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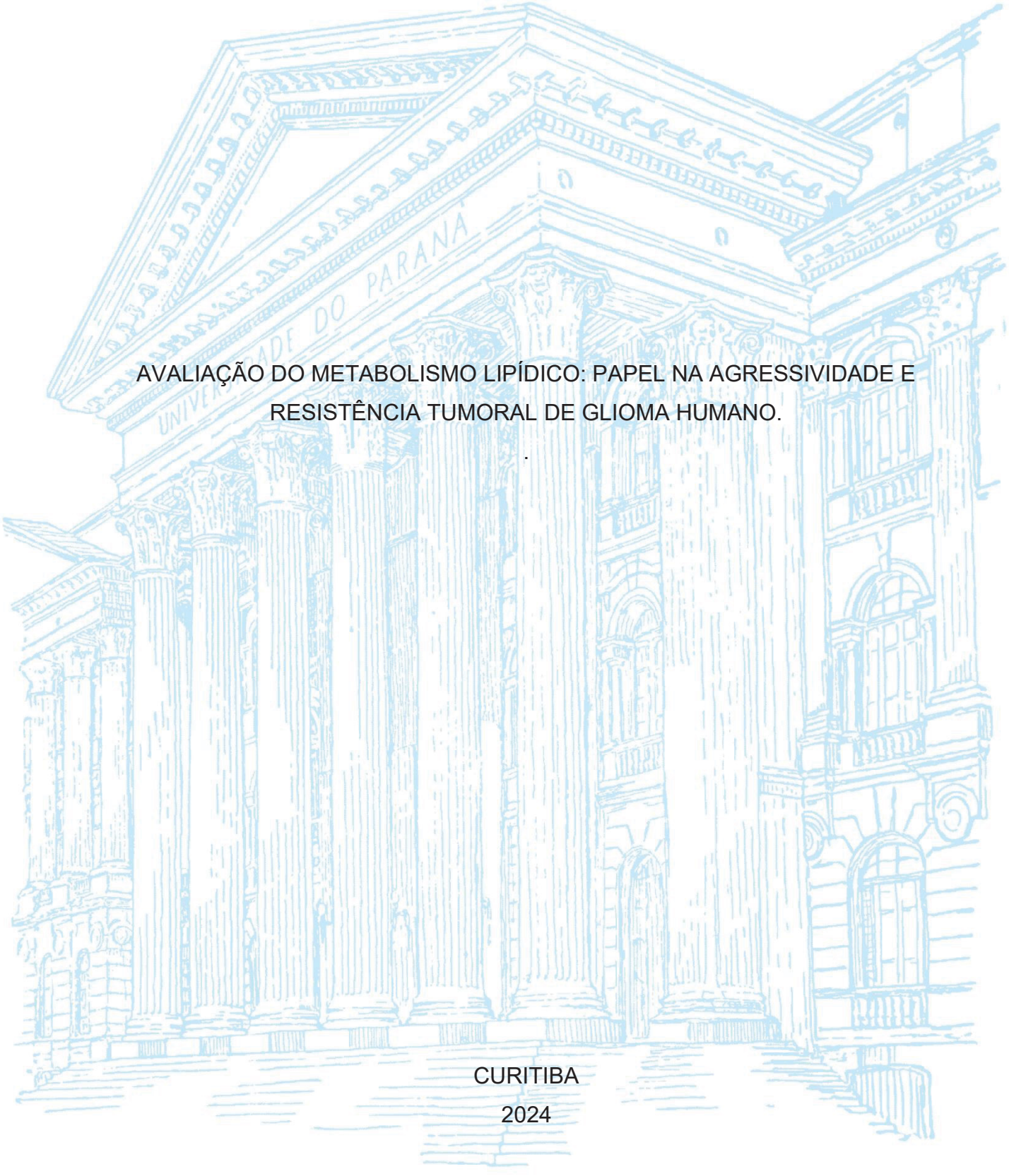

AVALIAÇÃO DO METABOLISMO LIPÍDICO: PAPEL NA AGRESSIVIDADE E RESISTÊNCIA TUMORAL DE GLIOMA HUMANO.

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CURITIBA

2024

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Tese apresentado ao curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências-Bioquímica. Matrícula: 201800079079 Data de Início: 01/04/2020.

Orientadora: Profa. Dra. Sheila M. B. Winnischofer

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **HENNRIQUE TABORDA RIBAS** intitulada: **AVALIAÇÃO DO METABOLISMO LIPÍDICO: PAPEL NA AGRESSIVIDADE E RESISTÊNCIA TUMORAL DE GLIOMA HUMANO**, sob orientação da Profa. Dra. SHEILA MARIA BROCHADO WINNISCHOFER, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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RESUMO

O glioblastoma (GBM) representa o tipo mais agressivo de tumor do sistema nervoso central, marcado por desafios terapêuticos persistentes. Embora haja avanços na compreensão da biologia do glioma, a eficácia das terapias atuais permanece limitada. A exploração de novos marcadores moleculares para a classificação do glioma é crucial. Portanto, considerando o papel crucial do metabolismo lipídico, destaca-se a importância de desvendar as vias relacionadas aos lipídios no glioma, potencialmente revelando alvos diagnósticos e terapêuticos inovadores. Nesse contexto, este estudo teve como objetivo avaliar o papel dos lipídios na agressividade tumoral e na resistência ao tratamento quimioterápico padrão, temozolomida (TMZ). Para isso, integraram-se dados clínicos de transcriptoma obtidos em repositórios públicos com dados experimentais de células tumorais, analisando o impacto do metabolismo lipídico nesses fenótipos.A análise abrangeu 743 genes relacionados a lipídios, comparando sua expressão em dados públicos de RNAseq de pacientes com glioma com análises lipidômicas de linhagens celulares de glioblastoma humano. Foi possível identificar vias lipídicas correlacionadas com prognóstico e com resistência à TMZ, revelando alterações na via de esfingolipídios e na composição da membrana em ambos os fenótipos. Destaca-se principalmente o papel crucial do eixo envolvendo a Esfingomielinase, Esfingomielina fosfodiesterase e Esfingosina Quinase, em que se demostrou que, o aumento de esfingosina-1-fosfato está relacionado tanto com o ganho de agressividade, quanto com a resistência à TMZ. Ainda, foi observado que com o ganho de agressividade, há redução da taxa de insaturação de ácidos graxos; aumento dos níveis de fosfolipídios; e redução dos níveis de colesterol, impactando nas propriedades físicas da membrana. Em contrapartida, no contexto de resistência à TMZ, observou-se que o aumento dos níveis de colesterol e plasmalogênio, aliado a níveis reduzidos de fosfolipídios e alterações em vias associadas a oxi-redução de lipídeos estão relacionados a resistência à TMZ. Os resultados enfatizam o papel fundamental da composição lipídica da membrana na agressividade do GBM, identificando marcadores lipídicos com potencial influência na progressão do glioma. Além disso, o estudo destaca a modulação do metabolismo lipídico no contexto da resistência à TMZ, proporcionando possíveis alvos para novas estratégias terapêuticas visando superar a resistência a medicamentos no glioblastoma.

Palavras-chave: Glioma; Esfingolipídios; Temozolomida; Agressividade; Resistência.

ABSTRACT

The glioblastoma (GBM) represents the most aggressive type of tumor in the central nervous system, marked by persistent therapeutic challenges. Although there have been advances in understanding glioma biology, the effectiveness of current therapies remains limited. The exploration of new molecular markers for glioma classification is crucial. Therefore, considering the crucial role of lipid metabolism, the importance of unraveling lipid-related pathways in glioma is emphasized, potentially revealing innovative diagnostic and therapeutic targets. In this context, this study aimed to evaluate the role of lipids in tumor aggressiveness and resistance to standard chemotherapy treatment, temozolomide (TMZ). To achieve this, clinical transcriptome data obtained from public repositories were integrated with experimental data from tumor cell lines, analyzing the impact of lipid metabolism on these phenotypes. The analysis covered 743 genes related to lipids, comparing their expression in public RNAseq data from glioma patients with lipidomic analyses of human glioblastoma cell lines. It was possible to identify lipid pathways correlated with prognosis and TMZ resistance, revealing changes in the sphingolipid pathway and membrane composition in both phenotypes. The crucial role of the axis involving sphingomyelinase and sphingosine-1-phosphate (S1P), mediated by SMPD1 and SPHK1, was particularly highlighted, demonstrating that an increase in S1P is related to both increased aggressiveness and TMZ resistance. Additionally, it was observed that increased aggressiveness is associated with a reduction in the rate of fatty acid unsaturation, an increase in phospholipids, and a reduction in cholesterol levels, impacting the physical properties of the membrane. Conversely, in the context of TMZ resistance, it was observed that increased cholesterol, plasmalogen, and pathways associated with oxidation-reduction, combined with reduced levels of phospholipids, are related to TMZ resistance. The results emphasize the fundamental role of membrane lipid composition in GBM aggressiveness, identifying lipid markers with potential influence on glioma progression. Furthermore, the study highlights the modulation of lipid metabolism in the context of TMZ resistance, providing possible therapeutic strategies to overcome drug resistance in glioblastoma.

Keywords: Glioma; Sphingolipids; Temozolomide; Aggressiveness; Resistance.

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LISTA DE ABREVIATURAS OU SIGLAS

Cer	- Ceramida
DMEN	- Meio Dulbecco MEM
DMSO	- Dimetilsufóxido
GBM	- Glioblastoma
LASS	- Ceramida sintase
LGG	- Glioma de baixo grau
GPI	- Glicosilfosfaditilinositol
INCA	- Instituto Nacional do Câncer
NIH	- National Institutes of Health
NPC2	- Proteína ligante ao colesterol
MGMT	- Metilguaninametiltransferase
MMP	- Metaloproteinase
MMR	- Reparo de mal pareamento do DNA
MTIC	- Monometil-triazenoimidazol-carboxamida
MTT	- brometo de [3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazolium
MSM	- Metilsulfonilmetano
RECK	- Proteína rica em cisteína, indutora de reversão, com motivos Kazal
PCR	- Reação em cadeia da polimerase
PBS	- Tampão fosfato salino
SFB	- Soro fetal bovino
SPHK	- Esfingosina quinase
SMPD1	- Esfingomielina fosfodiesterase
RMN	- Ressonância magnética nuclear
HSQC	- Correlação heteronuclear de quantum único
S1P	- Esfingosina-1-fosfato
SNC	- Sistema nervoso central
SM	- Esfingomielina
SMASE	- Esfingomielinase
SREBP	- Fator de Transcrição de ligação ao elemento regulador de esterol
TCGA	- *The Cancer Genome Atlas*
TMZ	- Temozolomida
UCGC	- Glucosiltransferase de ceramida

SUMÁRIO

1 INTRODUÇÃO

Os pacientes com tumores (CHEN et al., 2013). No Brasil, a razão mortalidade/incidência em tumores do SNC é de 92 % segundo o Instituto Nacional de Câncer (INCA). Além disso, foram estimados, no Brasil, 11.490 novos casos dessa neoplasia maligna que acomete o SNC para o ano de 2023.

O glioma, tipo mais comum de tumor do sistema nervoso central, e seu o grau mais maligno o glioblastoma (GBM) progride muito rapidamente e as terapias convencionais acabam não sendo eficientes, ocorrendo resistência às terapias na maioria dos casos. Esta resistência pode ocorrer através de diferentes mecanismos (HAAR et al., 2012), sendo que aquele que é modulado pelo sistema de reparo ao DNA é de particular importância para clínica, uma vez que a temozolomida (TMZ), um agente alquilante de DNA, é a droga padrão de escolha para o tratamento de glioblastoma, tipo mais agressivo de glioma (ZHANG; F.G. STEVENS; D. BRADSHAW, 2011). Ainda, novas estratégias terapêuticas que buscam explorar alvos mais específicos; como imunoterapia e terapia gênica, são bastante promissoras (RAJESH et al., 2017). A elucidação de mecanismos celulares e das biomoléculas envolvidas na patologia do câncer é de suma importância para que haja, não só a compreensão do processo de doença, mas também a identificação de alvos terapêuticos promissores.

O estudo da lipidômica é fundamental na elucidação dos papéis celulares dos lipídios em processos patológicos (SPENER, et al., 2003). Dentro do contexto de lipidômica, detaca-se a investigação da via metabólica de esfingolipídios. Essa via tem um papel importante na regulação de diversos processos celulares como crescimento celular, morte, senescência, adesão, migração e angiogênese (OGRETMEN, 2017).

Já no contexto estrutural dos lipídios, marcadores bioquímicos, como a colina, indicativos da síntese da membrana celular, possuem um valor clínico significativo nos diagnósticos que utilizam tecnologia de ressonância em glioma (BULIK et al., 2013; GORYAWALA et al., 2021; USINSKIENE et al., 2016). O colesterol, outro constituinte da membrana celular, possui um papel importante na modulação das propriedades biofísicas da membrana e seus baixos níveis diminuem a fluidez da membrana, podendo favorecer a invasão e metástase das células tumorais (BERNARDES; FIALHO, 2018).

Portanto, o presente estudo propõe uma investigação aprofundada sobre o papel da regulação do metabolismo de lipídios na progressão, agressividade e

resistência ao tratamento do glioblastoma humano analisando as assinaturas lipídicas específicas associadas a esses fenótipos. Ao identificar componentes lipídicos-chave, almejamos identificar os mecanismos complexos relacionados à progressão e resistência do glioma, explorando potenciais alvos terapêuticos. O objetivo final é aprimorar as estratégias de tratamento para o glioblastoma, elucidando a intrincada interação entre lipídios e a natureza agressiva do glioma, abrindo caminho para intervenções terapêuticas inovadoras e eficazes.

1.1 OBJETIVOS

Propomos investigar o papel do metabolismo lipídico no modelo de glioblastoma (GBM), com foco na identificação de marcadores lipídicos associados à agressividade tumoral e resistência à temozolomida (TMZ), visando contribuir para o desenvolvimento de estratégias terapêuticas inovadoras e aprimorar a compreensão da biologia do glioma.

1.2 OBJETIVOS ESPECÍFICOS

a) Analisar a expressão de genes relacionados a lipídios em dados públicos de RNAseq de pacientes com glioma, comparando esses resultados com análises lipidômicas de linhagens celulares de glioblastoma humano para identificar marcadores lipídicos correlacionados com a agressividade tumoral.
b) Avaliar o impacto do metabolismo lipídico na resistência à temozolomida (TMZ), identificando alterações específicas nas vias lipídicas e na composição da membrana relacionadas à resistência ao tratamento quimioterápico padrão.
c) Investigar as alterações nas vias lipídicas, com ênfase na composição da membrana celular associadas à agressividade do GBM, utilizando análises in sílico para compreender as implicações nas propriedades físicas da membrana.
d) Investigar o eixo envolvendo a esfingomielinase e a esfingosina-1-fosfato (S1P), mediado por SMPD1 e SPHK1, para elucidar o papel desse sistema na agressividade tumoral e na resistência à TMZ.
e) Validar experimentalmente os marcadores lipídicos identificados como potenciais influências na progressão do glioma, utilizando modelos *in vitro*, a fim de propor sua relevância como alvos terapêuticos.
f) Propor alvos de estratégias terapêuticas para superar a resistência à temozolomida (TMZ) por meio da modulação do metabolismo lipídico,

considerando intervenções farmacológicas e avaliando seu impacto na sensibilidade das células tumorais ao tratamento quimioterápico.

2 REVISÃO DE LITERATURA

2.1 CELULAS DA GLIA

O sistema nervoso central, extremamente fundamental para o funcionamento do organismo, integra todas as informações oriundas dos sistemas sensoriais ordenando determinadas respostas a serem realizadas (GUYTON; HALL, 2016). O neurônio é a unidade funcional desse sistema e é amparado por aproximadamente 10 a 50 células da glia (SNYDER et al., 2018). As células gliais se originam na fase embrionária e continuam sendo geradas no parênquima cerebral adulto através de células progenitoras. Como eventos neoplásicos estão mais associados com células que apresentam uma determinada capacidade proliferativa, acredita-se que a origem de células da glia tumorais está relacionada com as células progenitoras, as quais dão origem à células gliais (RUSZNÁK et al., 2016; SCHIFFER, 2006). As células da glia desempenham um papel importante na promoção da homeostase do sistema nervoso central e oferece suporte aos neurônios (EDGAR; NAVE 2009). O tipo celular mais abundante são astrócitos que conectam neurônios e vasos sanguíneos promovendo a barreira hematoencefálica, assim desempenham uma função fundamental no funcionamento neuronal (HERNDON; TOME; DAVIS, 2017). Já a mielina que recobre as células neuronais e auxilia na transmissão dos impulsos nervosos é formada por oligodendrócitos (Snyder et al., 2018). Por fim, células ependimárias são responsáveis pela circulação do líquido cefalorraquidiano recobrindo toda a superfície dos ventrículos (ROBINSON; NOONE; O'DOWD, 1996).

2.2 GLIOMA

Glioma é tipo de tumor do sistema nervoso central mais comum, quegeralmente apresenta um mau prognóstico (HANIF et al., 2017). Gliomas são classificados de acordo com características histológicas sendo divido em astrocitoma, oligodendogliomas e ependimomas e são classificadas de acordo com grau de malignidade (I a IV) (LAUG; GLASGOW; DENEEN, 2018). Baixa taxa proliferativa é

uma característica de gliomas de baixo grau (I e II), sendo o de grau II mais infiltrativo, podendo progredir para glioma de alto grau. Já gliomas com características histopatológicas malignas são classificados como de grau III e IV (alto grau) (LOUIS et al., 2007). Contudo, o diagnóstico e classificação desses tumores vem sofrendo algumas alterações pela inclusão de marcadores moleculares direcionando um diagnóstico e classificação tumoral não tão dependente de características histológicas como aquele apresentado na 4º edição da classificação de tumores do SNC da OMS de 2016 (LOUIS et al., 2021; RUSHING, 2021). Na nova classificação da OMS de 2021, gliomas difusos são agrupados em quatro categorias: gliomas difusos adultos, gliomas de baixo grau difuso pediátrico, gliomas de alto grau difusos pediátricos e gliomas astrocíticos circunscritos. Gliomas difusos adultos incluem astrocitoma com mutação no IDH, oligodendroglioma com mutação no IDH e deleção do 1p/19q, e glioblastoma com wildtype IDH. Astrocitomas difusos com mutação no IDH são agora classificados de 2 a 4 dentro do tipo, eliminando os termos "astrocitoma anaplásico" e "glioblastoma" com mutação no IDH. Adicionalmente, se um astrocitoma difuso com mutação no IDH apresentar deleção homozigótica do CDKN2A/B, é designado como neoplasia de grau 4 da OMS para o sistema nervoso central, mesmo na ausência de características histológicas de malignidade, como necrose e proliferação microvascular (OSBORN et al., 2022). A Associação Europeia de Neuro-Oncologia já recomenda a prática dessas novas atualizações e foram integradas na 5º edição da classificação de tumores do SNC da OMS (RUSHING, 2021; WELLER et al., 2021). Nota-se a importância na busca de marcadores moleculares que auxiliem na classificação, prognóstico e tratamento de glioma. Além disso, esclarecer as vias responsáveis pela diferenciação de células progenitoras para a geração de células gliais podem ser úteis para melhor compreensão da origem e progressão de gliomas (LAUG; GLASGOW; DENEEN, 2018).

O astrocitoma de grau IV, ou glioblastoma (GBM), apresenta, alta proliferação endotelial, necrose e alta densidade celular, levando a uma taxa de sobrevida em média de 14 meses, portanto sendo o mais agressivo dos gliomas (WESTPHAL; LAMSZUS, 2011). Glioblastoma pode ser divido em primário ou secundário, de acordo com características clínicas. Pacientes diagnosticados com glioblastoma secundário são aqueles que alguma vez já apresentaram algum tipo de astrocitomas de menor grau, enquanto que o primário é quando há diagnostico sem o conhecimento de algum glioma preexistente (HANIF et al., 2017).

O tratamento de gliomas depende do diagnóstico histológico e molecular, sendo a cirurgia realizada tanto para fim de diagnóstico quanto de tratamento (RUSHING, 2021). Porém, apesar dos avanços tecnológicos que visam facilitar os procedimentos cirúrgicos, a remoção total do tumor muitas vezes é comprometida pela localização em áreas vitais (ESQUENAZI et al., 2017). Já o uso de radioterapia reduz o risco de recidiva e é determinada de acordo com parâmetros clínicos e prognósticos do paciente, apesar de apresentar avanços, como a radioterapia hipofracionada, radioterapia tridimensional e radioterapia com modulação da intensidade do feixe; as tecnologias pioneiras acabam somente reduzindo toxicidades relacionadas ao tratamento e não promovem um aumento efetivo na sobrevida (BRADA; HAYLOCK, 2014; FROSINA, 2021). Por fim, a quimioterapia (além de ser limitada às substâncias que atravessam a barreira hematoencefálica) não é eficiente pelas características heterogêneas que favorecem a aquisição de resistência, tendo como principal droga de escolha a temozolomida TMZ (FRIEDMANN-MORVINSKI, 2014; WELLER et al., 2017). Essa complexidade heterogênea do glioma impõe uma variedade de características moleculares e celulares, contribuindo para a resposta variável à temozolomida (MALEKI; BAHRAMI; MATIN, 2024). Essa heterogeneidade não apenas influencia a eficácia inicial do tratamento, mas também cria um ambiente propício para o desenvolvimento de subpopulações resistentes ao longo do tempo (MALEKI; BAHRAMI; MATIN, 2024). Assim, a abordagem tradicional com TMZ enfrenta limitações diante da capacidade adaptativa e multifacetada das células glioma, destacando a necessidade premente de estratégias terapêuticas mais abrangentes e adaptativas para enfrentar a complexidade intrínseca desses tumores

2.3 TEMOZOLOMIDA E MECANISMOS DE RESISTÊNCIA

Temozolomida (TMZ) é uma pró droga lipofílica derivada de imidazotetrazina que quando convertida à 3-metil-(triazen-1-il)-imidazol-4-carboxamida (MTIC) apresenta capacidade antitumoral (BRANDNER et al., 2021; STEVENS et al., 1987). A propriedade alquilante ao DNA ocorre principalmente na guanina (G), o nucleotídeo que apresenta o potencial molecular mais eletronegativo ocorrendo em maior proporção na posição N7 (N7-MeG, 70%). No entanto, a resposta citotóxica ocorre pela metilação na posição O6 que ao gerar O6-MeG leva ao erro no pareamento de bases com a formação de O6-MeG/T (timina). O sistema de reparo de erro de

pareamento MMR (*mismatching repair*) é então induzido, e a persistência de mal pareamento leva eventualmente à morte celular e citotoxicidade (DRABLØS et al., 2004) (TISDALE, 1987). Portanto o efeito citotóxico e terapêutico de TMZ é dependente da atividade e do bom funcionamento do sistema de reparo efetuado por MMR. Além disso, O6-MeG pode ser demetilado através da atividade de uma proteína de reparo direto a MGMT (O6-methylguanine DNA methyltransferase). (LEE, 2016; SHARMA et al., 2009). Assim sendo, para que se obtenha uma boa resposta à temozolomida, MMR deve ser funcional e *MGMT* deve ser pouco expresso (ZHANG; F.G. STEVENS; D. BRADSHAW, 2011). Em modelo celular, por exemplo, foi descrito que o aumento da atividade de MGMT está relacionada com o ganho de resistência à temozolomida por células de glioma previamente sensíveis (ZHANG et al., 2010).

