive biomarker for glioma detection, though further research is needed to validate its clinical utility.

In vitro experiments demonstrated that HMB robustly inhibits glioma cell viability in both murine (GL261, CT2a, SB-28) and human (U-87 MG, GL-15) glioma cell lines. The reduction in cell viability was significant across all cell lines tested. This underscores HMB’s broad efficacy in targeting gliomas. These findings align with previous studies on HMB’s role in modulating protein

synthesis (Kaczka et al., 2019) and cellular metabolism (Holecek et al., 2009), though its effects on cancer cells have remained largely unexplored. HMB's ability to inhibit glioma cell migration and invasion, as demonstrated through wound healing and invasion assays, highlights its therapeutic potential. Given that migration and invasion are critical drivers of glioma aggressiveness(Vollmann-Zwerenz et al., 2020), HMB's inhibitory effects on both spontaneous and stimulus-driven motility suggest that it may interfere with key regulatory pathways that govern cytoskeletal dynamics and cell motility, such as the Rho/ROCK signaling axis. The involvement of Rho/ROCK signaling in glioma cell migration and proliferation has been well-established (Mertsch & Thanos, 2014), though this hypothesis should be further explored in glioma-specific contexts.

Perhaps the most promising aspect of our research is HMB's in vivo efficacy. Oral administration of HMB significantly reduced tumor volume in two distinct murine glioma models (GL261 and SB-28), demonstrating its potential as a non-invasive therapeutic option. The consistent reduction in tumor size across models suggests that HMB exerts strong antitumor effects, likely through metabolic modulation and direct inhibition of tumor cell proliferation. Importantly, no toxicity or adverse effects were observed in these models, indicating that HMB could be safely applied in combination with standard therapies such as temozolomide or radiotherapy. HMB is currently under clinical investigation for conditions such as sarcopenia and muscle damage (Su et al., 2024)Given the observed reduction of HMB in glioma-bearing mice, this metabolite presents a potential candidate for drug repositioning in glioblastoma treatment, warranting further investigation into its therapeutic relevance in this context.

Given the complexity of GBM, it is plausible that HMB exerts its antitumor effects through multiple mechanisms. HMB's known role in promoting muscle anabolism and preventing protein degradation may extend to cancer cells by inhibiting proteolytic pathways essential for tumor growth and invasion (H. J. Smith et al., 2004). Furthermore, HMB's influence on cellular metabolism could disrupt the metabolic reprogramming typically seen in cancer cells, such as the Warburg effect, where tumor

cells preferentially rely on glycolysis even in the presence of oxygen(Strickland & Stoll, 2017). By forcing glioma cells to revert to oxidative phosphorylation, HMB may create a less favorable metabolic environment for tumor growth. However, further studies are necessary to confirm these hypotheses and better define the molecular mechanisms involved.

While our findings are promising, several limitations must be acknowledged. The murine models used, though well-established in glioma research, do not fully capture the genetic mutations or immune complexity of human GBM. To address these issues, we used a variety of human and murine cell lines in vitro and two differentially immune-activating glioma cell lines in vivo, one with high MHC1 expression (GL261) and the other with very low MHC1 expression (SB-28)(Frederico et al., 2023). Despite these contrasting immunogenic effects, HMB consistently reduced tumor growth in both models, demonstrating its broad efficacy. Future studies should focus on exploring HMB's effects in patient-derived xenograft (PDX) models or genetically engineered mouse models that more closely represent human GBM heterogeneity. Additionally, while we have uncovered promising antitumor effects, the exact molecular mechanisms of HMB's action remain speculative. Further research is essential to fully understand its therapeutic potential, optimize clinical applications, and explore its impact on metabolic pathways, cell cycle regulation, and immune responses.

Conclusion and Future Prospects

This study presents compelling evidence for the antitumor effects of β-Hydroxy β-Methylbutyrate (HMB) in both murine and human glioma models, highlighting its potential as a novel therapeutic approach for glioblastoma. HMB's multifaceted effects on tumor growth, cell viability, migration, and invasion, offer a promising avenue for glioma treatment. The significant reduction in fecal HMB concentrations in glioma-bearing mice provides early evidence of a tumor-associated metabolic shift, suggesting that HMB could serve as a non-invasive biomarker for glioma detection. Moreover, the observed antitumor activity of HMB in both in vitro and in vivo models, alongside its ability to impair glioma cell motility, supports its further exploration as a therapeutic candidate. Notably, the oral administration of HMB, which significantly reduced tumor volume in murine models, underscores its translational potential for human use, especially given its safety profile (Prado et al., 2022) and feasibility of delivery. The broad efficacy across different glioma subtypes and species suggests that HMB may target conserved pathways in glioma biology. Furthermore, its potential to modulate metabolic reprogramming, such as disrupting the Warburg effect, and its likely interference with cytoskeletal dynamics, provides a strong mechanistic foundation for further study.

Future directions include elucidating the molecular mechanisms behind HMB's effects, particularly its role in metabolic reprogramming and immune modulation. Further research should optimize dosing, explore combination therapies, and assess its efficacy in patient-derived and genetically engineered glioblastoma models

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