A expressão de *MGMT* é regulada pela metilação de seu promotor, que quando metilado tem sua expressão suprimida. Por isso, a avaliação do status do promotor de MGMT é uma prática recomendada para auxiliar não só na escolha da estratégia terapêutica empregada mas também por ser um marcador de prognóstico (BRANDNER et al., 2021). A incidênica do promotor de *MGMT* demetilado é relativamente elevada, correspondendo a mais que 30 % dos pacientes diagnosticados com glioblastoma, no entanto mais de 50% dos pacientes não são responsivos ao tratamento com TMZ (BLANC et al., 2004; LEE, 2016). Outras vias foram descritas como possíveis candidatas na promoção de resistência independente da expressão de *MGMT*, como a regulação da via do ciclo celular e presença de célula tronco tumoral, indicando que outras possíveis vias podem relacionadas com a resistência a essa droga (LANG et al., 2021a). Assim, como marcadores de resposta ao tratamento ainda são limitados (restringindo-se basicamente ao status de metilação do promotor de *MGMT)*, a busca de novos marcadores e alvos envolvidos com essa resistência são necessários, de forma a aprimorar e auxiliar o tratamento e prognóstico (SINGH et al., 2021). Além disso, tumores podem eventualmente adquirir resistência à quimioterapia devido a sua característica evolutiva e heterogênea.

A heterogeneidade celular comumente presente em glioblastomas, aliado à alta complexidade genética oriunda da heterogeneidade genômica que tumores malignos apresentam podem ser fatores fundamentais para aquisição de resistência à temozolomida. (FRIEDMANN-MORVINSKI, 2014). Essa complexidade gênica originada pela alta taxa de mutações que células tumorais apresentam podem conter mutações que diferenciam determinadas células tumorais daquelas que lhe deram

origem, e assim, por pressão seletiva células aquelas mais adaptadas a um determinado microambiente tumoral são selecionadas (LOEB, 2011). Essa heterogeneidade pode acarretar uma dificuldade na terapia e até em resistência a tratamentos devido à seleção e expansão de subclones pré-existentes ou por evolução de células resistentes (DAGOGO-JACK; SHAW, 2018; RABÉ et al., 2022).

2.4 METABOLISMO LIPIDICO NO CONTEXTO TUMORAL

Os lipídios são uma ampla classe de biomoléculas orgânicas bastante diversificada quimicamente e funcionalmente. Apresentam funções que compreendem papeis estruturais, compondo as membranas biológicas, e do metabolismo energético sendo uma importante fonte de energia celular (NELSON; COX, 2008). Há uma grande variedade lipídica nas células e o gasto energético para manter o metabolismo e a síntese dessas moléculas é altamente custoso, portanto sugere-se uma vantagem evolutiva para que as células mantenham essa complexidade (VAN MEER; VOELKER; FEIGENSON, 2008). Além de doenças metabólicas como obesidade e doenças cardiovasculares, diferentes tipos de câncer são relacionados com disfunções no metabolismo de lipídios, além de que a obesidade pode elevar o risco ao desenvolvimento de 16 tipos de câncer, incluindo glioma (ULRICH et al., 2018; ZHAO et al., 2015). Neste contexto, exploraremos os principais lipídios envolvidos no metabolismo tumoral, além de destacar as principais técnicas para sua detecção.

2.4.1 Metabolismo de Colina

Fosfatidilcolina (PtdCho) é o lipídio mais abundante na membrana celular e é sintetizado através da via de Kennedy. Na primeira etapa dessa via ocorre a fosforilação de colina para formação de fosfocolina através da atividade de colina quinase, que pode ser codificada por dois genes *CHKA ou CHKB* (TAVASOLI et al., 2020). Em seguida a fosfocolina é transformada em CDP-colina na reação limitante para a síntese de PtdCho através da ação de fosfatidilcolina citidililtransferase (CORNELL; RIDGWAY, 2015). Por fim PtdCho é sintetizada pela atividade de CDP-coline fosfotransferase (SZLASA et al., 2020).

Diversos tipos de câncer apresentam alterações dos níveis de PtdCho, e esses níveis podem ser modulados através da atividade (CHKA), ou por fosfolipases, enzimas responsáveis pela sua degradação, fosfolipase C (PLC) e fosfolipase D (PLD)

(SZLASA et al., 2020). A colina é um indicador de formação de membrana celular e apresenta relevância clínica importante no diagnóstico de gliomas uma vez que pode ser facilmente detectado por ressonância magnética de imagem (^{1}H MRI) (BULIK et al., 2013; GORYAWALA et al., 2021; USINSKIENE et al., 2016). Níveis elevados de colina estão relacionados com tumores de maior grau e com maior taxa de proliferação (BULIK et al., 2013; SU et al., 2021). Além disso o acúmulo de fosfolipídios contendo colina foi descrito em diversos tipos de câncer, uma vez que seu metabolismo tem um papel pró-tumoral importante por promover a proliferação celular (SAITO et al., 2022). Em modelo *in vitro*, o tratamento de células de glioblastoma humano (U87MG) com colina induziu efeitos anti-apoptótico e proliferativo através da ativação das vias de AKT e ERK (PUCCI et al., 2021).

Apesar de tumores apresentarem altos níveis de colinas totais, os níveis das diferentes moléculas contendo colina podem estar associadas a um diferente grau tumoral como mostraram as análises de ressonância avaliando biopsias de pacientes. Gliomas de alto grau apresentam maiores níveis de fosfocolina associado ao aumento da expressão de ChoK, PLC e redução da expressão de fosfocolina citidililtransferase B (PCYT1B); enquanto gliomas de baixo grau apresentam altos níveis de glicerofosfocolina (RIGHI et al., 2009). Outro trabalho comparou amostras teciduais de pacientes com gliomas de diferentes graus, além de amostras teciduais de pacientes com tumores primários e suas respectivas recorrências, e demonstrou que o aumento dos níveis de PtdCho correlaciona com ganho de malignidade em gliomas (LEHNHARDT et al., 2001).

O metabolismo da fosfatidilcolina tem sido associado à resistência à quimioterapia em vários tipos de câncer, tornando os níveis de PtdCho um potencial marcador crucial para avaliar a resposta à terapia. No entanto, permanece uma incerteza se a redução ou aumento desses níveis favorece fenótipos resistentes, pois ambas as possibilidades são descritas na literatura. (DALY et al., 1987; SAITO et al., 2022). A sua redução em células resistentes pode ser explicada, pelo menos em parte, pelo fato de linhagens resistentes expressarem mais receptores de resistência múltipla a drogas (MDR), uma vez que, PtdCho já é descrito como um substrato para o receptor MDR1 em modelo de leucemia (BOSCH et al., 1997). No entanto, em modelo de glioblastoma, em que se usou linhagens de U87MG com resistência adquirida à doxorubicina e cisplatina foi observado, além do aumento de expressão de MDR1, o aumento da captação de colina aliado ao aumento de expressão de colina

quinase (ChoK) o que sugeriria um aumento da síntese de fosfatidilcolina (VANPOUILLE et al., 2009). No entanto, Vanpouille et. al 2019 não mensuraram os níveis de PtdCho nesse modelo. Ainda, a inibição de ChoK foi capaz de induzir apoptose e reduzir o crescimento tumoral de glioblastoma tanto em modelo *in vitro* quanto em modelo *in vivo*. Esse efeito antitumoral mediado pela inibição de ChoK foi observado concomitante com a redução dos níveis intracelulares de PtdCho e aumento dos níveis de ácidos graxos poli-insaturados avaliados por ^{1}H MRI (KUMAR et al., 2015). O uso de inibidores da via de PI3K (terapias promissoras ao tratamento de glioma) também reduz os níveis de colina total e fosfocolina acompanhada de redução dos níveis de ChoK em células de glioblastoma humano, enquanto o tratamento com TMZ induziu o aumento de glicerofosfocolina, colina total e fosfocolina (AL-SAFFAR et al., 2014). Percebe-se que o papel de PtdCho e do metabolismo de colina na resposta a quimioterapia e na resistência não estão totalmente claros e mais trabalhos que visem elucidar esse papel são necessários

2.4.2 Esfingolipídios

Os esfingolipídios apresentam função estrutural e também desempenham funções de sinalização celular (ERNST; BRÜGGER, 2014). Ceramida e esfingosina-1-fosfato são os esfingolipidios mais explorados dentro da oncologia, e são a base para o paradigma que explica a participação dos esfingolipídios em tumores. A teoria de reostase consiste em explicar efeitos pró-tumorais ou antitumorais de acordo com o balanço dos níveis desses esfingolipídios. Quando há um desbalanço favorecendo os níveis de ceramida há uma promoção de efeitos antitumorais e apoptóticos. Em contrapartida, quando há altos níveis de esfingosina-1-fosfato ocorre o favorecimento de proliferação e tumorigênese (VAN BROCKLYN; WILLIAMS, 2012).

A ceramida apresenta um papel importante na regulação de morte celular, progressão tumoral e resistência à quimioterapia (HANNUN; OBEID, 2008). Sua síntese pode ocorrer por duas vias distintas, a via *de novo* ou pela via de “reaproveitamento” (BARTKE; HANNUN, 2009). Em tumores cerebrais os níveis desses esfingolipídios são significativamente baixos, e esses níveis reduzidos estão associados à progressão de malignidade em astrocitomas, além de estarem relacionados com prognósticos ruins (RIBONI et al., 2002).

O papel antitumoral e apoptótico das ceramidas é amplamente descrito em estudos que utilizam modelos celulares (QUADRO 1). Porém percebemos na literatura

uma generalização equivocada desses efeitos, uma vez que, nesses modelos somente as ceramidas de cadeias curtas (C2 ou C6) são avaliadas. Isso ocorre pois em modelos celulares somente essas são passíveis de atravessar a membrana celular.

QUADRO 1- Diferentes ceramidas e as respostas geradas em modelos de glioma

Ceramida (Cadeia)	*Modelo*	*Resposta*	*Referência*
C2	Celular - U251MG, U87MG, T98G, H4, C6, A172 e U-118. Tratamento com Cer-C2	Morte Celular	(WATANABE et al., 2020) (CHANG et al., 2018)(KUŞ et al., 2018)(KIM et al., 2005)(DESAI; VYAS; AMIJI, 2008)
C16	Celular - U251MG Indução de apoptose com estaurosporina	Aumento de Cer-C16 após indução da apoptose	(MIGNARD et al., 2020)
C6	Celular – U87MG Tratamento com CerC6	Morte celular autofágica	(THAYYULLATHIL et al., 2020)
C18	Celular – A172 e U251 Tratamento com CerC18	Morte celular por ativação de estresse de retículo e autofagia letal	(WANG et al., 2017)

Porém, por não se limitarem a avaliar as ceramidas de cadeia curtas, o perfil esfingolipídico em modelos clínicos aponta que ceramidas podem apresentar um papel pró-tumoral. Em pacientes com glioma foi observado um aumento da ceramida

C16 quando comprados com pacientes saudáveis, porém esse resultado não foi explorado pelos autores (ABUHUSAIN et al., 2013). RIBONI et al. (2002) quantificaram as ceramidas em pacientes com glioma e concluíram que nessa doença há uma redução de ceramida totais, porém o estudo realizado não permitiu avaliar os diferentes tipos de ceramidas.

Considerando outros tipos tumorais, estudos apontam para o aumento de ceramidas de cadeia muito longa (C16 e C24) em tecido tumoral de mama e de ovário (HARTMANN et al., 2012; KOZAR et al., 2018). Corroborando esse resultado, análises metabolômicas de soro de pacientes com câncer de ovário identificaram níveis aumentados de ceramidas de cadeia muito longa e esfingomielinas em comparação com os níveis encontrados no soro de pacientes controle (KOZAR et al., 2018). Destaca-se a importância de realizar metodologias como a de metabolômica a fim de identificar de maneira mais abrangente as diferentes biomoléculas. É importante ressaltar, ainda, que as ceramidas C16 e C24 impactam nas propriedades da membrana celular, tais como redução de fluidez e formação de domínios de membrana independentes de colesterol (GUPTA et al., 2020; PINTO et al., 2014; VENTURA et al., 2020). Além disso, o tamanho da cadeia das ceramidas depende da ação de diferentes isoformas da ceramida sintase (STIBAN; TIDHAR; FUTERMAN, 2010)

As ceramidas podem ser geradas a partir de esfingomielinas que também apresentam um papel importante nas propriedades das membranas (TANIGUCHI; OKAZAKI, 2014). Foi demonstrado que em tecidos de pacientes com glioma há um aumento de determinadas esfingomielinas. E de maneira interessante quando há inibição da enzima esfingomielinase (SMASE) que converte esfingomielina à ceramida, a ativação da via de PI3K/Akt é reduzida em resposta a insulina em modelo celular de glioma utilizando a linhagem U373MG (ZHAI et al., 2019). Porém nesse trabalho não foram quantificados níveis das diferentes ceramidas e esfingomielinas possivelmente afetadas pelo tratamento com inibidor, para que se pudesse relacionar quais esfingolipídios estão envolvidos na resposta avaliada.

Dados de transcriptoma de pacientes demonstram que o aumento da expressão do gene que codifica para SMASE é mais elevado de acordo com a agressividade de gliomas (GRAMATZKI et al., 2013). De forma interessante, em trabalho prévio do grupo foi verificado que a geração de resistência ao quimioterápico

padrão TMZ, na linhagem U87MG, foi acompanhada do aumento da expressão de SMASE(RIBAS, 2020).

Por outro lado, a esfingosina-1-fosfato (S1P), produzido pela enzima esfingosina quinase SPHK1 e SPHK2 a partir da esfingosina, de maneira geral, têm efeitos opostos daqueles mediados pela ceramida (SNIDER; GANDY; OBEID, 2010). Aumento dos níveis dessa biomolécula está relacionado com uma menor taxa de sobrevida em pacientes diagnosticados com glioblastoma (VAN BROCKLYN et al., 2005). Utilizar SPHKs como alvo terapêutico demonstra ser promissor para o tratamento de gliomas (ABUHUSAIN et al., 2013; CHEN et al., 2019; DAI et al., 2020). A inibição de SPHKs pode ser realizada por inibidores que inibem ambas isoformas ou com inibidores específicos para cada isoforma. Em glioma foi demostrado que a inibição por inibidores não específicos potencializa a resposta à morte celular por TMZ (NOACK et al., 2014; OANCEA-CASTILLO et al., 2017a), e promove inibição do crescimento celular e indução de apoptose em modelo celular de glioma humano (DAI et al., 2020). Já a inibição da isoforma SPHK1 induziu apoptose tanto em linhagens de glioblastoma humano sensíveis quanto com resistência adquirida à TMZ (BEKTAS et al., 2009), além de inibir angiogênese em glioblastoma (ABUHUSAIN et al., 2013).

A inibição de SPHK1 é capaz de induzir morte celular, não obstante, sua atividade é importante para o processo de angiogênese em modelos celulares de glioma (ABUHUSAIN et al., 2013; DAI et al., 2020). De fato S1P é capaz de regular invasão de glioblastomas, e seus altos níveis estão relacionados com uma menor taxa de sobrevivência em pacientes com glioma (VAN BROCKLYN et al., 2005; YOUNG; PEARL; VAN BROCKLYN, 2009). S1P também medeia resposta ao aumento de PLD, elevando MMP-2 , que mostrou por consequência aumento da invasão celular de gliomas *in vitro* (PARK et al., 2009).

2.4.3 Colesterol

Outro componente importante das membranas celulares é o colesterol, que pode ser sintetizado a partir da acetil-CoA através da via do mevalonato por qualquer célula, e sua quantidade nas membranas regula propriedades biofísicas modulando a fluidez das membranas e atividades de proteínas de membrana (IKONEN, 2008). A síntese de colesterol pode ser regulada através da ativação transcricional de Proteínas de Ligação a Elemento Regulador de Esterol (SREBPs), que promove a

síntese de proteínas como HMG-CoA reductase, enzima envolvida na etapa limitante na síntese de colesterol (SHIMANO; SATO, 2017).

Alterações nos níveis desse lipídio vem sendo associado em diversos tipos de cânceres, porém diferentes estudos clínicos demostram que tanto o aumento quanto a redução de colesterol pode estar relacionado com fenótipos de agressividade tumoral, como aumento de proliferação, invasão, metástase e resistência a terapia (DING et al., 2019; MAYENGBAM et al., 2021). A redução nos níveis de colesterol promove um aumento na fluidez de membrana favorecendo a invasão e metástase de células tumorais, enquanto que seu aumento pode promover resistência à quimioterapia por reduzir a permeabilidade de drogas (BERNARDES; FIALHO, 2018).

Alguns trabalhos demonstram que a via de colesterol está relacionada com glioma e sua inibição seria um bom alvo terapêutico (AHMAD et al., 2019; GUO et al., 2022). Utilizando dados de trancriptoma do *Cancer Genome Atlas* (TCGA) foi demonstrado que a via do mevalonato e o aumento da biossíntese de colesterol estão associados a piores prognósticos em pacientes com glioma, e ainda observou-se efeito citotóxicos em células de glioma e não de astrócitos quando tratados com inibidores da síntese do colesterol (KAMBACH et al., 2017). Porém Kambach e colaboradores não apresentaram quais genes dessas vias foram utilizados para gerar o metagene score utilizado no cálculo de sobrevida. Contrapondo essa observação, no entanto, um trabalho mais recente demonstrou que alta expressão de esqualeno mooxigenase (SQLE), uma outra enzima importante para a biossíntese de colesterol, foi relacionada com melhor prognóstico em pacientes com glioblastoma e a redução dessa expressão foi relacionado com resistência a morte após tratamento com TMZ em modelo celular (YAO et al., 2022). Nota-se que em glioma a síntese de colesterol é realmente importante para manter fenótipos tumorais, porém pouco sabe-se sobre a modulação dos níveis desse esterol na malignidade tumoral que pode apresentar efeitos opostos.

Colesterol também demonstrou importância para regulação dos níveis de esfingomielinas, além de ter um papel importante na degradação de alguns esfingolipídios mediados pela proteína transportadora de colesterol NPC2 (CHEN; XU; DUAN, 2015; ONINLA et al., 2014). De fato, NPC2 é estimulada a retirar colesterol dos endossomos quando há formação de ceramida, o que facilita ainda mais tal degradação (ENKAVI et al., 2017). A depleção de colesterol demonstrou também capacidade de reduzir a formação de agregados de gangliosídios na membrana

(FUJITA et al., 2007). Esses dados sugerem que compostos capazes de alterar o conteúdo de colesterol das células possam modular a formação de ceramida podendo ter um benefício antitumoral.

2.4.4 Lipidômica

A lipidômica é o estudo que compreende a identificação e quantificação de substâncias lipídicas, considerando, além de suas funções bioquímicas, a expressão e regulação gênica de proteínas importantes para o metabolismo lipídico. A lipidômica vem cada vez mais sendo introduzida em novos estudos, e apresenta um papel importante na elucidação do metabolismo celular de determinadas doenças (SPENER et al., 2003; TABORDA RIBAS et al., 2024). Porém, são poucos os trabalhos que analisam em modelo celular a composição lipídica total com intuito de esclarecer o envolvimento dessas moléculas na malignidade, manutenção, desenvolvimento e resistência a medicamentos em glioma.

Talvez a técnica mais amplamente utilizada no estudo da lipidômica seja a espectrometria de massas. Nessa técnica a amostra pode ser previamente separada por método cromatográfico ou ser injetada diretamente à fonte de íons. Essa metodologia compreende diferentes tecnologias que variam conforme a fonte de ionização e devem ser utilizadas de maneira a conhecer suas limitações, e as espécies que podem ser identificadas por tais métodos (WANG; WANG; HAN, 2019). Outra técnica importante é a ressonância magnética nuclear (RMN), apesar de apresentar menor sensibilidade, não é destrutiva e apresenta um menor tempo de análise (GEBREGIWORGIS; POWERS, 2012). RMN é uma técnica analítica fundamental utilizada no crescente campo da lipidômica, apesar de menor sensibilidade em comparação com a espectrometria de massa, suas características, como alta reprodutibilidade, capacidade quantitativa, não invasiva, além da habilidade de identificar metabólitos desconhecidos em misturas complexas em modelos ex vivo, *in vivo* ou *in vitro*, oferecem numerosos benefícios para a lipidômica (NAGANA GOWDA; RAFTERY, 2021). Vale ressaltar que ambas as técnicas apresentam certas limitações que devem ser consideradas e a utilização de diferentes técnicas podem ser úteis para haver complementação dos resultados.

3 RESULTADOS

Os resultados e métodos utilizados nesta tese serão apresentados por meio de dois artigos científicos. No primeiro manuscrito, focamos em entender como o metabolismo lipídico está intrinsecamente ligado à agressividade do glioma, explorando as complexas interações entre lipídios e as características distintas desse tipo de tumor. O segundo manuscrito concentra-se na relação específica entre o metabolismo lipídico e a resistência à temozolomida (TMZ) no glioblastoma, abordando a necessidade crucial de compreender esses mecanismos para aprimorar as estratégias terapêuticas. Essa abordagem integrada visa fornecer o entendimento mais aprofundado do papel dos lipídios na biologia do glioma.

4 MANUSCRITO I

Lipid metabolism as a marker for glioma aggressiveness.

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Running head: Lipid Metabolism and Glioma Aggressiveness

Highlights:

- Glioma aggressiveness is linked to lipid composition.
- Membrane alterations drive glioma aggression.
- Sphingolipid signaling is crucial in glioma.

Abbreviation list

APL - Area per lipid
CDP - Cytidine diphosphate
DMEM - Dulbecco MEM media
DMSO - Dimethyl sulfoxide

ECM - Extracellular matrix
EDTA - Ethylenediaminetetraacetic acid
FBS - Fetal bovine serum
GBM - Glioblastoma
GO - Gene Ontology
HSQC - Heteronuclear single quantum coherence spectroscopy
LGG - Low Grade Glioma
MSM - Methylsulfonylmethane
NMR - Nuclear Resonance Magnetic
PCR - Polimerase Chain Reaction
PI - Phosphatidylinositol
PTEN - Phosphatase and tensin homolog
PUFA - Polyunsaturated fatty acid
SM - Sphingomyelin
SMASE - Sphingomyelinase
SMPD - Sphingomyelin Phosphodiesterase
SPHK - Sphingosine Kinase
SQLE - Squalene epoxidase
S1P - Sphingosine-1-phosphate
TCGA - The Cancer Genome Atlas
TSP - Trimethylsilylpropanoic acid
UPR - Unfold protein response

Clinical Significance

This study holds significant importance on unraveling the lipid metabolism linked to glioma by integrating clinical and experimental data. Exploring the impact of lipid profiles on glioma aggressiveness unveils potential biomarkers, diagnostic tools, and therapeutic targets. The presented NMR data could serve as a tool for non-invasive Magnetic Resonance Spectroscopy in patients. Furthermore, the exploration of sphingolipid signaling as a key player in glioma progression provides valuable insights into the underlying mechanisms of this highly aggressive tumor. Understanding the lipid-related pathways and identifying relevant markers for glioma proliferation can ultimately contribute to more effective treatments and improved patient outcomes.

Abstract

Glioblastoma (GBM) is the most aggressive type of central system nervous tumor. There have been advances in glioma biology understanding, however, the current therapies are still inefficient. Additionally, new molecular markers are being explored for glioma grading, offering potential for novel diagnostic and drug targets. Considering the important role of lipid metabolism in tumorigenesis, understanding the lipid-related pathways in glioma could lead to new important markers. The aim of this study is to analyze lipid metabolism by integrating two different data sources, the transcriptome data from The Cancer Genome Atlas (TCGA), and experimental data from tumor cell lines; to investigate how lipid metabolism is regulated in glioma. We compared the expression of 743 lipid-related genes in public RNAseq data of glioma patients (n=681) to lipidomic analyses of glioblastoma cell lines (A172, U87MG, and T98G). We identified 29 lipid-related genes correlated to prognosis and constructed a risk-signature based on these genes. Extracellular matrix related genes were positively correlated to the risk score. Our findings revealed that aggressiveness is linked to alterations in membrane composition, characterized by increased fatty acid unsaturation, phospholipids, and cholesterol, which impact membrane physical properties. Modulation of critical signaling lipids, specifically sphingolipids, was also observed. The converging axis involving sphingomyelinase and sphingosine-1-phosphate, mediated by the actions of sphingomyelinase SMPD1 and sphingosine-kinase SPHK1, emerged as a pivotal factor in glioma. This study suggests that lipid membrane composition is pivotal for the GBM aggressiveness. Furthermore, it identifies lipid markers that may play a significant role in glioma.

Keywords: Lipid metabolism, glioma, lipidomic, plasma membrane

4.1 INTRODUCTION

Gliomas are classified according to their malignancy, being low-grade glioma (LGG) the less aggressive 1, and the GBM the more aggressive. Glioblastoma (GBM) is the most aggressive type showing a high lethality. This aggressiveness is explained by its high cellular density, necrosis, and endothelial proliferation, which leads to a poor prognosis with a survival rate of 14 months 2. Studying GBM could lead to a better biochemical understanding of how lipid mechanisms could be involved in aggressiveness.

Biochemical markers as choline, an indicator of cellular membrane could present a significant clinical value for glioma imaging-based diagnosing3–5. Choline/creatine measurements by magnetic resonance spectroscopy have shown that this ratio rises as the tumoral grade and proliferation index increase 3,6. Accumulation of choline-containing phospholipids has been already described in several cancers, and its metabolism plays an important pro-tumoral role supporting proliferative phenotype 7.

Cholesterol levels modulation has been described in several studies; however, it is not clear whether its decrease or increase that would be related to malignancy 8,9. Cholesterol level modulates biophysical properties of the membrane, and, with low levels decreasing membrane fluidity and favoring invasion and metastasis of tumor cells 10. Furthermore, it was shown that SQLE, an important enzyme to the cholesterol biosynthesis, is negatively correlated to aggressiveness and its overexpression leads to the inhibition of migration and invasion in GBM cells 11.

Sphingolipids have been extensively described to modulate tumoral behavior, and play an important role in glioma malignancy 12. Higher levels of ceramide are related to antitumoral effects, and several studies demonstrated that its treatment promotes glioma cell death 13–17. Ceramide can be generated by either de novo synthesis or by degradation of other sphingolipids as sphingomyelinase, that converts sphingomyelin to ceramide 18. On the other hand, sphingosine-1-phosphate (S1P) has shown to promote pro-tumoral effects being correlated to glioma malignancy 12. Here we explore the whole lipid metabolism in glioma to investigate which lipid would be related to aggressiveness in glioma.

4.2 MATERIALS AND METHODS

4.2.1 Bioinformatic analysis

The gene set “REACTOME_METABOLISM_OF_LIPIDS” was obtained from the Human Molecular Signatures Database (MSigDB), and 743 lipid metabolism-related genes were extracted. The TCGA RNAseq normalized gene expression data of 743 lipid-related genes from 681 glioma (LGG and GBM) patients were collected from Firebrowse (http://firebrowse.org/). The expression was compared between groups by calculating T-test using the statistical function of the scientific computation library SciPy in Python. The data regarding survival and PTEN status were collected from the Gliovis website (http://gliovis.bioinfo.cnio.es/), and the survival analysis were

calculated by using the KaplanMeierFitter function of the Lifelines library in Python. Further, the up and down-regulated genes were separated according to their mean values. The modulated genes were subjected to gene ontology (GO) and function cluster annotation analysis using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp) to determine significantly enriched genes. All the graphs were generated in Python by using the data visualization library Seaborn.

To identify genes with significant prognostic value, gene expressions that showed statistical differences between LGG and GBM underwent a univariate Cox analysis, where those with a P value < .001 were considered prognostically relevant. Subsequently, these chosen genes were integrated into a Cox regression model utilizing the least absolute shrinkage and selection operator (LASSO) technique. The resulting coefficients for each gene were employed to compute the risk score: riskscore=∑(Coefi×Xi). The threshold for categorizing individuals into the high-risk and low-risk groups was set at the median risk score. The difference in survival outcomes between the high-risk and low-risk groups was evaluated through the creation of survival curves, followed by the application of the log-rank test.

4.2.2 Cell culture

The A172, U87MG and T98G human GBM cell lines were kindly provided by Professor Mari C. Sogayar, University of São Paulo. Cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and 25 µg/ml gentamicin (Gibco), at 37ºC in a humid atmosphere containing 5% CO_2 and maintained at approximately 80% confluency. For growth curves, 1×10^4 cells were seeded in 48-wells plates. Triplicates were collected on days 1, 3, 5, and 7, using trypsin, fixed in 3.7% formaldehyde and counted using the Neubauer chamber. The graph was generated in python by using the data visualization library Seaborn.

4.2.3 Lipid extraction

Lipid extraction was performed as previously described 19. 2.3 x 10^6 cells were seeded in a 150 mm plate (8 plates) for 24 hours, then collected using 0.1% trypsin solution containing 1 mM EDTA and centrifuged at 2000 g for 5 minutes (4 °C). The cell pellet was washed thrice with phosphate buffer, and the supernatant was discarded. The cell pellets were subjected to lysis, by freezing into liquid nitrogen and returned to a 37 °C water bath several times. After being freeze-dried overnight,

10 mg, of each cell line, was weighed and transferred to a glass tube with a screw Teflon cap (volume of 4 mL). The pellet was extracted using $CHCl_3$:MeOH 1:1 (v/v) (1 mL), vortexed for 1 minute, and then kept for 10 min at room temperature in an ultrasonic bath. The supernatant was collected, and the solvent dried under a stream of nitrogen.

4.2.4 NMR spectroscopy

$CDCl_3$ was purchased from Cambridge Isotope Laboratories, Inc. (Miami, U.S.A.) and from Sigma-Aldrich (St. Louis, MO). The samples were deuterium exchanged by repeated dissolution in MeOD-D_2O (2:1). Both were freeze-dried overnight. Their spectra were obtained in $CDCl_3$-MeOD (3:1, 600 µL) at 30 °C, using methylsulfonylmethane (MSM) as internal standard (δ = 3.03 ppm). Spectra were obtained on a Bruker 600 MHz ASCEND equipped with a QXI probe (Bruker Biospin, Germany). 1D ^{1}H-NMR was carried out using enough scans to give a Signal/Noise (S/N) ratio of at least 2000/1 (90° pulse, relaxation delay = 4.0 s, number of time domain points = 65536, spectral width = 10.6541 ppm and acquisition time = 7.7 s). Experiments were performed without tube rotation and with the MSM or TSP signal at a. medium half line width ranging from 0.6–1.0 Hz). For multivariate analysis and estimation of metabolites concentration, the spectra were acquired using the noesygppr1d.2 pulse sequence, with 128 transients and a delay of 10.0 s (2.0 s on the analysis of lipid samples). 2D NMR experiments were carried out using multiplicity-edited ^{1}H-^{13}C HSQC, heteronuclear correlation via double inept transfer with decoupling during acquisition, using trim pulses in inept transfer with multiplicity editing during the selection step (hsqcedetgpsp.3). The signals were assigned according to previous described[19].

4.2.5 Simulation

The GROMACS 2019.1 MD (ABRAHAM et al., 2023, 2015) engine and MARTINI 2.2 (MARRINK et al., 2007) coarse-grain forcefield were used for simulations. Three membranes (15 × 15 × 10 nm) included polarizable MARTINI water and 150 mM NaCl were constructed using the insane package(WASSENAAR et al., 2015). Each membrane was energy-minimized with steepest descent, following 50 ns equilibration at 310 K (with a coupling constant of 1.0 ps) using a 10 fs timestep and constant temperature and pressure. Pressure was maintained at 1 bar with a Berendsen

barostat (3×10^{-4} bar^{-1}, 12 ps). Production simulations were performed for 20 μs in triplicate, with each replicate initiated from different velocities. Temperature maintained same as for equilibration, while semi-isotropic Parrinello-Rahman pressure coupling (1 bar, 3×10^{-4} bar^{-1}, 12 ps) was used to maintain pressure(WILSON et al., 2020). All simulations were conducted with a timestep of 20 fs, according to standard MARTINI configuration recommendation (DE JONG et al., 2016). The screenshot images were generated using the 3D viewer MOL*(SEHNAL et al., 2021) from the RCSB PDB (https://www.rcsb.org/)(BERMAN; HENRICK; NAKAMURA, 2003; BERMAN et al., 2000).

Biophysical properties were analyzed using ROH, GL1, and GL2 beads as references for cholesterol and phospholipids, respectively. Reported values include SEM between replicates. The area per lipid (APL) was calculating according to its definition: the cross-sectional area (AXY) of the whole system along the bilayer surface plane (XY -plane), divided by half the total number of lipids (NL) present in the bilayer, i.e., AL = AXY/(NL/2). Membrane thickness, flip flop rate and self-diffusivities were calculated using lipyphilic package (SMITH; LORENZ, 2021).

4.2.6 Quantitative gene expression assays

Reverse transcription was performed with 1 μg total RNA and Random primers, with ImProm II Reverse Transcriptase Kit (Promega, Madison, USA). qPCR was carried using the GoTaq® Real-Time PCR Systems (Promega) on a StepOne Plus thermal cycler (Applied Biosystems), according to the recommended protocol. Data were normalized according to Vandesompele et al. (VANDESOMPELE et al., 2002), using as reference genes GAPDH, HMBS and HPRT being the HMBS selected as the best normalizer.

Primers sequences used were: SPHK1 sense: 5'-ATTATGCTGGCTATGAGCAGG–3'; SPHK1 Antisense: 5'-TGCAGAGACAGCAGGTTCAT–3'; SPKK2 sense:5'-CCAGACAGAACGACAGAACCAC–3'; SPHK2 Antisense: 5'CTCCCGAGACCGTGACGATG3'; SMPD1 Sense:5 ACCGAATTGTAGCCAGGTATGA3'; SMPD1 Antisense: 5'AGAAGACCTCAAATTCATCCACA3'; GAPDH sense: 5-'ACCCACTCCTCCACCTTTGA-3'; GAPDH antisense: 5'-CTGTTGCTGTAGCCAAATTCGT-3); HPRT sense: 5'-

GAACGTCTTGCTCGAGATGTGA-3'; HPRT antisense: 5'-TCCAGCAGGTCAGCAAAGAAT-3'; HMBS sense: 5'-TGGACCTGGTTGTTCACTCCTT-3'; HMBS antisense: 5'-CAACAGCATCATGAGGGTTTTC-3').

4.2.7 Statistical analysis

All experimental data were obtained with three independent experiments. The ANOVA was complemented with Tukey's test, and the calculations were performed with Python software using stastmodel module. All statistical tests were considered at the predetermined level of significance of 5%. All the graphs were generated in Python by using the data visualization library Seaborn.

4.3 RESULT

4.3.1 Identification of lipid metabolism-related genes with prognostic value in glioma

To evaluate how lipids could be related to glioma aggressiveness we first assessed the expression data of 743 lipid-related genes in public RNAseq data TCGA of glioma patients (n=681). We compared the expressions between low-grade glioma (LGG, n=530) and glioblastoma (GBM, n=171) by t-test and select the genes that are up and downregulated ($p < 0.05$,; $-\log10(p\text{-value})<1.3$) in the most aggressive subtype to perform enrichment analysis (Figure 1A and 1B). High-grade glioma showed higher expression of genes related to the synthesis of membrane lipids such as glycosphingolipids, phosphatidylcholine, and the signaling lipids, sphingolipids. Whereas reduced expression of genes related to phosphatidylinositol and cholesterol synthesis was present in GBM.

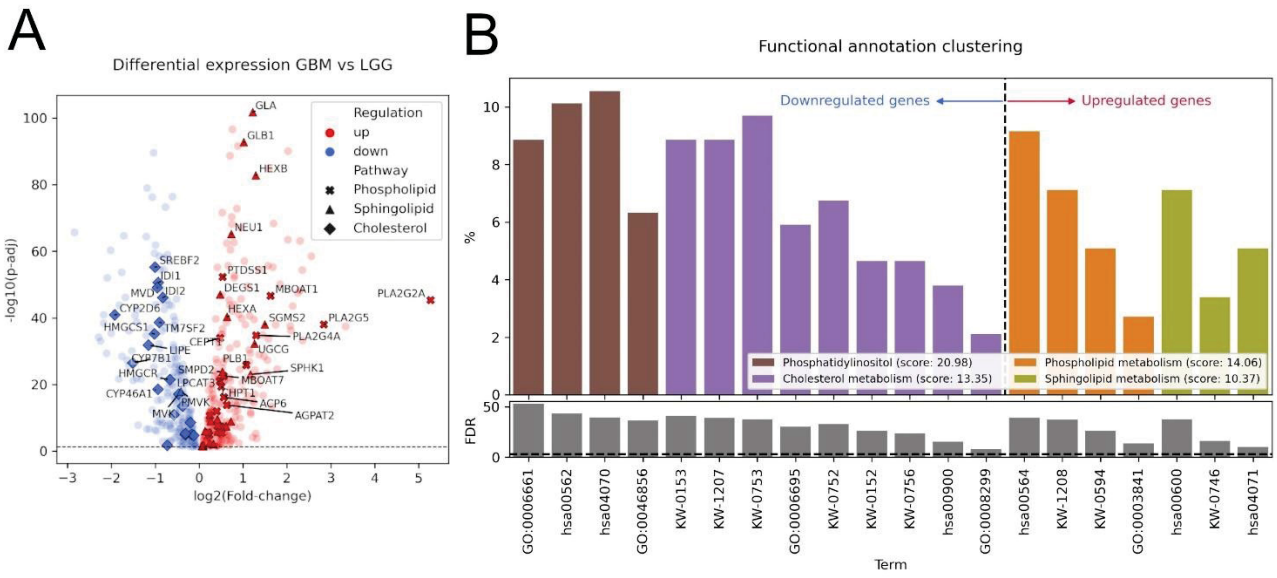


Fig 1. Lipid metabolism pathways related to glioma aggressiveness. A) Lipid metabolism related DE genes (LGG vs GBM) are represented on volcano plot, genes involved in the most deregulated pathways are highlighted. B) Enrichment analysis, identifying related genes by measuring the similarity of their global annotation profiles, of down and up-regulated lipid metabolism-related genes using t-test ($p<0.05$) comparing expression data of GBM to LGG patients (n=681); the genes were classified into three functional gene groups based on the functional similarity scores. Statistical values of -ln(FDR) to rank their biological significance is shown.

To evaluate the relationship between lipid metabolism-related genes and glioma patients' survival we first collected the most differently expressed (DE) genes ($p < 0.05$, $|logFC| > 1$) to further perform univariate Cox regression analysis. We identified 109 DE genes between LGG and GBM samples and after univariate Cox regression analysis, 29 genes were significantly associated with patient prognosis ($p<0.001$). Subsequently, the chosen genes were integrated into a Cox regression model resulting coefficients for each gene, which were then employed to calculate the risk score (Supplementary Table1). The median risk score was used as a cutoff to separate patients into the groups, high-risk group, and low-risk group. Consistently, Kaplan-Meier analysis showed a significant difference in overall survival between high-risk and low-risk patients (Figure 2 A).

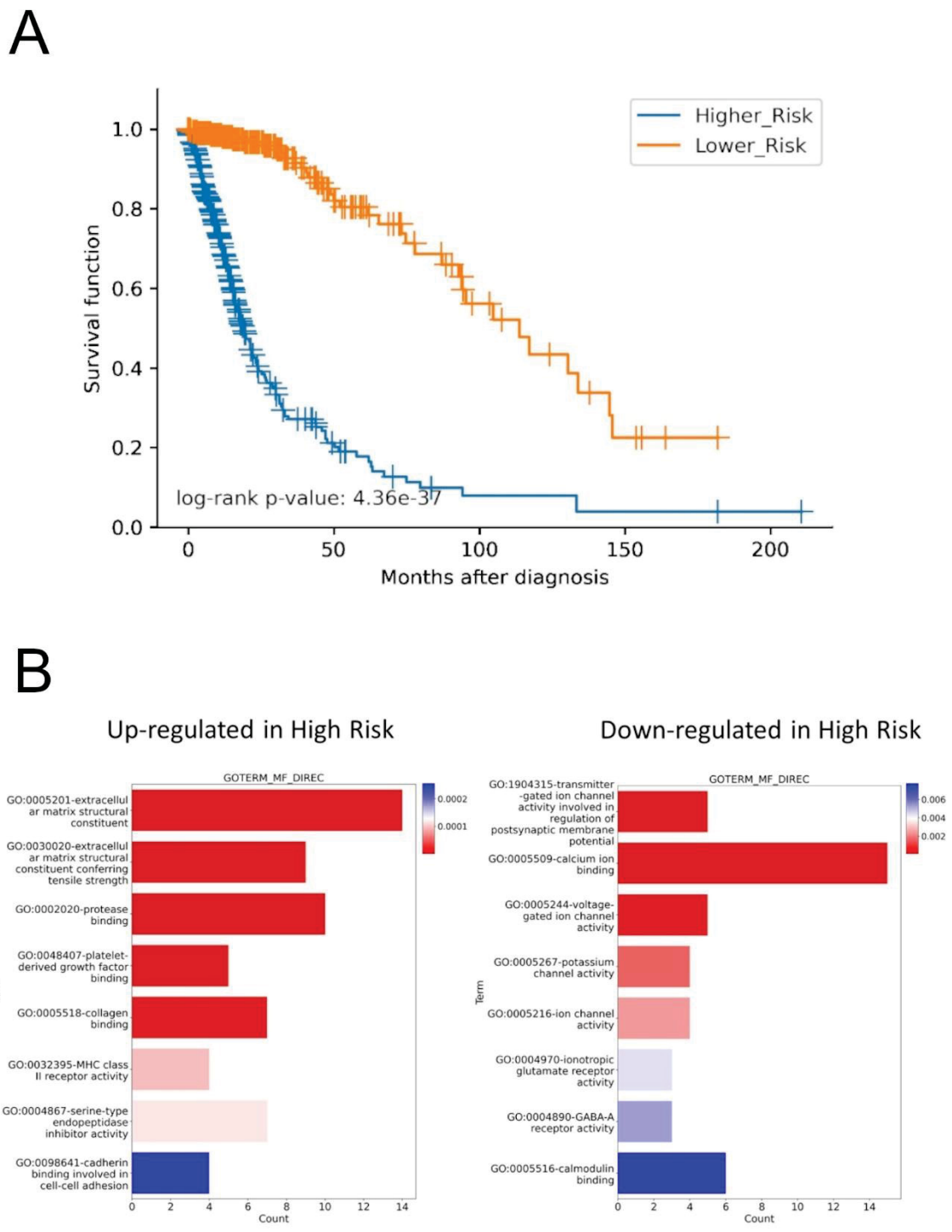


Fig. 2 - Lipid-associated risk score establishment. A) Survival curves of patient according to the lipid-associated risk score using the median risk value as cutoff. B) Enrichment analysis, using molecular functional (MF) annotation from gene ontology (GO), of down and up-regulated genes using $p < 0.05$ and $|logFC| > 2.5$ comparing expression data of high-risk low-risk patient groups. Statistical values to rank their biological significance are represented by color scale.

Furthermore, we analyzed DE genes related to risks by comparing High to Low lipid-related risk groups and performed an enrichment analysis of significant gene expressions ($p < 0.05$, $|logFC| > 2.5$). As shown in figure 2B, genes related to extracellular matrix and its regulation are up-regulated in high-risk patients, while genes related to ion transportation are down-regulated.

Taking together the data indicates that there is a crosstalk between membrane composition and extracellular matrix, and ion transportation, which could play an important role on the tumor aggressiveness phenotype.

4.3.2 Cholesterol, phospholipid, and sphingolipid metabolisms are modulated in high grades gliomas

To better understand how lipid metabolism pathways changed according to the aggressiveness phenotype, we further analyzed the differentially expressed lipid metabolism related genes. Important genes to phosphatidylcholine and phosphoethanolamine synthesis are more expressed in GBM and related to lower survival rates; whereas, in LGG, lower levels of these phospholipids are suggested since higher expression of genes involved with their degradation are presented (Figure 3 and S1). Although, it has been shown that the two initial steps of phospholipid biosynthesis responsible to convert choline to CDP-choline are related to a less aggressive type of glioma, as shown by the higher expression of *CHKA* and *PCYT1B* in LGG as well as by the higher survival rate in patients overexpressing these genes (Figure 3 and S1). The data also suggested that GBM might have higher levels of the pro-tumoral sphingolipid sphingosine-1-phosphate generated from different sources such as *de novo* synthesis and sphingomyelin degradation (Figure 3). Taken together, the result shows that higher levels of phosphatidylcholine, phosphoethanolamine, and sphingosine-1-phosphate could play an important role in glioma aggressiveness.

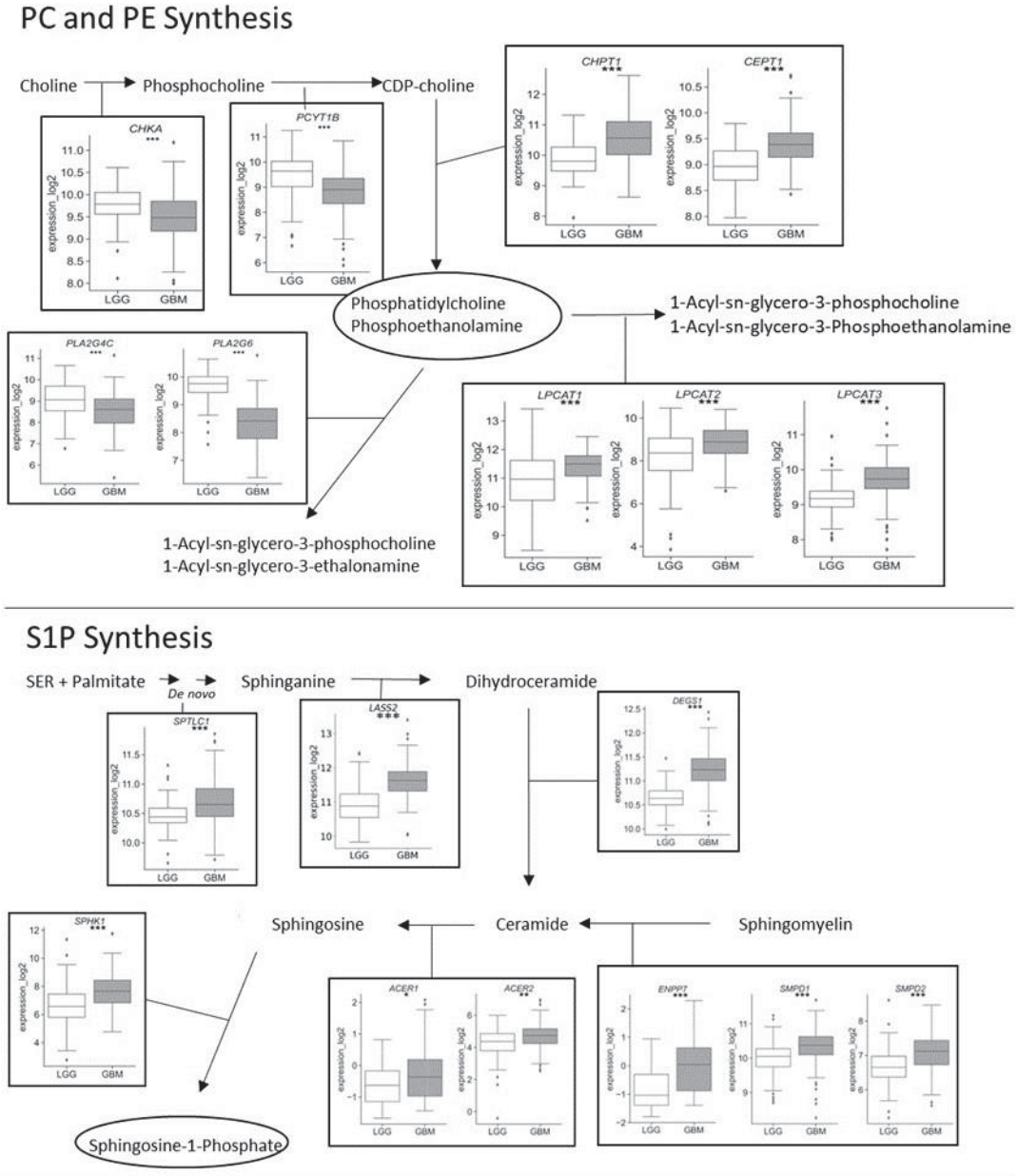

PC and PE Synthesis
Choline
Phosphocholine
CDP-choline
CHKA
PCYT1B
CHPT1
CEPT1
Phosphatidylcholine
Phosphoethanolamine
1-Acyl-sn-glycero-3-phosphocholine
1-Acyl-sn-glycero-3-Phosphoethanolamine
PLA2G4C
PLA2G6
LPCAT1
LPCAT2
LPCAT3
1-Acyl-sn-glycero-3-phosphocholine
1-Acyl-sn-glycero-3-ethalonamine
S1P Synthesis
SER + Palmitate
De novo
Sphinganine
Dihydroceramide
SPTLC1
LASS2
DEGS1
SPHK1
Sphingosine
Ceramide
Sphingomyelin
ACER1
ACER2
ENPP7
SMPD1
SMPD2
Sphingosine-1-Phosphate
Cholesterol Synthesis
Acetyl-CoA
Acetoacetyl-CoA
ACAT1
HMGCS1
HMGCS2
3-hydroxy-3-methylglutaryl-coa
HMGCR
SQLE
Mevalonate
Cholesterol
expression_log2
LGG
GBM

Fig. 3 Lipid metabolism pathways related to glioma aggressiveness. A) Representation of the three most important lipid metabolism pathways with the expressions of the genes that are being modulated according to aggressiveness, T-test was used to compare between groups *p<0,05; **p<0,01;***p<0,001 (n=681).

On the other hand, our analysis show that cholesterol metabolism might be prominent in LGG. Several genes important to the mevalonate pathway are differentially expressed in this subtype; furthermore, *HMGCR* and *SQLE*, two important enzymes that regulates the cholesterol biosynthesis, are related to higher survival rate suggesting that increased levels of this sterol might be important to sustain LGG (Figure 3 and S1). The phosphatidylinositol pathway was also shown to be less active in GBM. However, since PTEN mutation could be related to higher-grade glioma, we assessed whether the phosphatidylinositol pathway modulation would be due to a higher PTEN mutation prevalence in this group. Indeed, PTEN mutation is more incident in GBM patients as shown in Figure S2, furthermore, comparing patients with PTEN mutant (PTENmut) with PTEN WT shows that mutation in PTEN is correlated to lower expression of genes involved in the phosphatidylinositol (PI) pathway (Figure S2). Interestingly, PTENmut patients also showed lower expression of genes related to the cholesterol pathway, suggesting that also in the more aggressive phenotype by considering PTEN status, the cholesterol pathway is also downregulated. PTEN mutation is related to a worse prognosis, therefore, the cholesterol pathway is downregulated in a more aggressive phenotype not only considered by tumor grade but also by the PTEN status.

4.3.3 Cholesterol and phospholipids metabolisms are modulated in glioblastoma cells with different aggressiveness phenotypes.

To investigate the lipid metabolism in the aggressiveness phenotype and confirm the clinical data, we used three PTEN mut glioblastoma cell lineages with different pre described tumorigenic phenotypes (A172, U87MG, and T98G)(CORRÊA et al., 2006; LOUCA et al., 2019). A172 is the least aggressive cell line with the slowest proliferation rate reaching the saturation density of $10.24x10^4$ cells/cm^2 (SD = 1.22 $x10^4$) 1at the 9^{th} day, while U87MG presented a saturation rate of $29.7x10^4$ cells/cm^2 (SD = 1.61 $x10^4$) (Figure S3). The most proliferative cell line, T98G, reaches the stationary phase in 7 days with a similar saturation density as U87MG, $28.7x10^4$ cells/cm^2 (SD = 1.28 $x10^4$). Then, we performed a lipidomic analysis of the lipid extract

of those cells by using standardized ^{1}H NMR spectra (Figure 4A). The ^{1}H NMR analysis showed that the three glioblastoma linages presented similar lipid profiles, with the exception of the phosphocholine-related lipid profile (Figure 4B). That suggests different levels of choline, phosphocholine, and phosphatidylcholine among the cell lines. Furthermore, quantification of this data showed that A172, which presents the less aggressive phenotype, contains lower levels of total phosphocholine-related lipids, ethanolamine lipids, and higher levels of cholesterol (Figure 4C). These results are in accordance to the bioinformatics data, suggesting that phospholipid and cholesterol play opposite roles in glioblastoma aggressiveness.

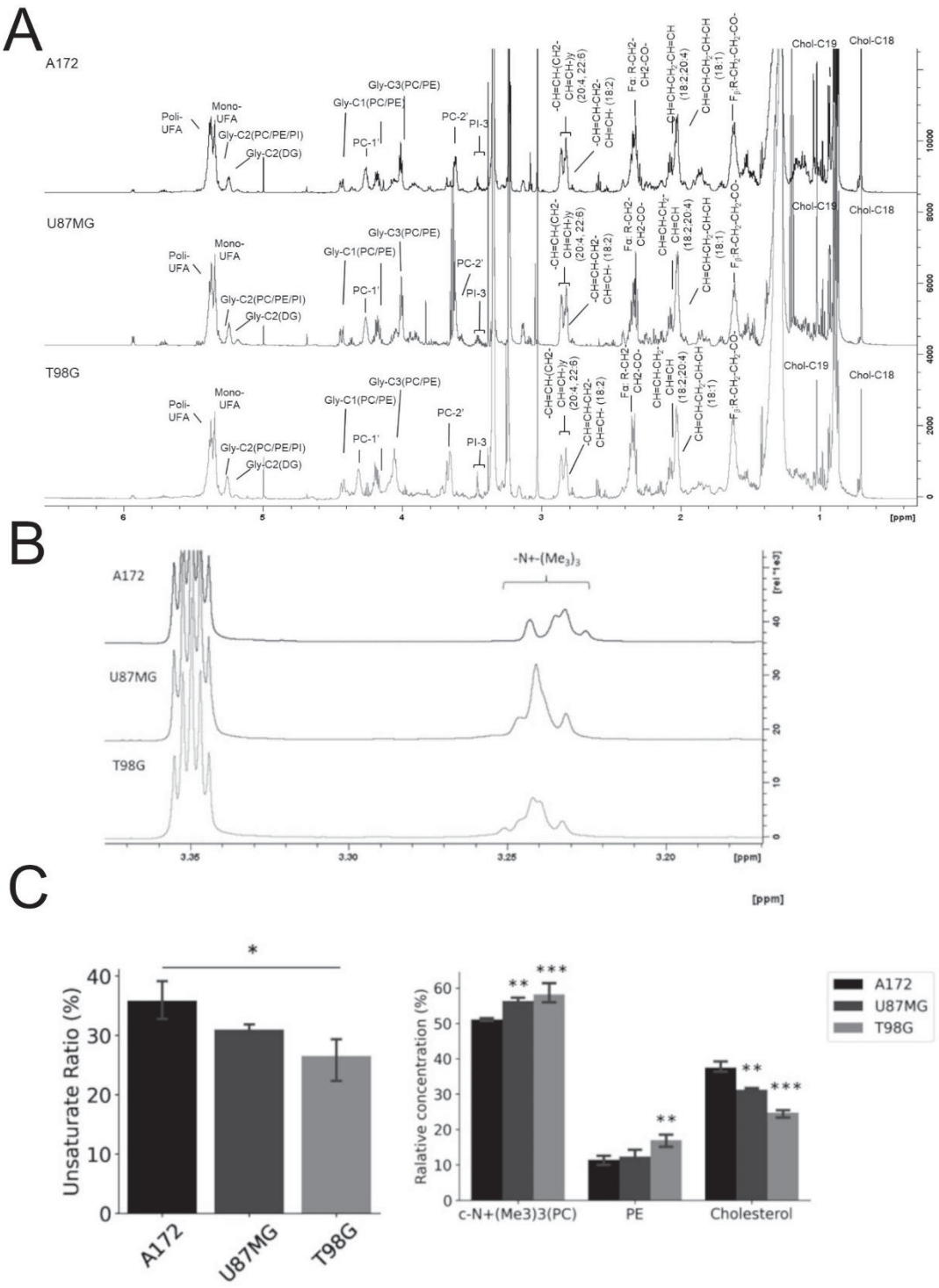


Fig. 4. Lipid profile related to aggressiveness *in vitro*. A) 1H NMR spectra of the lipid extract of the GBM cells. B) 1H NMR spectra showing the different profiles of lipids containing choline between the cells.C)The relative quantification of the main lipid groups using MSM as an internal standard, data related to three independent experiments. Anova test complemented with Tukey's test were used for statistical analysis among groups. Data are represented as mean ± SEM. ** represents $p < 0.01$; *** represents $p < 0.001$.

4.3.4 Membrane fluidity could be modulated according to the glioblastoma aggressiveness.

We also observed different levels of fatty acid unsaturation rates according to the aggressiveness phenotype in the glioblastoma cell lines (Figure 4C). The less aggressive cell line, A172, presented higher levels of unsaturated to saturated fatty acids, furthermore, the unsaturated ratio decreases as the aggressiveness increases. To further asses the relationship between malignancy and fatty acid unsaturation we also assessed the expression of genes related to fatty acid biosynthesis in patients with different malignant grades (Figure S4A). Interestingly, high grade patients showed lower expression of desaturase genes also suggesting that a decreased levels of unsaturated fatty acids is related to malignancy. Since fatty acid acyl chain length also impacts the membrane fluidity, we evaluated the expression of genes related to fatty acid elongation. Higher expression of these genes associated to lower expression of desaturase genes suggest that biophysical properties of membranes change according to aggressiveness in gliomas (Figure S4B).

Since cholesterol levels also impact membrane physical properties, we use the MARTINI force field for coarse-grained molecular dynamics simulations to evaluate the impact of unsaturation rate and cholesterol levels on mechanical properties of the membrane by using three membranes that decrease cholesterol levels and unsaturated ratio as observed in our experimental data (Figure 5A). The membrane 1, membrane 2 and membrane 3 share similar composition of these lipids to A172, U87MG and T98G, respectively. Area per lipid (APL) slightly increases from membrane 1 to membrane 3, even though no changes on bilayer thickness were observed (Figure 5B). Interestingly, the lipid composition related to more aggressive phenotype is related to decreased cholesterol flip-flop rate (Figure 5C). Changes on lipid composition observed on glioma cells impact the membrane mechanical property and cholesterol trafficking suggesting that biophysical properties of membrane are modulated according to aggressiveness.

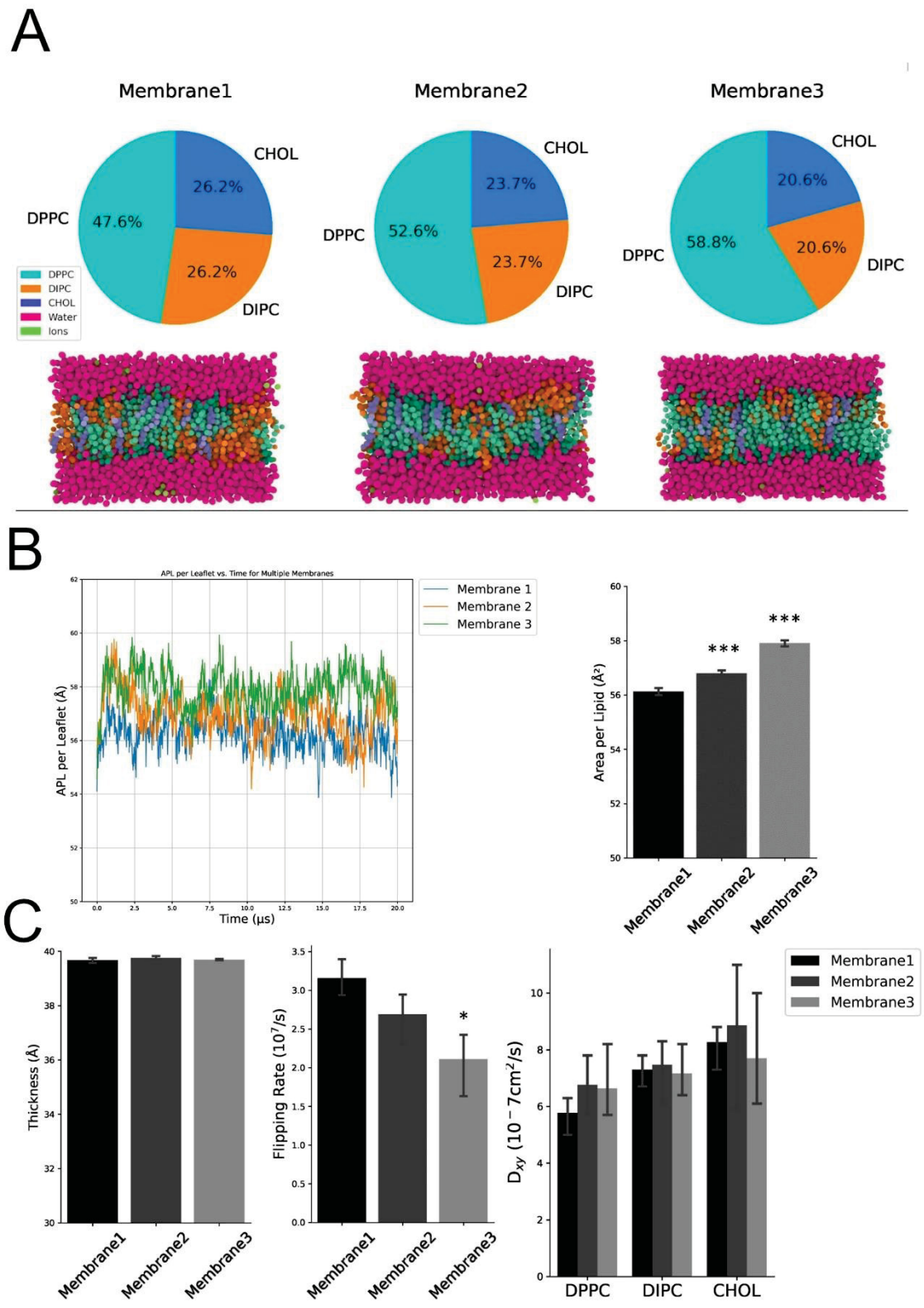


Fig. 5. Lipid modulation affects biophysical properties of membranes. A) PM lipid distributions. Pie charts with the overall distribution of the lipids, as well as snapshots of the membranes of the simulations after 20 μs. The membrane 1, membrane 2 and membrane 3 share similar composition of these lipids to A172, U87MG and T98G, respectively. B) APL over 20 μs simulation and the average APL. C) Membrane Thickness; average CHOL flipping rate and lipid lateral self-diffusivities for each

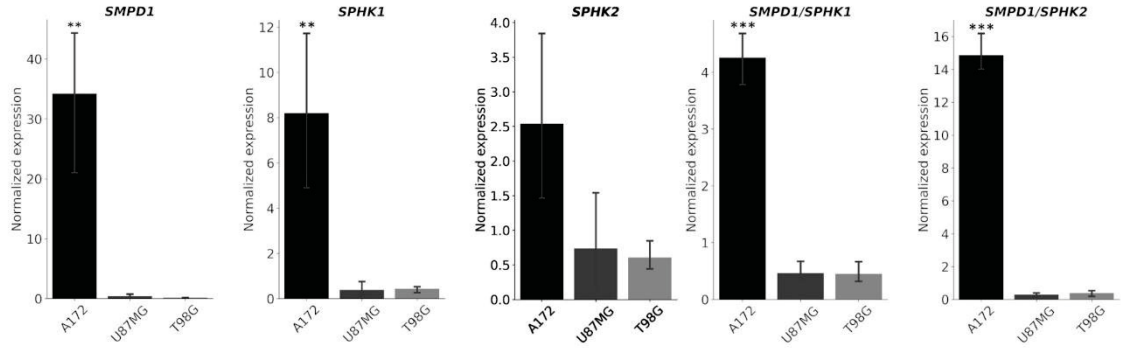
membrane. Anova test complemented with Tukey's test were used for statistical analysis between groups. Data are represented as mean ± SEM. *p<0,05;***p<0,001.

4.3.5 Sphingomyelin to sphingosine-1-phosphate conversion dictates glioma proliferation and prognosis.

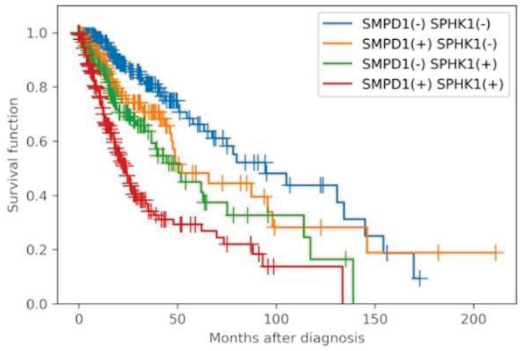
Since the patient data showed that the sphingolipid pathway could be modulated in malignancy, we performed a 2D NMR HSQC analysis to evaluate these lipids (Figure S5). We have noticed that the A172 cell lines showed no chemical shifts related to the sphingomyelin which are presented in more proliferative cells (Figure S5). Since sphingomyelin could be degraded into ceramides, which present antitumorigenic properties, we evaluated the expression of the sphingomyelinase gene (*SMPD1*) in the GBM cells. As shown in figure 6A, the A172 cell line presented a higher expression of *SMPD1*, which could explain the lower levels of sphingomyelin in this cell line.

Ceramides produced by sphingomyelin degradation could be used to generate S1P, which presents tumorigenic properties. Furthermore, as we noticed, higher-grade patients present higher levels of both *SMPD1* and *SPHK1* gene expression (Figure 3). We also evaluate the expression of *SPHK1* and *SPHK2* in the cells to assess the S1P synthesis pathway. Even though, the less proliferative cell line presents a higher expression of *SMPD1* and *SPHK1* isoform the ratio *SMPD1/SPHK* is much higher in the less proliferative cell line (Figure 6A). Therefore, the synthesis of S1P via the conversion of sphingomyelin to ceramide and then to S1P might be important for a more proliferative phenotype. Kaplan Meier analysis has shown that higher expression of *SMPD1* and *SPHK1* highly decreases the patient's survival, whereas there are not very significant differences when only one gene is higher expressed (Figure 6B). The same is not observed with *SMPD1* and *SPHK2,* where only a higher expression of *SPHK2* is related to decrease survival, meaning that SMPD1/SPHK1 and not SMPD1/SPHK2 axis is related to aggressiveness in patient. Furthermore, higher *SMPD1/SPHK1* ratio was related to better overall survival, suggesting that that SM to S1P via SMPD1/SPHK1 axis would be related to aggressiveness and patient's prognosis.

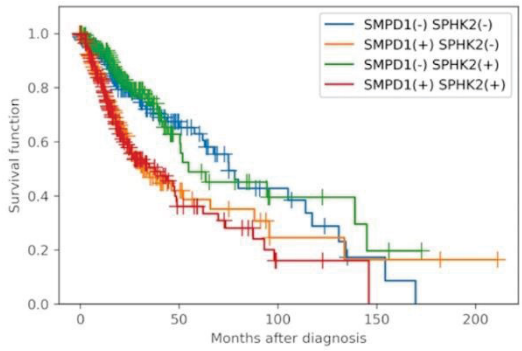
A



B



	logrank_test (p)
SMPD1(-) SPHK1(-) vs SMPD1(+) SPHK1(-)	0.029
SMPD1(-) SPHK1(-) vs SMPD1(-) SPHK1(+)	5.598e-05
SMPD1(-) SPHK1(-) vs SMPD1(+) SPHK1(+)	9.806e-16
SMPD1(+) SPHK1(-) vs SMPD1(-) SPHK1(+)	0.160
SMPD1(+) SPHK1(-) vs SMPD1(+) SPHK1(+)	1.838e-06
SMPD1(-) SPHK1(+) vs SMPD1(+) SPHK1(+)	0.0004



	logrank_test (p)
SMPD1(-) SPHK2(-) vs SMPD1(+) SPHK2(-)	0.0025
SMPD1(-) SPHK2(-) vs SMPD1(-) SPHK2(+)	0.57
SMPD1(-) SPHK2(-) vs SMPD1(+) SPHK2(+)	0.0001
SMPD1(+) SPHK2(-) vs SMPD1(-) SPHK2(+)	0.0002
SMPD1(+) SPHK2(-) vs SMPD1(+) SPHK2(+)	0.69
SMPD1(-) SPHK2(+) vs SMPD1(+) SPHK2(+)	4.994e-05

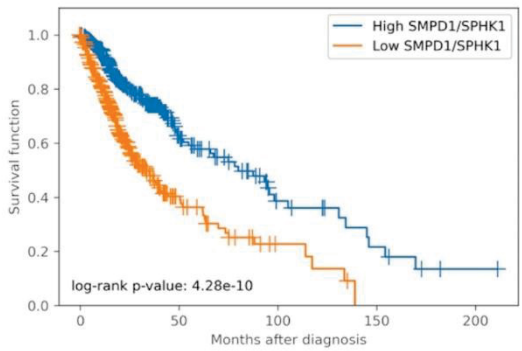


Fig.6 Impact of the SM to S1P in glioma aggressiveness. A) Relative expression levels of *SMPD1*, *SPHK1* and *SPHK2* normalized to *HMBS* in each glioma cell line, and the expression ratio between *SPHK1/SMPD1* and *SPHK2/SMPD1*. ANOVA test was used for statistical analysis between groups. Data are represented as mean ± SEM. * represents $p < 0.05$; ** represents $p < 0.01$ and *** represents $p < 0.001$. B) Kaplan-Meier curves displaying the estimated survival probability for 4 different groups of

patients according to the expressions of *SPHK1* and *SMPD1* and the log rank test calculated for each comparison.

Taken all together, the expression of *SMPD1* could be related to a higher grade of glioma, however, the high expression of these should be associated with higher expression of the *SPHK*1 gene.

4.4 DISCUSSION

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We could identify 29 lipid-related genes that are correlated to prognosis in patient data. And from the risk-signature constructed based on the expression of these genes, we showed a significant difference in overall survival according to the risk. Extracellular matrix related genes were positively correlated to the risk score, while the gene expression data suggested that structural and signaling lipid pathways could be involved in glioma aggressiveness, because GBM patients showed more expression of genes important to the synthesis of phosphatidylcholine and phosphoethanolamine, very important components of the plasmatic membrane 33. Taking all together, the data indicates that a crosstalk between membrane composition and its role to regulated extracellular matrix (ECM) would play an important role on the aggressiveness phenotype. Mechanical and physical properties of ECM controls cell proliferation and it was shown that also regulates lipid metabolism trough different pathways 34,35. Phosphoethanolamine plays an important role promoting membrane protein folding 36, interestingly ER lipid associated pathways are also up regulated in the GBM patients suggesting that high levels of PE could be due to a response to ER stress. It has been shown that GBM highly express unfold protein response (UPR) markers, therefore, high levels of PE could sustain oncogenic ER stress and promote glioblastoma survival37.

Accumulation of choline-containing phospholipids has been already described in several cancers, and its metabolism plays an important pro-tumoral role supporting proliferative phenotype 7. Here, we demonstrated in glioblastoma cell lineages with different tumorigenic phenotypes (A172, U87MG, and T98G) 31,32, that levels of choline-containing lipids increase according to the aggressiveness of GBM cells confirming what the expression data analysis suggests. Higher expression of genes involved in the last step of phosphatidylcholine synthesis, along with lower expression

of genes involved in phosphatidylcholine degradation in GBM patients could be related to higher levels of this lipid, therefore, being an important pathway to glioma aggressiveness maintenance. Moreover, according to the NMR profile the analyzed cell lines present different levels of choline, phosphocholine, and phosphatidylcholine. The different composition observed in cell lines could also suggested that they would be related to glioma proliferation and aggressive phenotype.

PTEN mutation is strongly related to worse prognosis and malignancy in glioma 38. Interestingly, we have noticed that lower expressions of several phosphatidylinositol-related genes are correlated to higher grade gliomas. Since PTEN mutation is more common in higher grade gliomas we evaluated whether the PI pathway modulation would be due to a higher PTEN mutation prevalence. PI pathway associated genes are downregulated in PTEN mutated patients regardless of the glioma grade which could be explained by a negative feedback regulation of gene expression. Cholesterol metabolism was negatively associated with PTEN mutation. Since PTEN mutation is related to worse prognosis and more aggressive type of glioma 38, it would reenforce that cholesterol metabolism is more related to less aggressive type of gliomas. Here, we showed that several genes important to cholesterol biosynthesis are downregulated in GBM. Furthermore, important enzymes that regulates the cholesterol biosynthesis, HMGCR and SQLE, are related to higher survival rate. We have determined the cholesterol profile in our cellular model, in which cholesterol decrease is related to higher proliferation rate in PTENmut glioblastoma cells.

The MARTINI molecular dynamics simulations shows that a lower unsaturation rate and lower levels of cholesterol similar to more proliferative glioblastomas cells decrease cholesterol flipping rate and increase APL suggesting that physical membrane properties changes could be related to higher malignancy. APL is a measure of membrane packing and is closely related to the phase and fluidity of the membrane. It has been described that decreased levels of cholesterol in the membrane of cancer cells lead to an increase in APL which could be explained by a lower ordering of the lipid chains 39–41. Furthermore, cholesterol flip-flop rate could be modulated by its own levels and unsaturation rate 42,43. Here, we showed that lipid composition of more aggressive cells leads to a more fluid membrane and changed cholesterol trans-bilayer distribution that would impact the organization of cell membrane. More fluid membranes were already described in cancer cells and promote

a more aggressive phenotype by facilitating tissue invasion and metastasis33,44. Furthermore, the cholesterol efflux receptor was related to control of the membrane fluidity in cancer, and by decreasing that fluidity, the cell motility and the epithelial-mesenchymal transition can be reduced as well as metastasis inhibition45.

It was described that increased polyunsaturated fatty acid (PUFA) regulates domains of the membrane by its low affinity to cholesterol which would modulate proteins signaling 46, therefore, the levels of PUFA and cholesterol could not only be modulated to regulate malignant membrane biophysical properties but also tumoral signaling pathways in more proliferative glioblastoma cells.

Cholesterol intracellular trafficking is tightly correlated to sphingomyelin levels by acid SMase activity 47–50. Here, we showed that less aggressive glioblastoma cell line present higher levels of cholesterol and lower levels of sphingomyelin suggesting dual role of those lipids in aggressiveness.

The less proliferative glioblastoma cell (A172) shows higher expression of acid sphingomyelinase *SMPD1* suggesting that this cell linage has higher rate of conversion of sphingomyelin to ceramide. On the other hand, S1P was shown to promote pro-tumoral effects being correlated to glioma malignancy12. According to that, we showed from TCGA data that sphingolipid related genes are upregulated in higher grade glioma, and it suggests that S1P would be more presented in this more aggressive type. Furthermore, since ceramide formed from sphingomyelin could be further converted to S1P, we analyzed the SMPD1 to SPHKs ratios (SMPD1/SPHK1 and SMPD1/SPHK2) in the glioblastoma cells. The less proliferative cell line showed higher SMPD1/SPHK1 and SMPD1/SPHK2 ratios suggesting more conversion of sphingomyelin to ceramide, whereas the more proliferative cells presented ratios lower than 1 suggesting more conversion to S1P. Finally, higher expression of both genes SMPD1 and SPHK1 drastically decreases overall survival rate of glioma patients. Taken all together, the conversion of sphingomyelin to S1P has shown to be important for glioma growth and aggressiveness.

Acknowledgments

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

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ANEXO 1 – FIGURAS SUPLEMENTARES DO MANUSCRITO I

Supplementary Table 1 – Coefficients of genes selected to calculate the risk score.

Gene	Coefficients	log2_FC	log_p-value
ACAA2	-0.0935	1.367013	55.48938
AHR	-0.1006	1.446046	35.54551
ANGPTL4	0.1411	1.478769	29.01995
ARSD	0.1821	1.679046	56.29028
CYP27B1	0.1714	2.07444	38.25337
FHL2	-0.0508	1.23676	21.58101
GLB1	0.5115	1.01869	94.98539
GLIPR1	0.0907	1.571266	56.78365
MBOAT1	0.1402	1.627579	47.54003
OSBPL10	0.0698	1.844473	51.06522
PIK3CG	0.2923	1.113422	17.00089
PIK3R6	-0.0645	1.001002	18.63014
PLA2G4A	0.1130	1.297086	35.39656
PLEKHA4	0.1025	1.557474	27.85773
PNPLA4	0.1472	1.328711	23.17168
PTGS2	-0.0608	1.77689	25.14031
SEC24D	-0.1531	1.586115	86.6135
TSPO	-0.0842	1.062964	26.16885
UGCG	-0.0364	1.263183	32.87129
CYP21A2	-0.0178	-1.32213	22.05727
FASN	0.1210	-1.20442	65.73473
HMGCS1	-0.1421	-1.01714	35.87066
NEU4	-0.0021	-2.00266	36.05982
PLEKHA5	-0.1042	-1.02761	25.83747
PLEKHA6	0.1062	-1.45869	41.49036
PPM1L	-0.0743	-1.42011	39.46134
SCD	-0.1464	-1.71394	65.98175
SLCO1A2	-0.0629	-2.83647	67.0512
SMPD3	0.0451	-2.01484	61.52312

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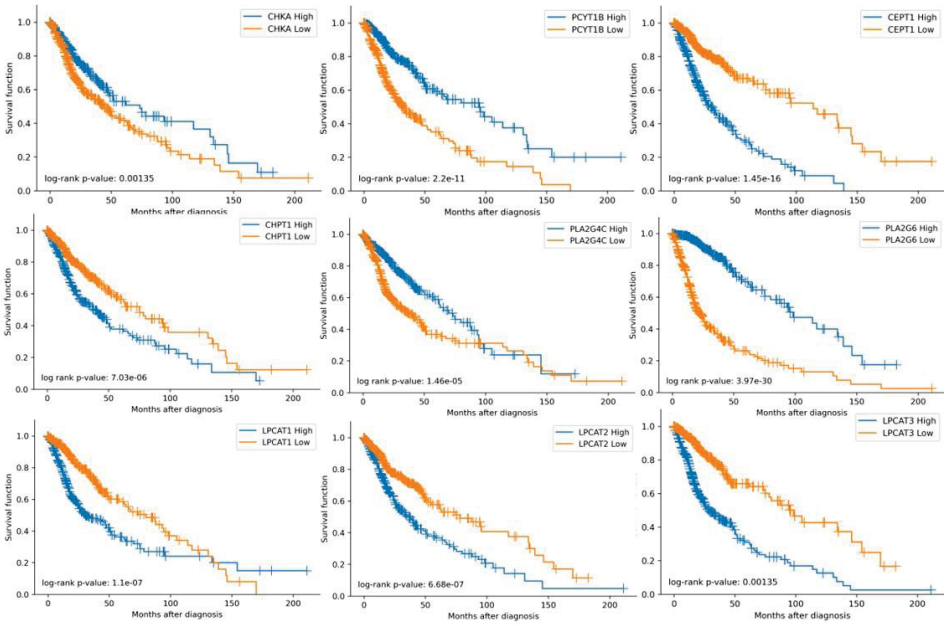
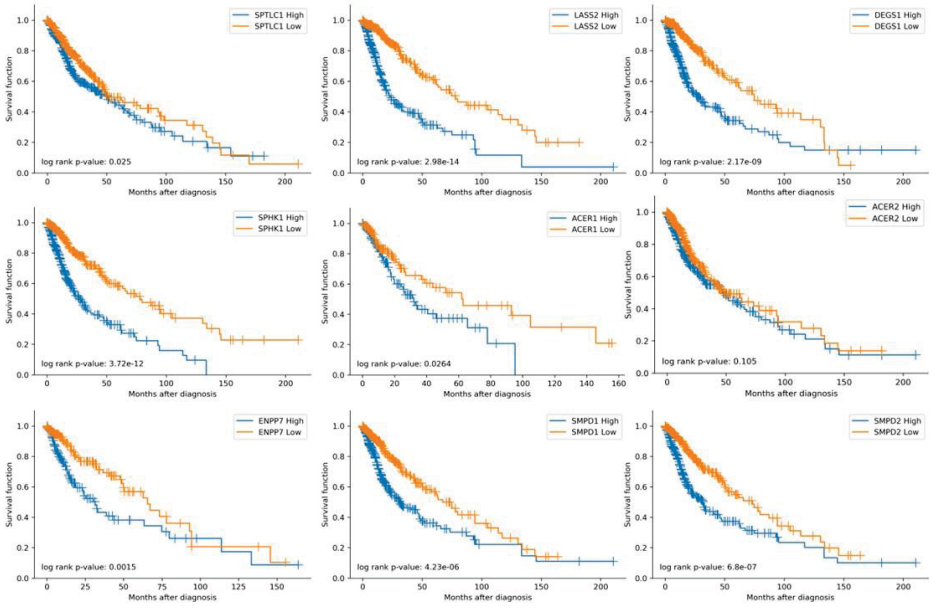
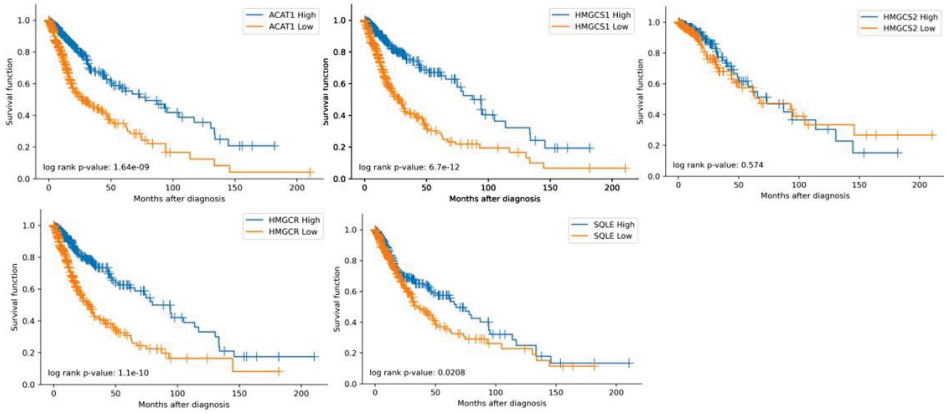
PC and PE Synthesis



S1P Synthesis

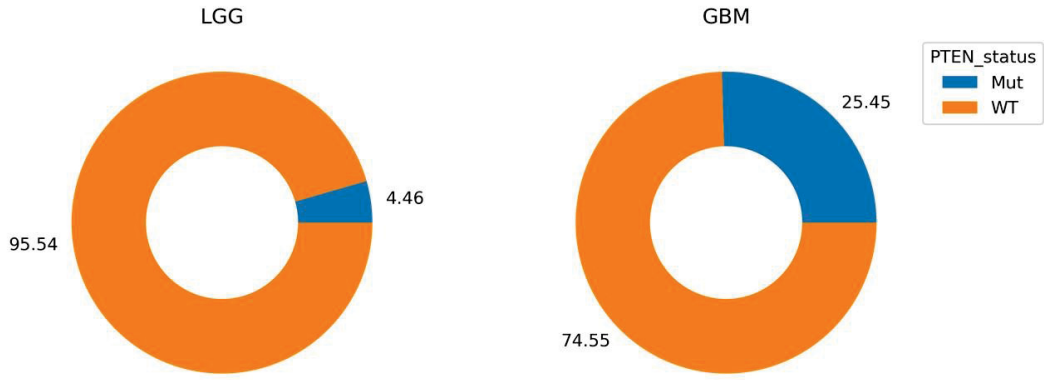


Cholesterol Synthesis

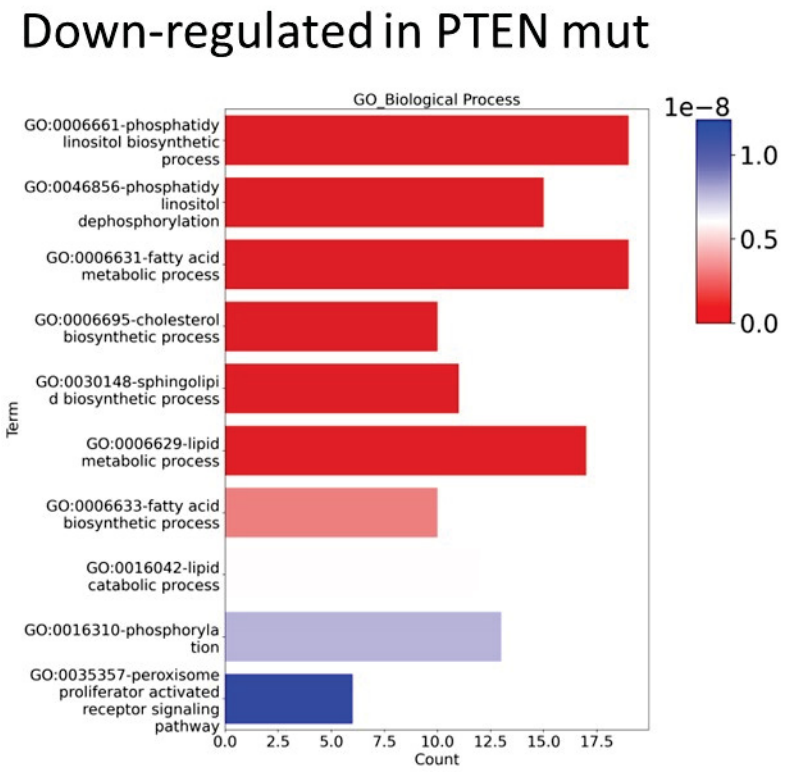


FigS1. Lipid related genes linked to glioma survival. Survival curves of genes important for metabolism are represented, the median value of each gene expression was used as cutoff (n=672).

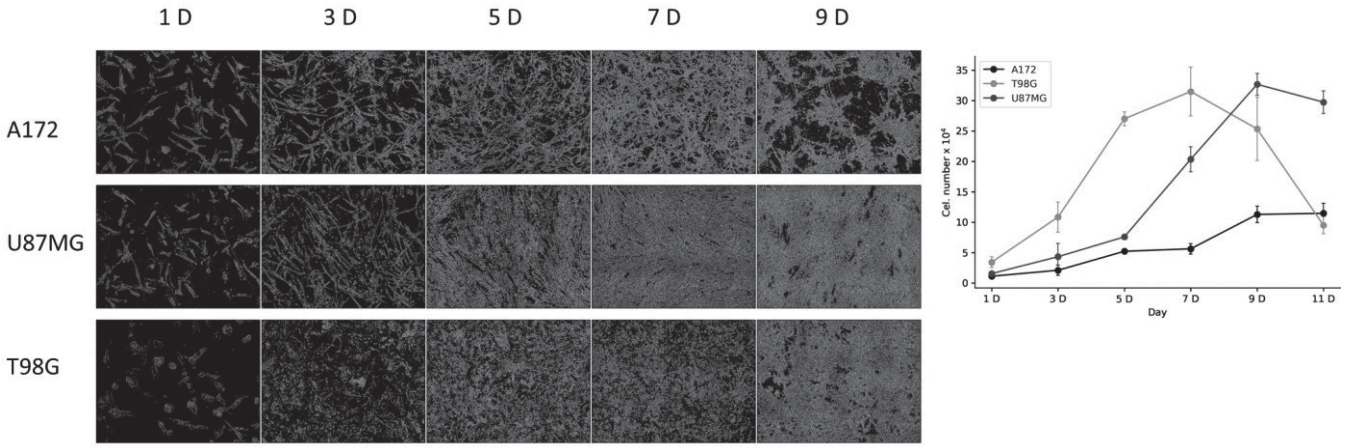
A



B.

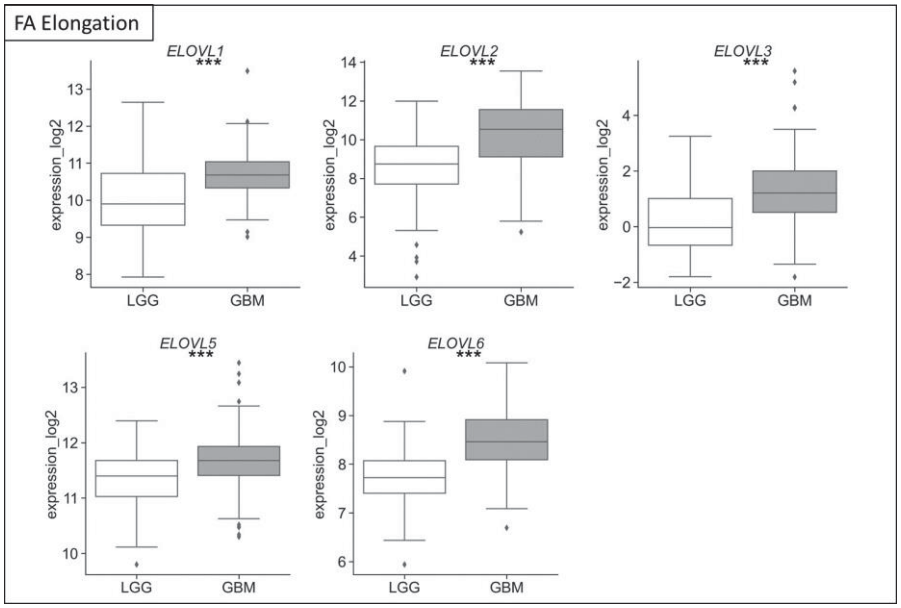


FigS2. Lipid metabolism related to PTEN mutation. Comparing the PTEN mut vs PTEN WT population in the glioma patients and the enrichment analysis, using molecular biological process (BP) annotation from gene ontology (GO) of lipid pathways that are downregulated in the PTEN mut population. Statistical values to rank their biological significance are represented by colors.



FigS3. Glioblastoma cells with distinct aggressive phenotype. Illustrative and the proliferation curve of the three GBM cells A172, U87MG, and T98G.

A



B

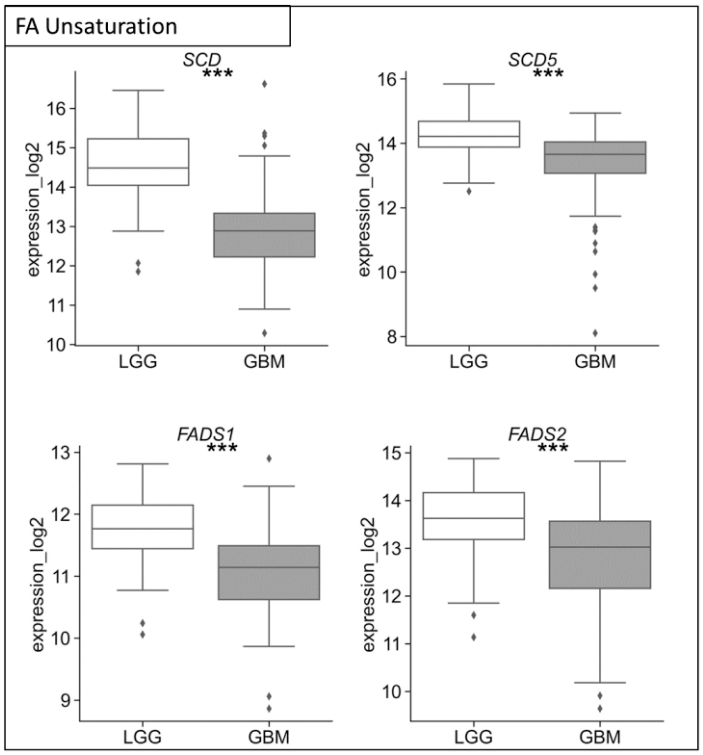


Fig S4. The fatty acid composition according to aggressiveness.

Comparing expression data of modulated genes important for FA elongation and unsaturation between GBM to LGG patients (n=681). T-test was used to compare between groups *p<0,05; **p<0,01;***p<0,001.

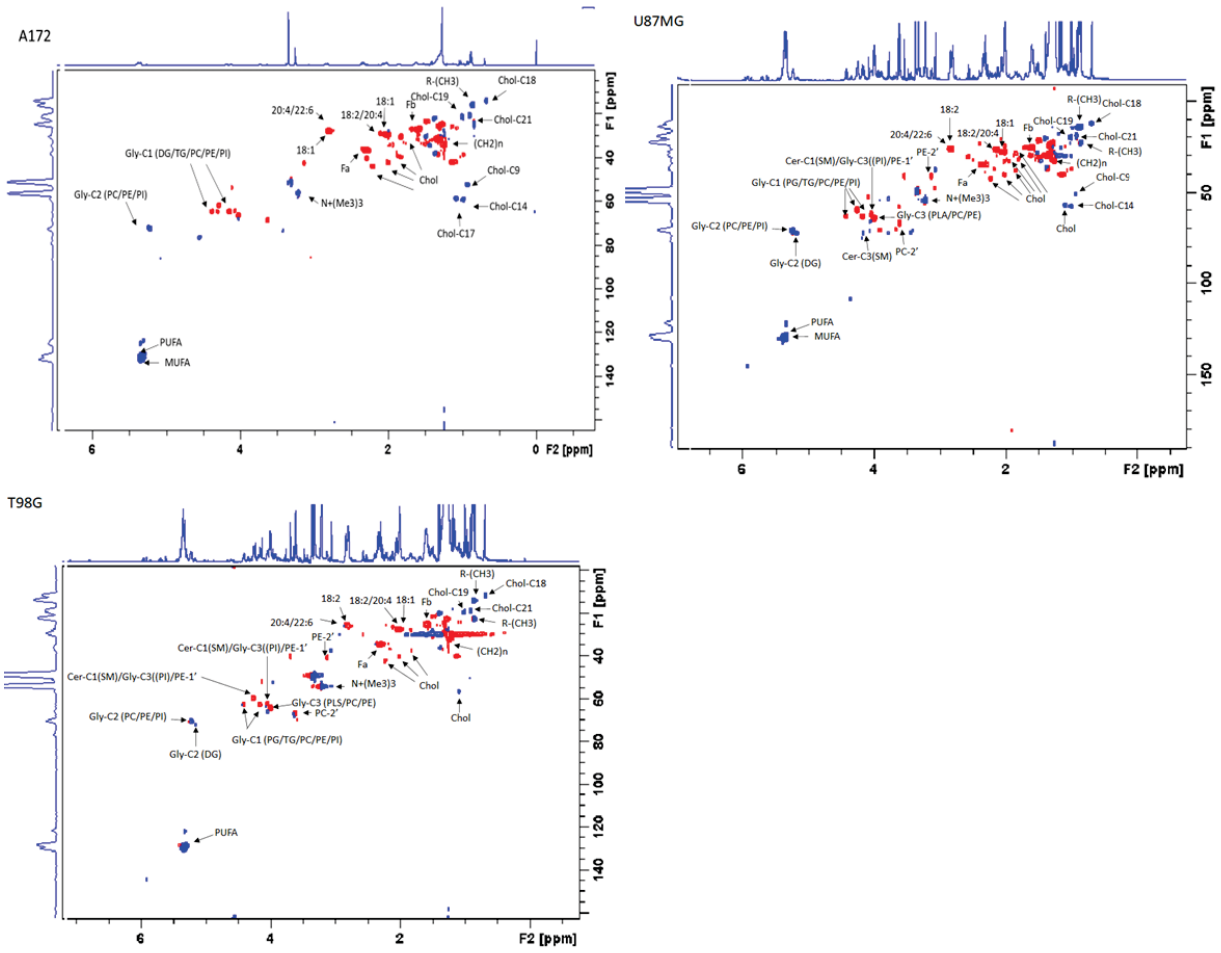


Fig S5. Partial 2D 1H-13C multiplicity-edited HSQC NMR correlation map. 2D-NMR of the lipid extract obtained from each glioma cell line with the principal lipid assignments. The positive phase (blue) corresponds to CH and CH3 correlations, and the negative phase (red) corresponds to CH2 correlations.

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5 MANUSCRITO II

Breaking the Membrane Barrier: Unraveling Lipid Metabolism Dysregulation in Temozolomide-Resistant Glioblastoma Multiforme.

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Running head: Lipid Metabolism and Glioma Resistance

Keypoints:

- Glioma resistance is linked to lipid composition.
- Membrane alterations drive TMZ resistance.
- Sphingolipid signaling is crucial in TMZ response.

Keywords: Lipid metabolism, glioma, lipidomic, TMZ resistance, plasma membrane

Abbreviation list

APL - Area per lipid
CDP - Cytidine diphosphate
DMEM - Dulbecco MEM media
DMSO - Dimethyl sulfoxide
ECM - Extracellular matrix
EDTA - Ethylenediaminetetraacetic acid
FBS - Fetal bovine serum
GBM - Glioblastoma
GO - Gene Ontology
HSQC - Heteronuclear single quantum coherence spectroscopy
LGG - Low Grade Glioma
MSM - Methylsulfonylmethane
NMR - Nuclear Resonance Magnetic
PCR - Polimerase Chain Reaction
PI - Phosphatidylinositol
PTEN - Phosphatase and tensin homolog
PUFA - Polyunsaturated fatty acid
SM - Sphingomyelin
SMASE - Sphingomyelinase
SMPD - Sphingomyelin Phosphodiesterase
SPHK - Sphingosine Kinase
SQLE - Squalene epoxidase
S1P - Sphingosine-1-phosphate
TCGA - The Cancer Genome Atlas
TMZ - Temozolomide
TSP - Trimethylsilylpropanoic acid
UPR - Unfold protein response

Abstract

Glioblastoma (GBM) is the most aggressive type of tumor the CNS, even though advances have been made to understand better this pathology, therapies are still not very efficient. Increased *MGMT* expression due to MGMT promoter unmethylation is related to temozolomide (TMZ) resistance in patients. Since lipid metabolism has

shown to play an important role in tumorigenic phenotypes, understanding the crucial lipid-related pathway for glioma progression could lead to important new markers. By identifying and quantifying lipids, lipidomic studies could reveal new biomarkers, as well as the roles of different lipids in the biological context they are involved. Therefore, lipidomic approaches have shown to play an important role in elucidating cellular mechanisms in pathological processes. The aim of this study is to analyze lipid metabolism by integrating two different data sources, the transcriptome data from The Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA), coupled with experimental data from human glioblastoma cells to investigate how lipid metabolism is related to TMZ resistance. We compared the expression of 743 lipid-related genes in public RNAseq data of glioma patients (n=681) with different MGMT expression status or recurrence phenotype (n=984) to lipidomic analyses of human glioblastoma cell lines with different TMZ responsiveness (A172, U87MG, U87MGR (TMZ-acquired resistance cell line) and T98G). We have observed that acquired resistance modulates membrane composition with a decrease in phospholipids and increase in cholesterol and plasmalogen as well as modulation of the important signaling lipids, sphingolipids, suggesting that the sphingomyelinase SMPD1 and SPHK1 could play an important role in TMZ response.

5.1 INTRODUCTION

Glioblastoma (GBM) is a very aggressive type of tumor, showing a high lethality with high cellular density, necrosis, and endothelial proliferation, which is associated to a poor prognosis with a rate survival of 14 months 1. Glioblastoma has a fast growth rate and the first-line treatment using temozolomide (TMZ) is not efficient due high microenvironment complexity and acquirement of resistance to treatment 2. High *MGMT* expression is related to TMZ resistance, and its transcription is inhibited when its promoter is methylated, therefore, *MGMT* promotor methylation status has been demonstrated to be a predictive factor of response to TMZ 3. However, other pathways have been described as potential candidates in promoting resistance independent of *MGMT* expression, such as the regulation of the cell cycle pathway and the antioxidant protection pathways4,5. Investigating lipid-related pathways becomes particularly crucial in this context, as the interplay of lipids with different pathways important to tumorigenesis have been described 6.

Lipids play important roles in the cell, not only structurally, but also in cellular signaling. Also, keeping the large variety of lipid in the cells demands energy, therefore, an evolutionary advantage to maintain this metabolism might be important 7. Therefore, studying lipid dynamics is crucial to comprehend the intricacies of drug resistance and advance promising therapeutic approaches for glioblastoma. Through the identification and quantification of lipids, lipidomic investigations may unveil novel biomarkers and elucidate the diverse roles of lipids within their biological contexts. Consequently, lipidomic methods have proven instrumental in unraveling cellular mechanisms during pathological processes 8.

Lipidomic works revealed that several cancers presented alterations in choline metabolism, which is clinically relevant to glioma diagnosis 9–11. Choline metabolism has been also related to drug resistance, and levels of phosphatidylcholine are considered an important marker of drug response, although, there is controversy if its increase or decrease is related to resistance 12,13. In glioma, changes in choline-containing lipids and polyunsaturated fatty acids (PUFAs) levels have been already described after drug treatment, suggesting that cellular membrane modulation would be important to drug response 14,15. Indeed, membrane composition modulates its biophysical properties which in turn may affect TMZ bioavailability into the cells 16.

Changes in fatty acid composition was also described in acquired-resistant glioblastoma cells (U87MG) and PUFAs treatment promoted a decrease in the expression of the receptor ABCC1 17. Furthermore, higher levels of PUFAs predisposes cells to cellular death by lipid peroxidation, an oxidative chain reaction already described as a mechanism for TMZ response and development of resistance18,19.

Sphingolipids have been associated to drug resistance in several works, they were broadly described to be involved in cancer development and growth, playing an important role in glioma malignancy 20,21. Ceramide and sphingosine-1-phosphate (SP1) levels dictate anti-tumoral or pro-tumoral behaviors, respectively, therefore their balance, known as sphingolipid rheostat, is important to cancer malignancy 20. Specific glioma therapies, as gemcitabine, could increase ceramide levels and rapidly ceramide consumption by glioma cells could lead to drug resistance 21,22.

Here we focused on lipid metabolism to elucidate how lipids could be associated to TMZ resistance in glioblastoma and how could be used as target to improve glioblastoma treatment.

5.1.1 MATERIALS AND METHODS

5.1.2 Bioinformatic analysis

The TCGA RNAseq normalized gene expression data of 743 lipid-related genes from 681 glioma (LGG and GBM) patients were collected from Firebrowse (http://firebrowse.org/). While the RNAseq normalized gene expression data from primary and recurrent patients from CGGA (n=984) were collected from GlioVis data portal for visualization and analysis of brain tumor expression datasets (http://gliovis.bioinfo.cnio.es/)20. The resistance phenotype in TCGA patients was obtained based on *MGMT* expression using the media value as cutoff, where higher expression was related to TMZ resistant patients. The expression was compared between groups by calculating T-test using the statistical function of the scientific computation library SciPy in Python. Gene expressions that showed statistical differences between groups were filtered. Further, the up and down-regulated genes were separated according to their mean values. The modulated genes were subjected to gene ontology (GO) and function cluster annotation analysis using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp) to determine significantly enriched genes. All the graphs were generated in python by using the data visualization library Seaborn.

5.1.3 Cell culture

The A172, U87MG and T98G human glioblastoma cell lines were kindly provided by Professor Mari C. Sogayar, University of São Paulo. Cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and 25 µg/ml gentamicin (Gibco), at 37°C in a humid atmosphere containing 5% CO_2 and maintained at approximately 80% confluency.

U87MGR was generated based as previous described 24,25. Briefly, U87MG was seed in 25 m² flask and it was treated with crescent TMZ concentrations (10 µM, 20 µM, 40 µM, 60 µM, 80 µM e 100 µM) for 3 months. The concentration was increased approximately every 3-4 days, and the treatment added when the cells reach approximately 80%.

5.1.4 Lipid extraction

Lipid extraction was performed as previously described 23. 2.3 x 10^6 cells were plated in a 150 mm plate (8 plates) for 24 hours, then collected using 0.1% trypsin solution containing 1 mM EDTA and centrifuged at 2000 g for 5 minutes (4 °C). The cell pellet was washed thrice with phosphate buffer, and the supernatant was discarded. The cell pellets were subjected to lysis, by freezing into liquid nitrogen and returned to a 37 °C water bath several times. After being freeze-dried overnight, 10 mg, of each cell line, was weighed and transferred to a glass tube with a screw Teflon cap (volume of 4 mL). The pellet was extracted using $CHCl_3$:MeOH 1:1 (v/v) (1 mL), vortexed for 1 minute, and then kept for 10 min at room temperature in an ultrasonic bath. The supernatant was collected, and the solvent dried under a stream of nitrogen.

5.1.5 NMR spectroscopy

$CDCl_3$ was purchased from Cambridge Isotope Laboratories, Inc. (Miami, U.S.A.) and from Sigma-Aldrich (St. Louis, MO). The samples were deuterium exchanged by repeated dissolution in MeOD-D_2O (2:1) and then freeze-dried overnight. Their spectra were obtained in $CDCl_3$-MeOD (3:1, 600 µL) at 30 °C, using methylsulfonylmethane (MSM) as internal standard (δ = 3.03 ppm). Spectra were obtained on a Bruker 600 MHz ASCEND equipped with a QXI probe (Bruker Biospin, Germany). 1D ^{1}H-NMR was carried out using enough scans to give a Signal/Noise (S/N) ratio of at least 2000/1 (90° pulse, relaxation delay = 4.0 s, number of time domain points = 65536, spectral width = 10.6541 ppm and acquisition time = 7.7 s). Experiments were performed without tube rotation and with the MSM or TSP signal at a medium half line width ranging from 0.6–1.0 Hz). For multivariate analysis and estimation of metabolites concentration, the spectra were acquired using the noesygppr1d.2 pulse sequence, with 128 transients and a delay of 10.0 s (2.0 s on the analysis of lipid samples). 2D NMR experiments were carried out using multiplicity-edited ^{1}H-^{13}C HSQC, heteronuclear correlation via double inept transfer with decoupling during acquisition, using trim pulses in inept transfer with multiplicity editing during the selection step (hsqcedetgpsp.3).

5.1.6 Quantitative gene expression assays

Reverse transcription was performed with 1 µg total RNA and Random primers, with ImProm II Reverse Transcriptase Kit (Promega, Madison, USA). qPCR was carried using the GoTaq® Real-Time PCR Systems (Promega) on a StepOne Plus thermal cycler (Applied Biosystems), according to the recommended protocol. Data were normalized according to Vandesompele et al. (VANDESOMPELE et al., 2002), using as reference genes GAPDH, HMBS and HPRT. GAPDH and HPRT were selected as the best normalizers.

Primers sequences used were: SPHK1 sense: 5'- ATTATGCTGGCTATGAGCAGG–3'; SPHK1 Antisense: 5'- TGCAGAGACAGCAGGTTCAT–3'; SPKK2 sense:5'-CCAGACAGAACGACAGAACCAC–3'; SPHK2 Antisense: 5'CTCCCGAGACCGTGACGATG3'; SMPD1 Sense:5 ACCGAATTGTAGCCAGGTATGA3'; SMPD1 Antisense: 5'AGAAGACCTCAAATTCATCCACA3'; FAR1 sense: 5'-TCCGGAGTTAATAGACCAAGAAACA3'; FAR1 Antisense: 5'-CGTCTGAAGGCCTGTTCGAG3'; FAR2 sense: 5'-TGTCTGAGCTGAGTCCTGAAGA3'; FAR2 Antisense: 5'-GGTAGTGAATATTTCGGAGCCTTT3'; GAPDH sense: 5-'ACCCACTCCTCCACCTTTGA-3'; GAPDH antisense: 5'-CTGTTGCTGTAGCCAAATTCGT-3); HPRT sense: 5'-GAACGTCTTGCTCGAGATGTGA-3'; HPRT antisense: 5'-TCCAGCAGGTCAGCAAAGAAT-3'; HMBS sense: 5'-TGGACCTGGTTGTTCACTCCTT-3'; HMBS antisense: 5'-CAACAGCATCATGAGGGTTTTC-3').

5.1.7 Cellular treatment and Cytotoxic assay

8x10³ cells per well were plated in 96-well plate and treated with 0.05 µM of 1-aminodecane-1,1-bisphosphonic acid (C10 bisphosphonate) (Avanti Polaris), 100 µMh TMZ (Sigma), or 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) for 72h. The control group was treated with vehicle DMSO 1%. After treatment, 3,4,5-dimethyl-2-thiazolyl2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/ mL diluted in PBS) was added. After 3h incubation at 37°C, 5% CO2, the supernatant was removed, and formazan crystals generated from the cleavage of

the tretazole ring by dehydrogenases, of the metabolically active cells, were resuspended in 200 µl of DMSO 100% (NEON). The absorbance was determined on a microplate reader (TECAN Infinite 200) using a 540 nm filter. The results were calculated from the mean absorbance values of the experimental triplicates. Three independent experiments were performed in quadruplicates.

5.1.8 Lipid Peroxidation

For detection of lipid peroxidation, glioblastoma cells were seeded in 24-well plates with 55,000 cells/well. After treatment cells were stained with BODIPY 581/591 C11 (Invitrogen, Karlsruhe, Germany) for 1 h at 37 °C in culture medium. After collecting and washing once with PBS, cells were re-suspended in an appropriate amount of PBS. Lipid peroxidation was analyzed by the detection of a fluorescence shift from green to red via FACS analysis. Excitation was performed at 488 nm and emission was recorded at 530 nm (green) and 585 nm (red). Data were collected from at least 10,000 cells.

5.2 RESULTS

5.2.1 Recurrent patients and with higher MGMT expression show sphingolipid, peroxisome, and oxi-reductase enriched pathways.

To assess the link between lipid metabolism and TMZ resistance, we analyzed expression data of 743 lipid-related genes from TCGA and CCGA public RNA-seq datasets. Since recurrence is correlated with TMZ resistance, we exploited this information from CGGA and first evaluate the lipid-related genes expression positively correlated with recurrent patients and subsequently perform enrichment analysis (Figure 1A). Further, we compared the lipid-related genes expression between patients showing higher and lower expression of the resistant marker gene *MGMT*, then select the up-regulated genes to perform enrichment analysis (Figure 1B). Interestingly, the modulated lipid pathways were very similar between datasets. Finally, we filtered genes upregulated in both analyses and conducted a combined enrichment analysis, revealing key lipid processes potentially involved in TMZ resistance (Figure 1C). Resistant-like gliomas showed higher expression of genes related to sphingolipid, mitochondria, oxi-reductase, phospholipid, cholesterol and peroxisome, suggesting that those pathways play an important role in TMZ resistance.

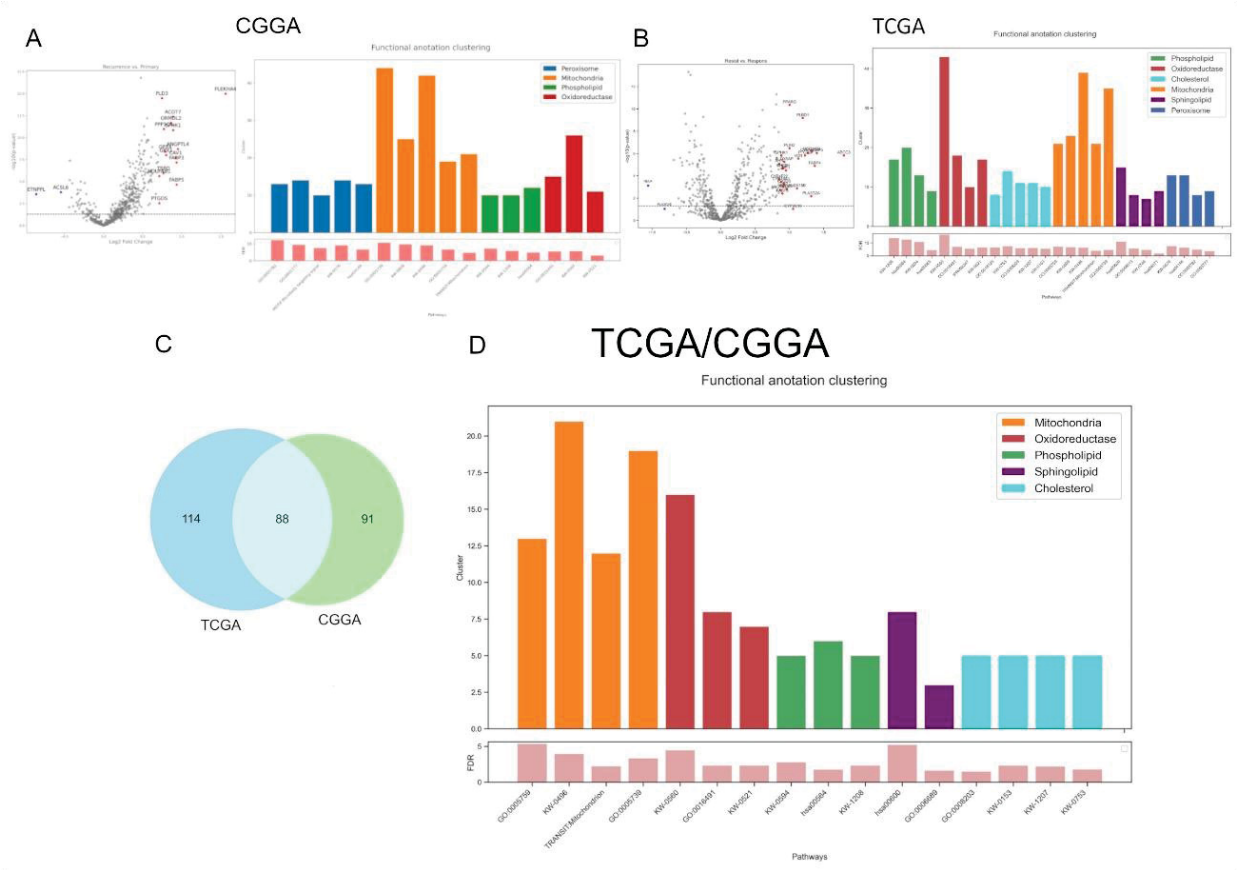


Fig. 2.1 – Lipid metabolism pathways related to resistance in glioma. **A.** Volcano plot of down and up-regulated lipid metabolism-related genes comparing expression data of recurrent and primary glioma from CGGA (n=984) and enrichment analysis, identifying related genes by measuring the similarity of their global annotation profiles, of up-regulated genes in recurrent patients **B.** Volcano plot of down and up-regulated lipid metabolism-related genes comparing expression data of MGMT+ and MGMT- patients (n=681) and enrichment analysis of up-regulated genes in MGMT+ patients. **C.** Venn diagram showing the number of common up-regulated genes from both datasets. **D.** Enrichment analysis of the common up-regulated genes of both datasets.

5.2.2 *MGMT* expression related resistance is linked to sphogosine-1-phosphate increasing by sphingolipid metabolism modulation.

Considering that sphingolipids play a pivotal role in tumorigenesis; we further analyzed the differentially expressed sphingolipid metabolism related genes to better understand how this pathway changed in the resistant phenotype. Genes important to ceramide synthesis either via *de novo* or by sphingomyelin degradation are upregulated in *MGMT*+ and recurrent patients (Figure 2.2A). Further, higher expression of sphingosine kinases (*SPHK1* and *SPHK2*) suggests a conversion of ceramide to the tumorigenic sphingolipid, sphingosine-1-phosphate (S1P).

Furthermore, to investigate how lipid metabolism could be important to TMZ resistance, we used three glioblastoma cell lineages (U87MG, T98G and A172) with different TMZ responsiveness and we generated a TMZ-resistant cell derived from the U87MG, the U87MGR. Resistance acquisition by U87MG did not promote proliferation rate changes (Figure S2.1). Results showed that U87MG and A172 are sensitive to TMZ treatment and express lower levels of *MGMT* comparing to the resistant cell lineage T98G (Figure 2.2 B and C). Induced-resistance cells U87MGR showed much higher expression of *MGMT,* and genes related to synthesis of SP1 (SPHK1, SPHK2 and SMPD1) (Figure 2.2D). Interestingly, these results were like what was showed in patients (Figure 2.2A).

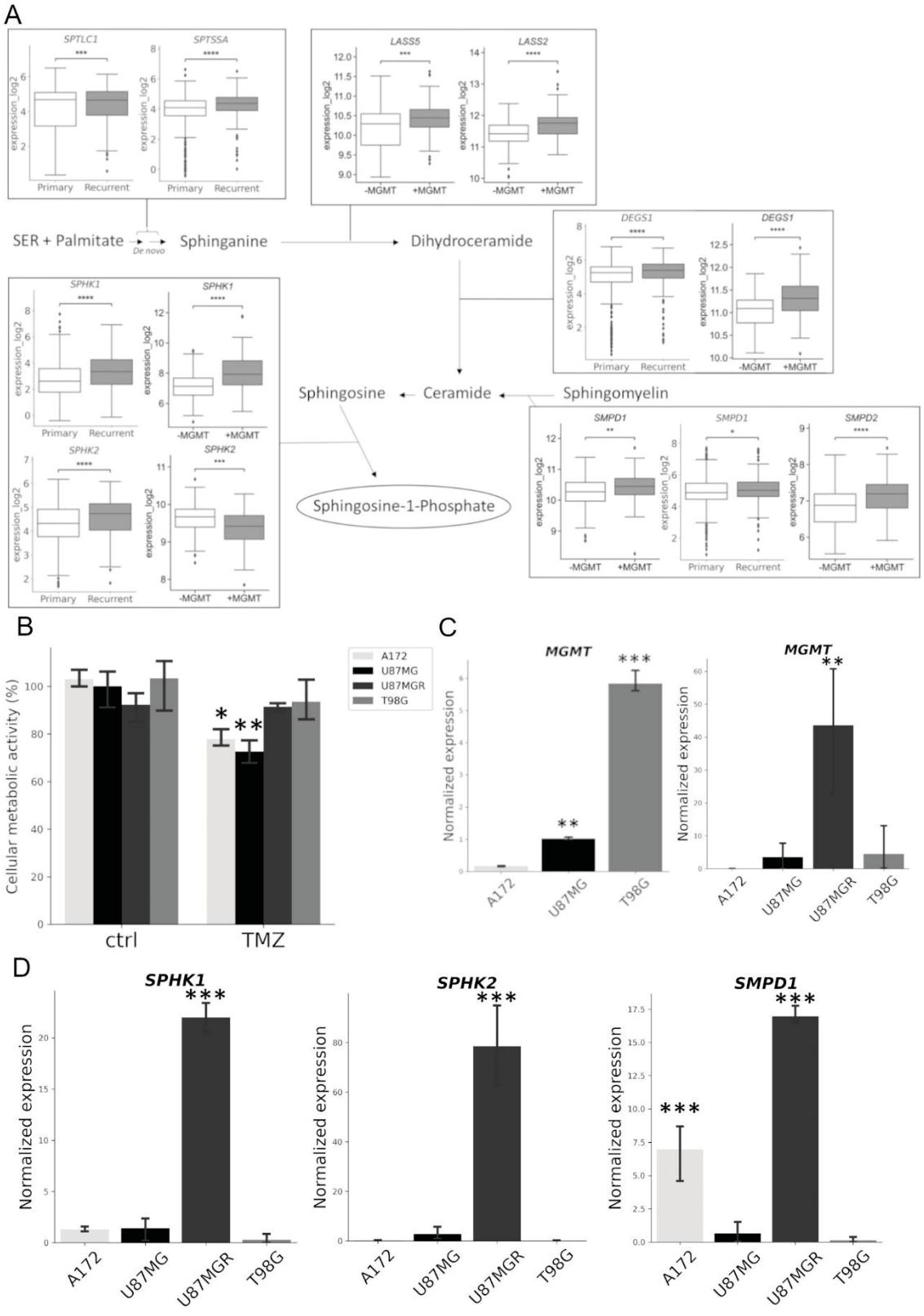
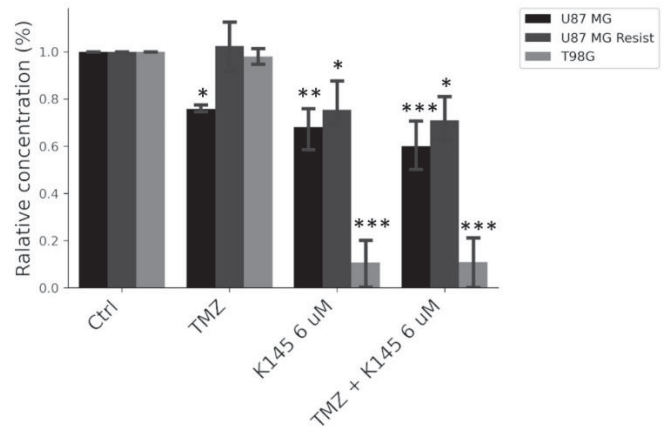
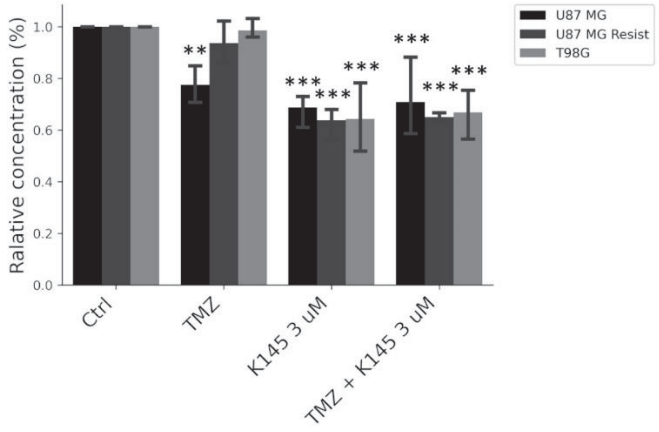


Fig 2.2 Sphingolipid metabolism related to resistance. **A** Sphingolipid pathway genes expression data of *MGMT+* and *MGMT-* patients (n=681) and primary and recurrent patients (n=984). T-test was used to compare between groups *p<0.05; **p<0.01;***p<0.001, ****p<0.0001. **B**. Different response to TMZ of the GBM cells T98G, U87MG, and U87MGR. **C**. Relative expression levels of MGMT normalized to HMBS in each glioma cell line. **D**. Relative expression levels of *SMPD1*, *SPHK1* and *SPHK2*

normalized to HPRT/GAPDH in each glioma cell line. Anova test complemented with Tukey's test were used for statistical analysis between groups. Data are represented as mean ± SEM. **p<0.05; **p<0.01;***p<0.001.

Our results showed that acquired-resistance cells exhibited increased expression of the sphingolipid related genes, *SPHK1*, *SPHK2* and sphingomyelinase (*SMPD1*). In fact, several studies have explored inhibition of SPHK1 in TMZ treatment to combat drug resistance in glioblastoma25–27. However, little is known about the impact of targeting SPHK2 and acid sphingomyelinases (SMases). Considering their enriched expression in resistant patients, we investigated the potential of inhibiting SPHK2 and Smases using K145 and C10, respectively, to restore drug sensitivity in resistant glioblastoma cells. As shown in Figure 2.3A and B, K145 in different concentrations did not enhance TMZ cytotoxicity. The combination of C10 and TMZ showed a modest synergistic effect in the resistant T98G cells. Notably, inhibiting *SPHK2* demonstrated a pronounced cytotoxic effect on T98G.





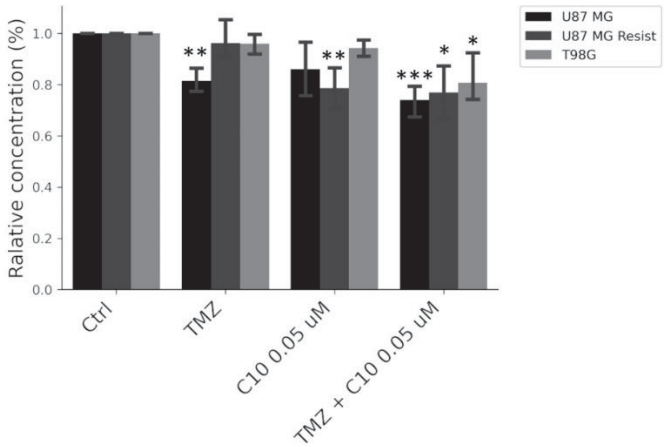


Fig. 2.3 Sphingolipid pathway inhibition. Cytotoxicity analysis after treatment with SPHK2 (K145) and SMASE (C10) inhibitors in combination with TMZ in glioma cells for 72 h. The percentage of metabolic activity of the cells was assessed by MTT assay. All bars are mean ± SEM of three biological replicates. Anova test complemented with Tukey's test were used for statistical analysis between groups. *p<0.05; **p<0.01;***p<0.001.

5.2.3 Resistance cells show enhanced metabolic flux and altered lipid metabolism impacting membrane composition.

To further investigate the potential significance of lipid metabolism in TMZ resistance, we performed comprehensive lipidomic analysis on the lipid extracts of U87MG and U87MGR by using standardized 1H NMR spectra to compare lipid compositions post-resistance acquisition (Figure 2.4A).

Quantitative analysis showed that resistant cells presented different levels of several membrane lipids. Following resistance acquisition, glioblastoma cells exhibited lower levels of choline-related lipids and phosphoethanolamine, alongside higher levels of cholesterol and plasmalogen (Figure 2.4B). Interestingly, these modulations are very similar to we have observed in clinical data (Figure 2.1).

While ^{1}H NMR spectra revealed a comparable lipid profile, we have noticed chemical shifts associated with creatine in U87MGR and absent in U87MG cells (Figure 2.4A). Subsequently, we evaluated the expression of the gene *GAMT* (Guanidinoacetate *N*-Methyltransferase), which is important to the creatine biosynthesis. Interestingly, the U87MGR cells showed higher expression of this gene suggesting an increase in metabolic flux in this pathway of these cells (Figure 2.4C). This corroborates with earlier findings suggesting that resistance correlates with an elevated metabolic ratio 28, further supported by clinical data indicating modulation of mitochondria-related genes (Figure 2.1).

Moreover, we assessed the expression of modulated genes related to the phospholipid pathway in both datasets and we noticed two key phospholipases (*PLA2G15* and *PLD3*) were higher in MGMT+ and in recurrent patients (Figure 2.4D). This result could indicate that phospholipid decreasing is associated in the developing condition of resistance.

Finally, to explore the potential link between increased plasmalogen levels and the upregulation of key biosynthetic genes of this biomolecule, we evaluated the expression of fatty acyl-CoA reductase FAR1 (Figure 2.4B and Figure S2.2). Resistant

cells exhibited elevated levels of FAR1, responsible for plasmalogen production in peroxisomes. Notably, peroxisomes related-pathways were enriched in the phenotype associated with resistance in patients, and the expression of FAR genes was also increased in these patients (Figure 2.4E).

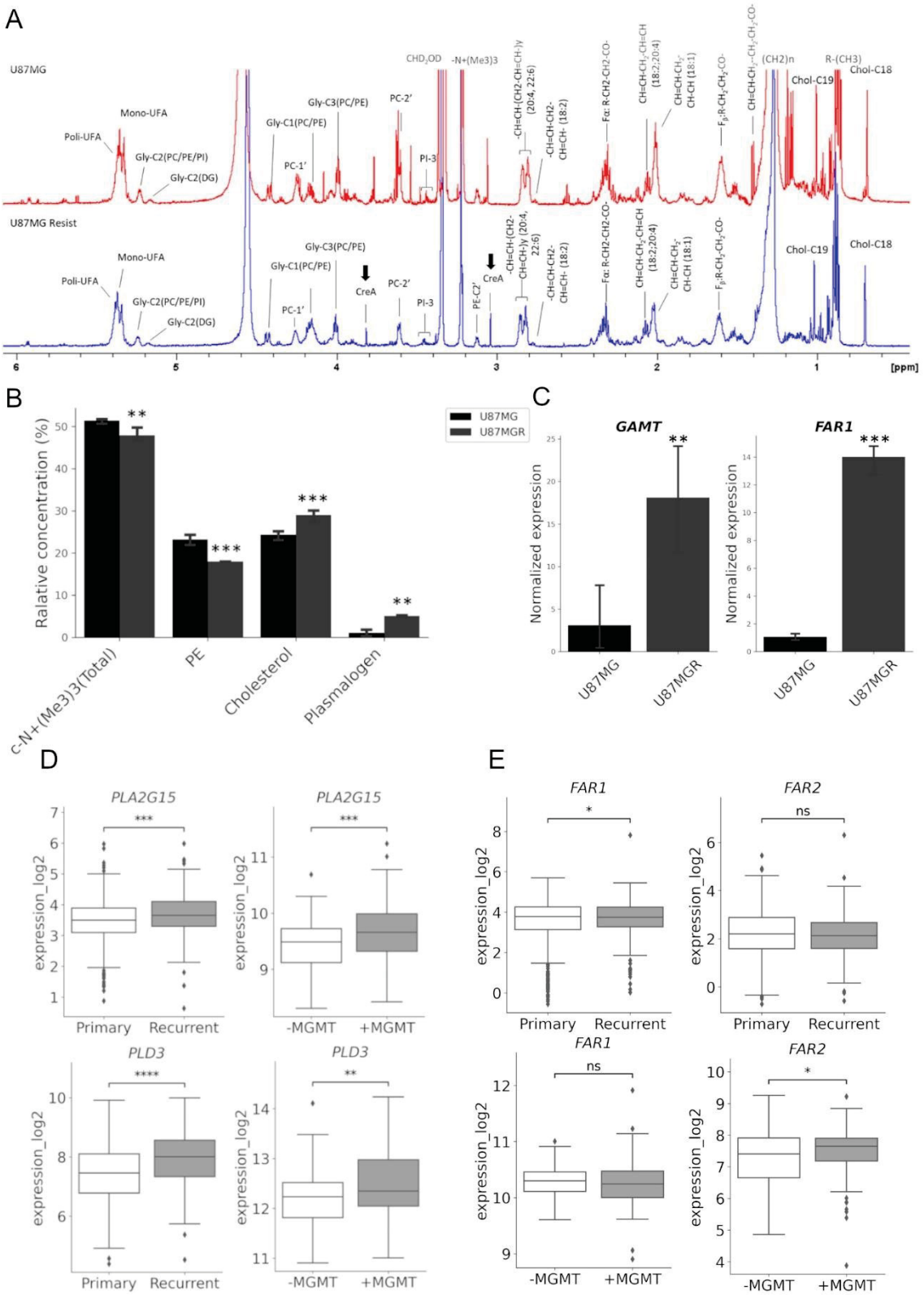

A
U87MG
U87MG Resist
Mono-UFA
Poli-UFA
Gly-C2(PC/PE/PI)
Gly-C2(DG)
Gly-C1(PC/PE)
Gly-C3(PC/PE)
PC-1'
PC-2'
PI-3
CHD2OD
-N+(Me3)3
CreA
PE-C2'
Chol-C19
Chol-C18
(CH2)n
R-(CH3)
[ppm]
B
U87MG
U87MGR
Ralative concentration (%)
c-N+(Me3)3(Total)
PE
Cholesterol
Plasmalogen
C
GAMT
FAR1
Normalized expression
U87MG
U87MGR
D
PLA2G15
PLD3
expression_log2
Primary
Recurrent
-MGMT
+MGMT
E
FAR1
FAR2
ns

Fig. 2.4 Lipid metabolism related to resistance *in vitro*. A. ^{1}H NMR spectra of the lipid extract of the GBM cells. **B.** Relative quantification of the main lipid groups by NMR ^{1}H spectra using MSM as an internal standard, data from three independent experiments. Anova test were used for statistical analysis between groups. Data are represented as mean ± SEM. ** represents p < 0.01; *** represents p < 0.001. **C**. Relative expression levels of *GAMT*, and *FAR1* normalized to HPRT/GAPDH in each glioma cell line. Anova test complemented with Tukey's test were used for statistical analysis between groups. Data are represented as mean ± SEM. **p<0.05; **p<0.01;***p<0.001 **D.** Key phospholipid pathway genes comparing expression data of *MGMT*+ and *MGMT*- patients (n=681) and primary and recurrent patients (n=984). T-test was used to compare between groups *p<0.05; **p<0.01;***p<0.001, ****p<0.0001. **E.** *FAR1* and *FAR2* comparing expression data of *MGMT*+ and *MGMT*- patients (n=681) and primary and recurrent patients (n=984). T-test was used to compare between groups *p<0.05; **p<0.01;***p<0.001, ****p<0.0001.

5.2.4 Resistance is linked to oxidative process and lipid peroxidation protection.

Given the established connection between plasmalogen and oxidative protection, coupled with the observed elevation of plasmalogen levels in U87MGR cells, and the modulation in the lipid oxi-reductase pathway in TMZ-resistant patients, we aimed to investigate oxi-reductase process within the context of TMZ resistance.

Notably, the phenotype associated with resistance in patients showed increased expression of glutathione peroxidase (GPX1 and GPX4), which plays an important role in maintaining redox homeostasis and protecting against lipid peroxidation (Figure 2.5A). Therefore, we next investigated the cellular response to the inhibitor of GPX4, RSL3 (Figure 2.5B). Significantly, U87MGR cells were less prone to reduce cell metabolism after RSL3 treatment, suggesting a plausible connection between development of resistance and increased oxidative protection. This connection is also supported by our results, where we tested if U87MGR would be more resistant to UFAs treatment, since more unsaturated fatty acids would be available to lipid peroxidation. To this end, cells were treated with varying concentrations of oleic and linoleic acids (Figure 2.5C). As shown, resistant cells could keep normal cellular metabolism to UFAs concentrations as high as 100 uM.

Interestingly, resistance cells presented higher levels of lipid peroxidation after TMZ treatment (Figure 2.5D and 2.5E), suggesting a heightened oxidative process in resistant cells. However, it is noteworthy that resistant cells demonstrated resistance to this increased peroxidation rate, highlighting a potential adaptive mechanism of protection against oxidative stress in the context of TMZ resistance.

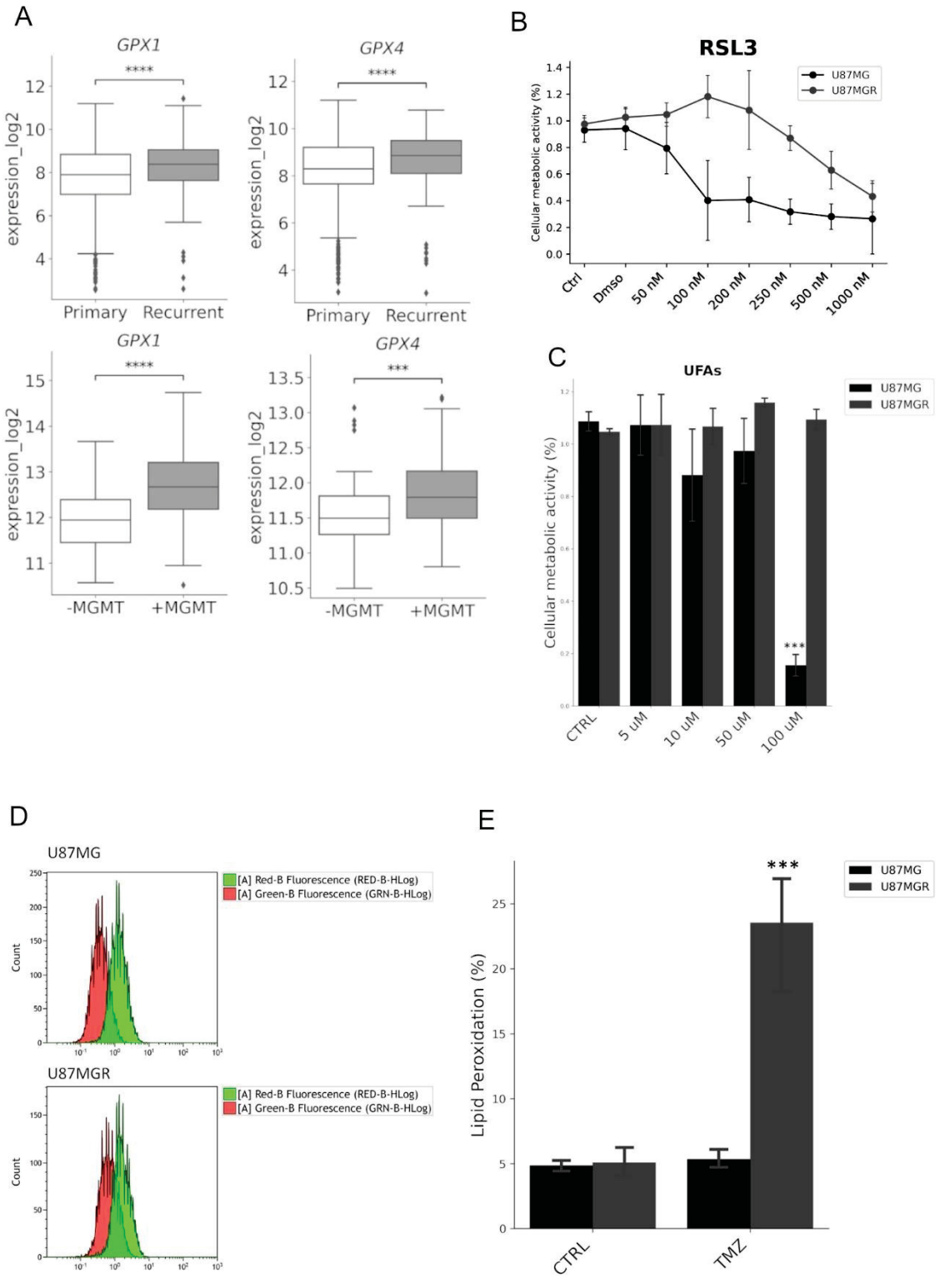


Fig. 2.5 Resistance is linked to oxidative process A. **.** Key oxi-reductase pathway genes comparing expression data of *MGMT*+ and *MGMT*- in patients (n=681) and primary with recurrent patients (n=984). T-test was used to compare between groups *p<0.05; **p<0.01;***p<0.001, ****p<0.0001. **B**. Cytotoxicity analysis of RSL3 at different concentration for 24 hours. The percentage of metabolic activity of the cells was assessed by MTT assay. **C.** Cytotoxicity analysis of oleic and linoleic acids in

glioma cells. **D**. Analysis of C11-BODIPY signal intensity based on flow cytometry experiments. Cells were stained with bodipy after 48 hours with treatment with TMZ. **E.** Lipid peroxidation of glioblastoma cells after TMZ treatment. All bars are mean ± SEM of three biological replicates. One-way ANOVA, *, $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5.3 DISCUSSION

The clinical data of glioma patients showed three important pathways that are enriched in patients according to the MGMT status and recurrence, suggesting that sphingolipids related genes, peroxisome related genes and genes involved in oxidoreductase reaction of lipids are involved in TMZ resistance related patients.

To further assess how lipid metabolism would be related to TMZ resistance, we performed lipidomic analysis in glioblastoma cells with different TMZ response as well as different expression of *MGMT*. It was shown that acquired resistance promote increased expression of *MGMT*, therefore, our cellular model is consistent to the clinical *MGMT*-related resistant data that we analyzed.

Our lipidomic analysis revealed that acquired resistance induces changes in the levels of choline-containing lipids, phosphoethalonamine, cholesterol and plasmalogen. Changes in cholesterol and plasmalogen levels could modulate membrane fluidity, and since alterations in the biophysical properties of the membrane could modulate TMZ bioavailability into the cells13.

Changes in choline-containing lipids have been already described after chemiotherapy treatment 9, and it is known that those changes are related to cellular death, however, the resistant cell developed the ability to survive even with these constitutive changes, furthermore U87MGR cells showed to be more resistant to the UFAs treatment, even in higher concentrations, than the parental U87MG 11,12.

Sphingolipids has been broadly described to be involved in cancer development and growth, playing an important role in glioma malignancy 17,18. Ceramide and SP1 levels dictate anti-tumoral or pro-tumoral behaviors, respectively, therefore their balance known as sphingolipid rheostat, it is important to cancer malignancy 17. In particular, glioma therapies could increase ceramide levels and rapidly ceramide consumption by glioma cells to convert into S1P could lead to drug resistance 18,19. Ceramide could be generated by *de novo* or by other sphingolipids degradation and sphingomyelin could be generated by the action of sphingomyelinases. The clinical

data showed that resistance in glioma is related to higher expression of SMPD1, SPHK1 and SPHK2. Even though, higher expression of SMPD1 could indicate higher levels of ceramide, higher expression of SPHKs, however, would indicate that ceramide is being further converted to S1P. Therefore, this data suggests that TMZ resistance is related to lower levels of ceramide and higher levels of S1P. Interestingly, it was also described in our previous work that glioblastoma cell presents higher expression of SMPD1, and both SPHKs isoforms (SPHK1 and SPHK2) after TMZ resistance acquisition. This data suggest that S1P is important to TMZ resistance and would be due to its ability to promote survival and also by the reduction of ceramide levels.

Inhibition of SPHKs has already been reported to promote glioma cell death in combination with TMZ as well as inducing cellular death in TMZ resistant glioblastoma. Here, we were interest in the SPHK2 isoform role in TMZ response, hence, we assessed whether the SPHK2 selective inhibitor would promote cellular death in combination with TMZ, further rescue sensitive to TMZ in resistance cells. SPHK2 inhibition promoted cellular death in both resistant and sensitive cells, however, it did not promote addition effect with TMZ. Interestingly the T98G, which is intrinsic resistant to TMZ, showed more cytotoxic effect to SPHK2 inhibition. Even though, SPHK2 inhibition did not rescue drug sensitive, it showed cytotoxic effect in resistant cells, which could be an alternative to TMZ resistant treatment.

Higher levels of PUFAs predisposes cells to cellular death by lipid peroxidation 15. Therefore, we also investigate whether resistant cell show more lipid peroxidation after TMZ. Surprisingly after TMZ treatment U87MGR presented higher levels of lipid peroxidation, even though this treatment did not provoke any cytotoxicity. This suggest that resistant cells became resistant to lipid alterations that would lead to cell death. Lipid peroxidation is one of the main mechanisms of the cellular death process known as ferroptosis, since we have noticed higher lipid peroxidation without cytotoxic effect in resistant cell, we evaluated the cytotoxic effect under ferroptosis induction 15. Curiously, we observed that resistance is also more resistance to ferroptosis. We hypothesize that resistant cell would present more antioxidants that will further protect cells against lipid peroxidation and ferroptosis. Interestingly, our *MGMT*-related resistant patient data showed enrichment of oxidoreductase pathways, as well as higher expression of GPX4, a glutathione peroxidase which protects de cell against lipid peroxidation, and it is the target of the ferroptosis inducer RSL3 30.

Here we have identified key pathways and lipidomic changes contributing to TMZ resistance in glioma. Clinical data highlighted sphingolipids, peroxisome-related genes, and lipid oxidoreductase pathways in resistant patients that were further confirmed in our cellular model, revealing lipid alterations associated with membrane. Sphingolipids, especially ceramide and S1P, play a crucial role, suggesting an imbalance in the sphingolipid rheostat linked to TMZ resistance. SPHK2 inhibition showed cytotoxic effects, indicating potential alternative treatment strategies. Surprisingly, TMZ-resistant cells adapted to lipid alterations associated with oxidative process, showing increased lipid peroxidation without cytotoxicity. Resistance to ferroptosis was observed, possibly due to elevated antioxidants, supported by clinical data showing oxidoreductase pathway enrichment and higher GPX4 expression. These findings offer insights into the complex molecular landscape of TMZ resistance, guiding the development of future targeted therapies.

ANEXO 2 - FIGURAS SUPLEMENTARES DO MANUSCRITO II

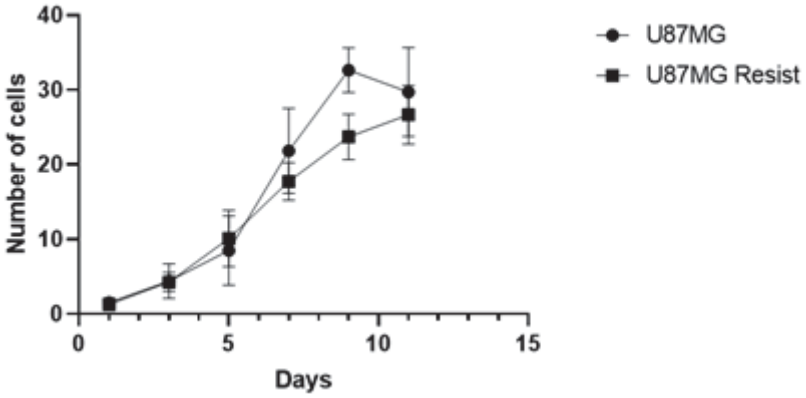


Fig S2.1 Proliferation rate of U87MG and U87MGR.

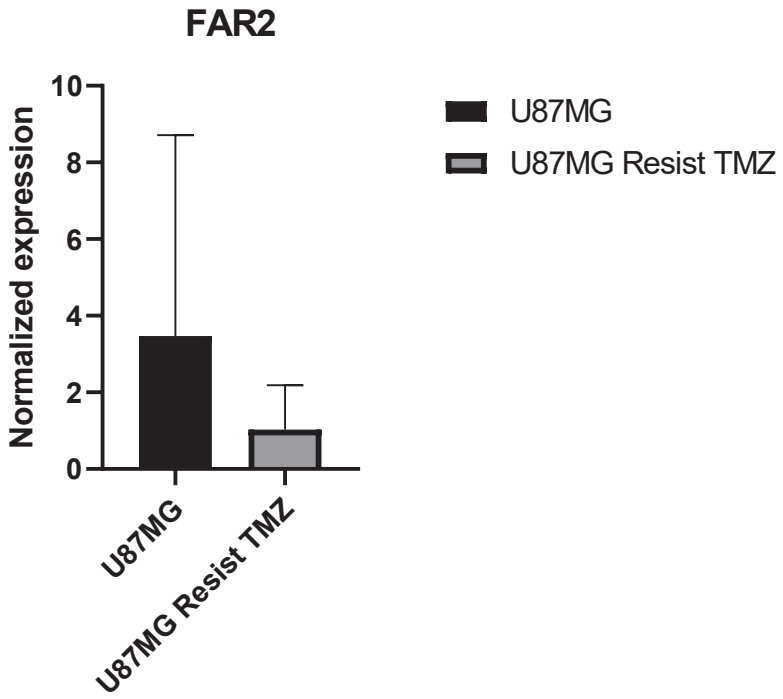


Fig S2.2. Relative expression levels of FAR2 normalized to HPRT/GAPDH in each glioma cell line. Here the PCR reaction was not efficient, due to the high Ct which explains the high error bar. To confirm this data another primer targeting FAR2 must be designed and tested.

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6 DISCUSSÃO

Na presente tese descrevemos como o metabolismo pode desempenhar um papel crucial na progressão tumoral e na resistência ao tratamento do glioblastoma. A partir da integração de dados clínicos de pacientes com dados experimentais usando modelo celular de glioblastoma pudemos evidenciar alguns mecanismos que podem impactar o desenvolvimento da agressividade tumoral, destacando sua interconexão com vias de resistência a tratamentos.

Foram identificados 29 genes relacionados a lipídios, cuja expressão está correlacionada ao prognóstico em dados de pacientes. Uma assinatura de risco foi construída com base na expressão desses genes, resultando em um score de risco que demonstrou, de fato, uma relação com o prognóstico (Figura 2A). Notavelmente, genes relacionados à matriz extracelular mostraram uma correlação positiva com esse escore (Figura 2B). É relevante destacar que diversos modelos celulares indicam que as propriedades mecânicas e físicas da matriz extracelular exercem controle sobre o crescimento tumoral e a invasão metastática, influenciando o metabolismo celular (GE et al., 2021). Isso inclui a regulação do metabolismo lipídico por meio das vias moduladas por SREBP, que em condições de reduzida contratilidade do actomiosina, ocorre a ativação dos fatores de transcrição SREBP, impulsionando a síntese e acúmulo de lipídios (GE et al., 2021; ROMANI et al., 2019). Portanto, esses dados, aliado ao fato de vias lipídicas associadas a membrana estarem relacionadas com maior agressividade, sugerem uma relação bidirecional entre a composição da membrana e a regulação da matriz extracelular (ECM), indicando uma possível contribuição mútua para a promoção do fenótipo de agressividade.

Em ambos os estudos, a composição da membrana teve seu papel destacado tanto na agressividade do glioblastoma quanto em sua resistência ao tratamento. No entanto, foram observadas distintas modulações em ambos os fenótipos; enquanto houve o aumento de fosfolipídios de colina e redução de colesterol no ganho de agressividade, no fenótipo de resistência observamos efeitos opostos, em que houve redução dos níveis de fosfolipídios de colina e aumento dos níveis de colesterol.

Alterações no nível de colesterol celular estão associadas a diversos tipos de tumores, porém diferentes estudos clínicos demonstram que tanto o aumento, quanto a redução de colesterol podem estar relacionados com fenótipos de agressividade tumoral, como aumento de proliferação, invasão, metástase e resistência a terapia (DING et al., 2019; MAYENGBAM et al., 2021). A redução nos níveis de colesterol

pode promover um aumento na fluidez de membrana favorecendo a invasão e metástase de células tumorais, enquanto que seu aumento pode promover resistência à quimioterapia por reduzir a permeabilidade de drogas (BERNARDES; FIALHO, 2018). Considerando que alterações nas propriedades biofísicas da membrana influenciam a biodisponibilidade de TMZ nas células, níveis elevados de colesterol poderiam gerar resistência a TMZ, ao menos em parte, ao modular a absorção do medicamento (RAMALHO et al., 2019). Curiosamente, esse efeito dual do colesterol, promovendo aumento de proliferação e resistência, foi observado tanto nos dados de pacientes (Figura 3 e Figura 2.1D) quanto no modelo celular (Figura 4C e Figura 2.4B), onde a redução esteve efetivamente relacionada à agressividade e seu aumento com resistência. Estratégias terapêuticas direcionadas a otimizar os níveis de colesterol, visando atingir uma faixa específica que minimize a fluidez excessiva da membrana sem comprometer a eficácia da quimioterapia, poderiam ser exploradas. Essa proposta tem sido explorada no grupo, evidenciando que a abordagem combinada de células de glioblastoma com TMZ e estatinas promove a sensibilização para a morte celular, com efeitos notáveis também na linhagem do subtipo mais agressivo (Dados em processo de submissão) (GOMES, 2018). Além disso, a combinação de agentes reguladores da via de colesterol com terapias convencionais poderia potencializar os efeitos antitumorais, visando tanto a redução da agressividade tumoral quanto a superação da resistência à quimioterapia. Essa abordagem oferece uma perspectiva promissora para o desenvolvimento de estratégias terapêuticas mais eficazes e personalizadas no tratamento de glioblastomas.

Vários trabalhos indicam que há acumulo de fosfolipídios contendo colina foi descrito em diversos tipos de câncer, uma vez que seu metabolismo tem um papel pró-tumoral importante por promover a proliferação celular (SAITO et al., 2022). A colina apresenta relevância clínica importante no diagnóstico de gliomas uma vez que pode ser facilmente detectado por ressonância magnética (^{1}H MRI) (BULIK et al., 2013; GORYAWALA et al., 2021; USINSKIENE et al., 2016). Níveis elevados de colina estão relacionados com tumores de maior grau e com maior taxa de proliferação (BULIK et al., 2013; SU et al., 2021). Outro trabalho comparou amostras teciduais de pacientes com gliomas de diferentes graus, demonstrou que o aumento dos níveis de PtdCho correlaciona com ganho de malignidade em gliomas (LEHNHARDT et al., 2001). Esses dados corroboram os resultados apresentados neste trabalho, uma vez que observamos o aumento dos níveis de lipídios de colina em nossos modelos mais

agressivos (Figura 3 e Figura 4C). No entanto, por apresentar diferentes espécies lipídicas sua modulação no contexto de agressividade pode ser dinâmica. Permanece incerta a influência da redução ou aumento dos níveis de fosfatidilcolina (PtdCho) em fenótipos resistentes, pois ambas as possibilidades são descritas na literatura (DALY et al., 1987; SAITO et al., 2022). Considerando que PtdCho é descrito como um substrato para o receptor de resistência múltipla a drogas (MDR), a redução dos níveis de PtdCho em células resistentes de leucemia foi explicada, pelo menos em parte, pelo fato das linhagens resistentes expressarem maiores níveis do receptor MDR1 (BOSCH et al., 1997). Por outro lado, foi descrito que o tratamento com TMZ induz o aumento de glicerofosfocolina, colina total e fosfocolina (AL-SAFFAR et al., 2014). Porém sabe-se que há redução em células resistentes que pode ser explicada, pelo menos em parte, pelo fato de linhagens resistentes expressarem mais receptores de resistência múltipla a drogas (MDR), uma vez que, PtdCho já é descrito como um substrato para o receptor MDR1 em modelo de leucemia (BOSCH et al., 1997).

Esses dados indicam que os componentes lipídicos da membrana desempenham um papel dinâmico e adaptativo, respondendo de maneira específica aos diferentes contextos do glioblastoma, influenciando na agressividade e na resistência ao tratamento. Essas observações ressaltam a complexidade da interação entre a composição da membrana e os fenótipos tumorais, indicando possíveis alvos terapêuticos para distintos fenótipos oferecendo estratégias mais precisas e eficazes.

As esfingomielinas apresentam também um papel importante nas propriedades das membranas (TANIGUCHI; OKAZAKI, 2014). De forma interessante, nossos dados destacam o papel dos esfingolipídios, particularmente a síntese de S1P através do eixo envolvendo a esfingomielinase, em que se há inicialmente a conversão de esfingomielina para ceramida e finalmente a conversão de ceramida a S1P. Esse eixo para a síntese de S1P, mediado por SMPD1 e SPHK1, demonstrou estar não só relacionado com o ganho de fenótipo de agressividade, mas também com a resistência ao quimioterápico padrão, TMZ (Figura 3, Figura 6 e Figure 2.2). Vale ressaltar que foi identificado o aumento de determinadas esfingomielinas em amostras teciduais de pacientes com glioma (ZHAI et al., 2019). Além disso, a inibição de SMPD1 em modelo *in vitro* de glioma foi capaz de promover morte celular (MEYER et al., 2021; ZHAI et al., 2019).

Ainda, S1P se destaca por promover proliferação e tumorigênese (ERNST; BRÜGGER, 2014). S1P é sintetizado pela enzima esfingosina quinase SPHK1 e

SPHK2, a partir da esfingosina, e seus elevados níveis correlacionam com menor sobrevida em pacientes com glioblastoma (VAN BROCKLYN et al., 2005). A partir dos dados de pacientes (TCGA) confirmamos que o aumento da expressão das *SPHK*s e de *SMPD1* de fato estão relacionados com pior prognostico em glioma. Na literatura, a utilização de SPHKs como alvos terapêuticos tem sido explorada como promissora no tratamento de gliomas, considerando que inibidores de SPHKs não específicos potencializam a resposta à morte celular por TMZ em glioma (NOACK et al., 2014; OANCEA-CASTILLO et al., 2017b). A inibição específica da isoforma SPHK1 induz apoptose em linhagens sensíveis e resistentes à TMZ (BEKTAS et al., 2009). Apesar da distinção das funções dessas isoenzimas ainda não estar clara, e as vezes SPHK1 e SPHK2 são consideradas com funções redundantes (HATOUM et al., 2017), foi observado que a supressão da expressão da isoforma *SPHK2* com RNA de interferência em modelo celular de câncer de mama mostrou-se mais eficiente para reduzir os fenótipos tumorais que a supressão de *SPHK1* (GAO; SMITH, 2011; HATOUM et al., 2017). Nesse contexto, nosso trabalho avaliou se a inibição específica de SPHK2 (utilizando o inibidor K145) poderia promover um efeito citotóxico aditivo quando associado à TMZ no tratamento de células de glioblastoma (Figura 2.3). Apesar de que o inibidor K145 não promoveu efeito aditivo quando combinado ao quimioterápico padrão, apresentou efeito citotóxico em diferentes linhagens de glioblastoma. Ainda, no contexto do eixo esfingomielina-S1P, avaliamos também os efeitos citotóxicos do uso de inibidor de SMASe (com o uso do inibidor C10) em combinação com TMZ. De maneira semelhante, apesar de não promover efeito aditivo significante quando combinado com o quimioterápico padrão (Figura 2.3), apresentou efeito citotóxico em diferentes linhagens de glioblastoma (com destaque para o intenso efeito citotóxico observado na linhagem T98G).

Portanto aqui avaliamos se a inibição de SPHK2 com o inibidor K145 poderia promover um efeito aditivo quando associada à TMZ em glioblastoma. Conduto, apesar de apresentar efeito citotóxico em linhagens de glioblastoma, o inibidor K145 não promoveu efeito aditivo quando combinado, indicando que, apesar que SPHK2 estar envolvido com agressividade, sua atividade possa não estar envolvida com mecanismo de resistência. Ainda, avaliando o contexto do eixo esfingomielina-S1P, avaliamos também o uso de inibidor de SMASe, C10, na combinação com TMZ. De maneira semelhante, apesar de apresentar efeito citotóxico em linhagens de glioblastoma destacando-se o efeito na linhagem T98G, o inibidor C10 não promoveu

efeito aditivo relevante quando combinado, indicando que, apesar que SMPD estar envolvido com agressividade, sua atividade possa não estar envolvida com mecanismo de resistência.

Considerando o fenótipo de resistência tumoral, nossos dados indicam três vias importantes do metabolismo lipídico que são enriquecidas em pacientes com glioma associados à resistência à TMZ (estabelecido de acordo com o *status* de MGMT e recorrência tumoral), sugerindo que a via do metabolismo de esfingolipídios, genes relacionados ao peroxissoma e genes envolvidos em reações de oxidorredutases estão envolvidos na resistência à TMZ (Figura 2.1).

É descrito que altos níveis de espécies reativas do oxigênio (EROs) é um dos mecanismos relacionados ao efeito citotóxico mediado por TMZ (LANG et al., 2021a). Portanto, vias antioxidantes como aquela mediada pelo sistema glutationa desempenham um papel significativo na proteção das células contra a toxicidade induzida pela quimioterapia (LANG et al., 2021a). Os níveis de glutationa reduzida (GSH) estão intimamente associados à resistência a TMZ em modelo celular de glioblastoma humano demonstrando níveis mais elevados de GSH em células resistentes (ZHU et al., 2018b). Enquanto que a redução de GSH por meio de inibidores sensibilizou células de glioma resistentes à TMZ, tanto *in vitro* quanto *in vivo* (ROCHA et al., 2014). O aumento elevado EROs é uma característica também presente no processo de morte celular programado, ferroptose e a resistência a esse tipo de morte é associado como um potencial mecanismo de resistência a TMZ (HU et al., 2020). A ferroptose pode ser induzida pela inibição de glutationa peroxidase 4 (GPX4), uma enzima antioxidante crucial que, ao utilizar GSH como substrato, reduz os produtos da peroxidação lipídica, e inibe a ferroptose (LUO et al., 2022). Foi relatada uma forte correlação entre a expressão elevada de GPX4 e um pior prognóstico de pacientes com glioma, além de sua expressão estar relacionado com proliferação e migração em modelo celular de glioma humano (ZHAO et al., 2017). Ainda foi demostrado que a redução da GPX4 por ALZ003, um análogo de curcumina, aumenta a sensibilidade à quimioterapia por meio da ferroptose em células de glioblastoma resistentes à TMZ (CHEN et al., 2020). Curiosamente, nossos dados demonstraram que as células resistentes apresentaram níveis elevados de peroxidação lipídica quando tratadas com TMZ, indicando resistência a alterações lipídicas que normalmente levariam à morte celular, mesmo sem efeitos citotóxicos (Figura 2.5D). Além disso, foi observado que pacientes com resistência relacionada

ao *MGMT* mostraram aumento da expressão de GPX4 (figura 2.5A), e que células resistentes tratadas com RSL3, inibidor de GPX4, apresentaram maior resistência aos efeitos citotóxicos (Figura 2.5B). Em conjunto, esses resultados corroboram os dados da literatura que apontam que gliomas apresentam uma maior dependência das vias antioxidantes para a sua sobrevivência, e que a expressão de GPX4 pode estar envolvida na promoção de resistência, reduzindo os efeitos citotóxicos mesmo com alta geração de peroxidação lipídica no tratamento com TMZ. Destaca-se a importância desse mecanismo na resistência do glioma a tratamentos quimioterápicos.

7 CONSIDERAÇÕES FINAIS

A presente tese, ao empregar a lipidômica no contexto do glioblastoma (GBM), proporciona discussões cruciais que elevam a compreensão e refinam as estratégias terapêuticas associadas a essa forma altamente agressiva de tumor do sistema nervoso central. Os estudos reconhecem não apenas a complexidade, mas também a ineficácia das terapias atuais, ressaltando a urgência de abordagens mais eficazes e personalizadas.

A análise integrada de dados transcriptômicos do TCGA e CGGA, assim como dados experimentais de células tumorais revelou uma rede intricada de alterações no metabolismo lipídico associadas tanto ao glioma quanto à resistência ao quimioterápico temozolomida. A identificação de genes de lipídios correlacionados com o prognóstico e a construção de uma assinatura de risco enfatizam a relevância clínica desses potenciais biomarcadores. A relação positiva entre genes relacionados à matriz extracelular e o escore de risco destaca a interconexão entre a composição lipídica e o microambiente tumoral. Além disso, demonstrou-se que a agressividade está relacionada com alterações na composição de membrana, especialmente no aumento da insaturação de ácidos graxos, fosfolipídios e colesterol. Já, a diminuição de fosfolipídios e o aumento de colesterol e plasmalogénio se destacaram como fatores cruciais na aquisição de resistência ao tratamento padrão, ressaltando o papel crítico da membrana celular na agressividade e resistência à temozolomida.

Por fim, destaca-se a importância significativa dos lipídios de sinalização, especificamente esfingolipídios, e a identificação do eixo envolvendo a

esfingomielinase SMPD1 e a esfingosina-1-fosfato (SP1) mediada por SPHK1. Essas descobertas ressaltam novos alvos moleculares promissores para intervenções terapêuticas em ambos os contextos explorados. Em síntese, esses estudos sublinham a relevância clínica da lipidômica no contexto do glioma, proporcionando novas perspectivas sobre a biologia tumoral e identificando alvos terapêuticos potenciais. Com a aplicação da tecnologia de ressonância magnética nuclear (RMN) utilizada neste estudo, que pode ser facilmente incorporada à prática médica, destacam-se implicações clínicas significativas. Essas implicações não apenas oferecem estratégias personalizadas de tratamento, mas também promovem avanços no diagnóstico e prognóstico. Este enfoque mais preciso e eficaz no enfrentamento do GBM destaca-se pela sua menor dependência de procedimentos cirúrgicos, representando assim um passo crucial para aprimorar a abordagem clínica dessa doença desafiadora e devastadora.

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